



UNIVERSITAT_{DE}
BARCELONA

Development of a biomimetic mechanical stimulation system to improve the maturation of human iPS-derived myocardial grafts

Juan Crespo Santiago

ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (www.tdx.cat) i a través del Dipòsit Digital de la UB (diposit.ub.edu) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX ni al Dipòsit Digital de la UB. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX o al Dipòsit Digital de la UB (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (www.tdx.cat) y a través del Repositorio Digital de la UB (diposit.ub.edu) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR o al Repositorio Digital de la UB. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR o al Repositorio Digital de la UB (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (www.tdx.cat) service and by the UB Digital Repository (diposit.ub.edu) has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized nor its spreading and availability from a site foreign to the TDX service or to the UB Digital Repository. Introducing its content in a window or frame foreign to the TDX service or to the UB Digital Repository is not authorized (framing). Those rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author.

Development of a biomimetic mechanical stimulation system to improve the maturation of human iPS-derived myocardial grafts

Barcelona, 7 de junio de 2016

Programa de doctorado H0101 BIOMEDICINA

Línea de investigación INGENIERÍA BIOMÉDICA

Desarrollada en el Centro de Medicina Regenerativa de Barcelona (CMRB) y el Instituto de Bioingeniería de Cataluña (IBEC)

Doctorando: Juan Crespo Santiago

Director: Ángel Raya Chamorro

Tutor: José A. Del Río

A Gabri,

a mis padres y hermana y familia,

a mis amigos.

A todos, gracias.

1.	THE HUMAN HEART	
1.1	<i>Anatomy of the heart</i>	1
1.1.1	Location and chambers	1
1.1.2	Layers of the heart	3
1.1.3	Histology of cardiac muscle	6
1.1.4	Electrical conduction	7
1.1.5	Mechanical contraction	8
1.1.6	The cardiac cycle	10
1.2	<i>Heart development</i>	12
1.2.1	Embryonic development	12
1.2.2	Cardiomyocyte maturation: fetal VS adult	13
1.3	<i>Epidemiology and myocardial infarction</i>	20
1.3.1	Cardiac function evaluation in myocardial infarction	22
2.	CARDIAC TISSUE ENGINEERING AND STEM CELLS	
	<i>Introduction</i>	25
2.1	<i>Components of a cardiac construct</i>	27
2.1.1	Cellular components	27
2.1.1.1	Human induced pluripotent stem cells (hiPSC) in cardiac tissue engineering	29
2.1.1.2	Human iPSC-derived cardiomyocytes	30
2.1.2	Biomaterials and strategies for cardiac scaffolds	32
2.1.3	Signaling molecules	39
2.1.4	Last generation cardiac constructs	40
2.2	<i>Bioengineering scaffold stimulation</i>	42
2.2.1	Electrical stimulation	42
2.2.2	Mechanical stimulation	43
2.2.3	Bioreactors	45
3.	CLINICAL TRIALS	47
4.	HYPOTHESIS & OBJECTIVES	49
5.	MATERIALS AND METHODS	
5.1	<i>Porcine myocardium mechanical assays</i>	50
5.2	<i>Design of a hydrogel scaffold</i>	51
5.2.1	Pluronic F127 preparation	51
5.2.2	Pluronic F127 rheological tests	51
5.2.3	Pluronic F127 culture with human foreskin fibroblasts (HFF)	52
5.2.4	Pluronic F127- gelatin hydrogel	52
5.2.5	Hydrogel of Poly-DL-lactic-co-glycolic acid (PDLLGA) with polyethylene glycol (PEG)	53
5.3	<i>Fabrication of engineered heart tissue (EHT)</i>	54
5.3.1	Isolation of neonatal rat heart cells	54
5.3.2	Fabrication of EHT scaffolds	54
5.4	<i>Fabrication of ring-shaped human engineered keratinocyte iPSC-derived cardiomyocyte (iPSC-CM) cardiac tissue</i>	56

5.4.1	In-vitro differentiation of <i>keratinocyte induced pluripotent stem cells (KiPSC)</i>	56
5.4.2	Fabrication of iPSC-CM ring-scaffolds	56
5.5	<i>Mechanical stimulation of rat engineered heart tissues (EHTs) and ring-shaped scaffolds with iPSC-CM</i>	58
5.6	<i>Mechanical stimulation of the biomimetic stretching device (BSD) with iPSC-CM cardiac tissues and rat cardiac tissues</i>	59
5.7	<i>Characterization of engineered iPSC-CM cardiac tissues and EHTs</i>	61
5.7.1	Immunohistochemistry	61
5.7.2	Morphological evaluation of the scaffolds	62
	Cell length	
	Cell alignment	
	Presence of Z-bands	
5.7.3	Transmission Electron Microscopy (TEM)	64
5.7.4	Quantitative Polymerase Chain reaction (q-PCR)	65
5.7.5	Electrical coupling of the scaffolds	65
5.7.6	Flow cytometry	68
5.7.7	Calcium imaging	68
5.7.8	Fractional Area Change	68
5.8	<i>Transplantation of iPSC-CM scaffolds in a mouse model of acute myocardial infarction (MI)</i>	69
5.8.1	Surgical procedure for induction of MI and tissue grafting	69
5.8.2	Histological evaluation	69
5.8.3	Cardiac function determination	70
5.8.4	Determination of the infarction size	70
5.9	<i>Statistical analysis</i>	71

6. RESULTS

6.1	<i>Analysis of the porcine myocardium stiffness</i>	72
6.2	<i>Generation of hydrogel scaffolds with different polymers</i>	75
6.2.1	Analysis of Pluronic F127 mechanical behavior	75
6.2.2	Generation of Pluronic F127 hydrogel scaffold with HFF	75
6.2.3	Generation of a Pluronic F127-gelatin hydrogel	76
6.2.4	Generation of PDLLGA with PEG hydrogel	77
6.3	<i>Generation of rat engineered heart tissues (EHTs)</i>	78
6.3.1	Morphological aspects of rat EHTs	78
6.3.2	Electrical coupling of EHTs	81
6.4	<i>Generation of membrane-based device scaffolds (BSD) with neonatal rat heart cells</i>	82
6.4.1	Morphological aspects of rat BSDs	82
6.4.2	TEM ultrastructural analysis of BSD compared to neonatal rat myocardium	89
6.4.3	Electrical coupling of BSDs with rat cardiac cells	89
6.5	<i>Cardiac differentiation of human keratinocyte iPSC-derived cardiomyocyte (iPSC-CM)</i>	90
6.6	<i>Fabrication of ring-scaffolds with human iPSC-CM</i>	93
6.6.1	<i>Morphological aspects of ring-shaped scaffolds with iPSC-CM</i>	93

6.6.2	Electrical coupling of ring-scaffolds with human iPSC-CM	96
6.6.3	Ultrastructural analysis of ring-scaffolds with human iPSC-CM	96
6.6.4	Expression of cardiac genes for iPSC-CM ring-scaffolds	97
6.7	<i>Fabrication of BSD scaffolds with human iPSC-CM</i>	98
6.7.1	Morphological evaluation of BSD scaffolds	98
6.7.2	Ultrastructural analysis of BSDs with human iPSC-CM	99
6.7.3	Electrical coupling of BSDs with human iPSC-CM	100
6.7.4	Expression of cardiac genes for BSDs with iPSC-CM	100
6.7.5	Contractile behavior of BSDs with human iPSC-CM	102
6.8	<i>Regenerative potential of BSD scaffolds with human iPSC-CM in a mouse model of myocardia infarction (MI)</i>	104
6.8.1	Echocardiographic evaluation of left ventricular function in a mouse model of MI	104
6.8.2	Measurement of left ventricle remodeling	105
6.8.3	Tissue engraftment	106
7.	DISCUSSION	110
8.	CONCLUSIONS	119
9.	BIBLIOGRAPHY	120

1. The human heart

1. THE HUMAN HEART

The heart is one of the three main components of the cardiovascular system with the blood vessels and the blood. Its function is to pump blood through the blood vessels to all body systems and is constantly pumping blood since the very first stages of embryo development until the end of our lives. Blood must be constantly pumped through the blood vessels so that it can reach body cells and exchange materials with them. To accomplish such a task, the heart beats about 100,000 times every day, which adds up to 35 million beats in a year and about 2,500 million times in an average lifetime. Even at a relaxed state, the heart pumps 30 times its own weight each minute, about 5 liters, that is about 14,000 liters a day. And this rate increases during an active state, such as the physical effort.

1.1 Anatomy of the heart

1.1.1 Location and chambers

The heart is located on top of the diaphragm, near the midline of the thoracic cavity. It lies in the mediastinum, an anatomical region that extends from the sternum to the vertebral column, the first rib to the diaphragm, and between the lungs (Fig.1), (Principles of Anatomy and Physiology, Tortora & Derrickson, 12th Ed., 2008). It is about 12 cm long, 9 cm wide at the broadest point and 6 cm thick, with an average mass of 250 g in adult females and 300 g in adult males. About two thirds of the mass of the heart lies to the left of the body's midline. The pointed apex is formed by the tip of the left ventricle (a lower chamber of the heart) and rests on the diaphragm. The base of the heart is its posterior surface, formed by the atria (the two upper chambers).

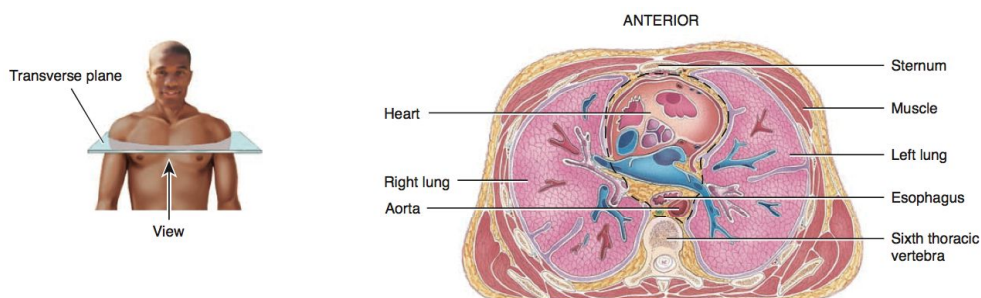
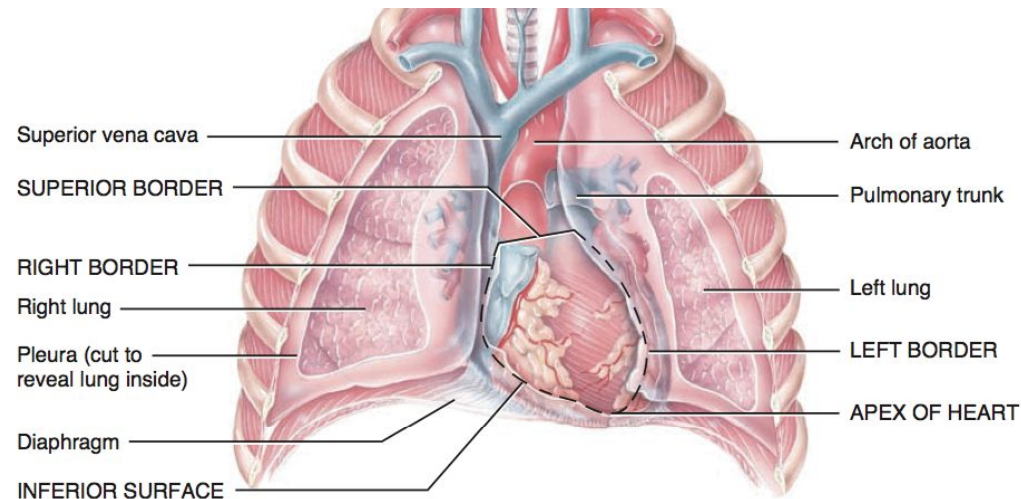


Fig. 1

(A) Anterior view of the heart in the thoracic cavity. (B) Anterior transversal view of the heart in the thoracic cavity. (Tortora & Derrickson, 2008)

The heart is composed of four chambers: the upper atria and the two lower ventricles (Fig.2). On the anterior surface of each atrium is a wrinkled pouch-like structure called auricle. Each auricle slightly increases the capacity of the atrium allowing to hold a greater volume of blood. Also on the surface of the heart are a series of grooves, called sulci that contain coronary blood vessels and a variable amount of fat. Each sulcus marks the external boundary between two chambers of the heart. The deep coronary sulcus encircles most of the heart and marks the external boundary between the superior atria and inferior ventricles. The anterior interventricular sulcus is a shallow groove on the anterior surface of the heart that marks the external boundary between the right and left ventricles.

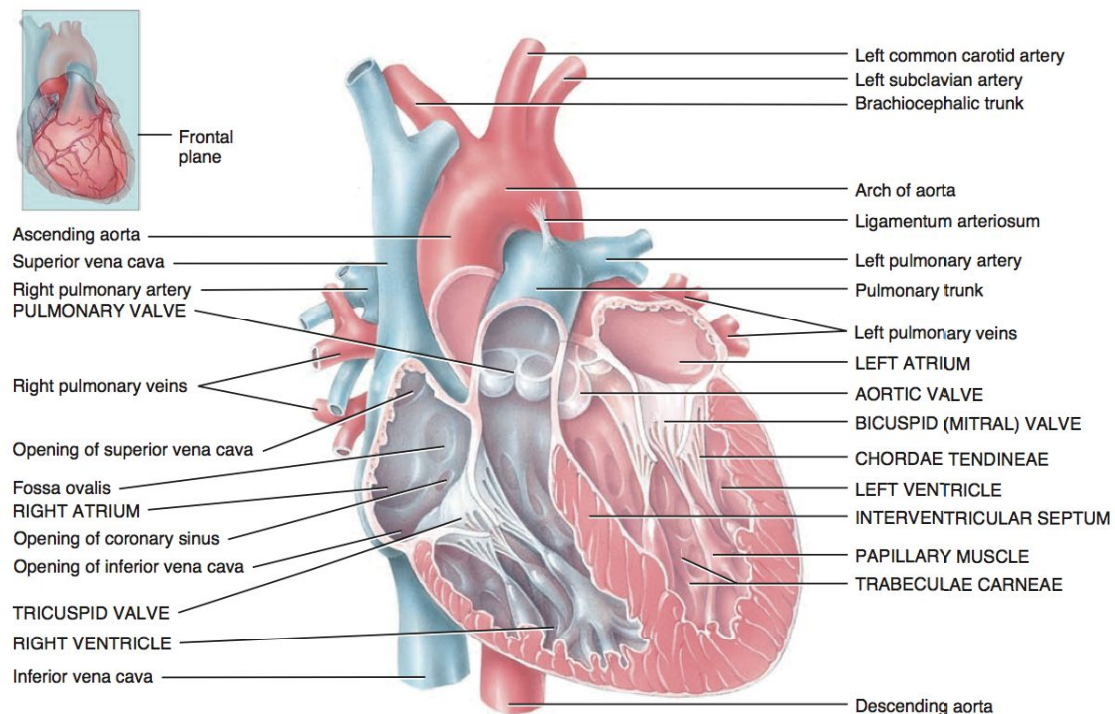


Fig. 2

Anterior view of frontal section of the heart showing internal anatomy. (Tortora & Derrickson, 2008)

The blood circuit in the heart

- The right atrium receives the blood from three veins: the superior vena cava, inferior vena cava, and coronary sinus. Blood flows from the right atrium into the right ventricle through a valve called the tricuspid valve, formed with 3 leaflets.
- The right ventricle pumps blood through the pulmonary valve into a large artery called the pulmonary trunk, which divides into right and left pulmonary arteries. The pulmonary trunk contains a series of ridges formed by raised bundles of cardiac muscle fibers called trabecular carneae. Some of these trabecular carneae convey part of the conduction system of the heart. The cusps of the tricuspid valve are connected to tendonlike cords, the chordae tendineae, which in turn are connected to cone-shaped trabeculae carneae called papillary

muscles. Internally, the right ventricle is separated from the left ventricle by a thin wall called the interventricular septum.

- The left atrium receives blood from the lungs through four pulmonary veins. Blood passes from the left atrium into the left ventricle through the bicuspid valve. Ventricles are separated by a partition called the interventricular septum.
- The left ventricle is the thickest chamber of the heart, averaging 10 to 15 cm and forms the apex of the heart. Blood passes from the left ventricle through the aortic valve into the ascending aorta. Some of the blood flows into the coronary arteries, the remainder of the blood passes into the arch of the aorta and descending aorta. Like the right ventricle, the left ventricle contains trabeculae carneae and has chordae tendinae that anchor the cusps of the bicuspid valve to papillary muscles.

1.1.2 Layers of the heart

The membrane that protects and surrounds the heart is called pericardium. It confines its position in the mediastinum while allowing sufficient freedom to move for vigorous and rapid contraction (Fig.3). The pericardium consists of 2 parts: the fibrous pericardium and the serous pericardium. The superficial fibrous pericardium is composed of tough, inelastic, dense irregular connective tissue; its open end is fused to the connective tissues of blood vessels entering and leaving the heart. This tough layer prevents overstretching of the heart, provides protection, and anchors the heart in the mediastinum. The deeper serous pericardium is a thinner, more delicate membrane that forms a double layer around the heart. The outer parietal layer of the serous pericardium is fused to the fibrous pericardium. The inner visceral layer of the serous pericardium is one of the layers of the heart wall and adheres tightly to the surface of the heart. Between the parietal and visceral layers of the serous pericardium is a thin film of lubricating serous fluid. This secretion of the pericardial cells, known as pericardial fluid, reduces friction between the layers of the serous pericardium as the heart moves. The space that contains the few milliliters of pericardial fluid is called the pericardial cavity.

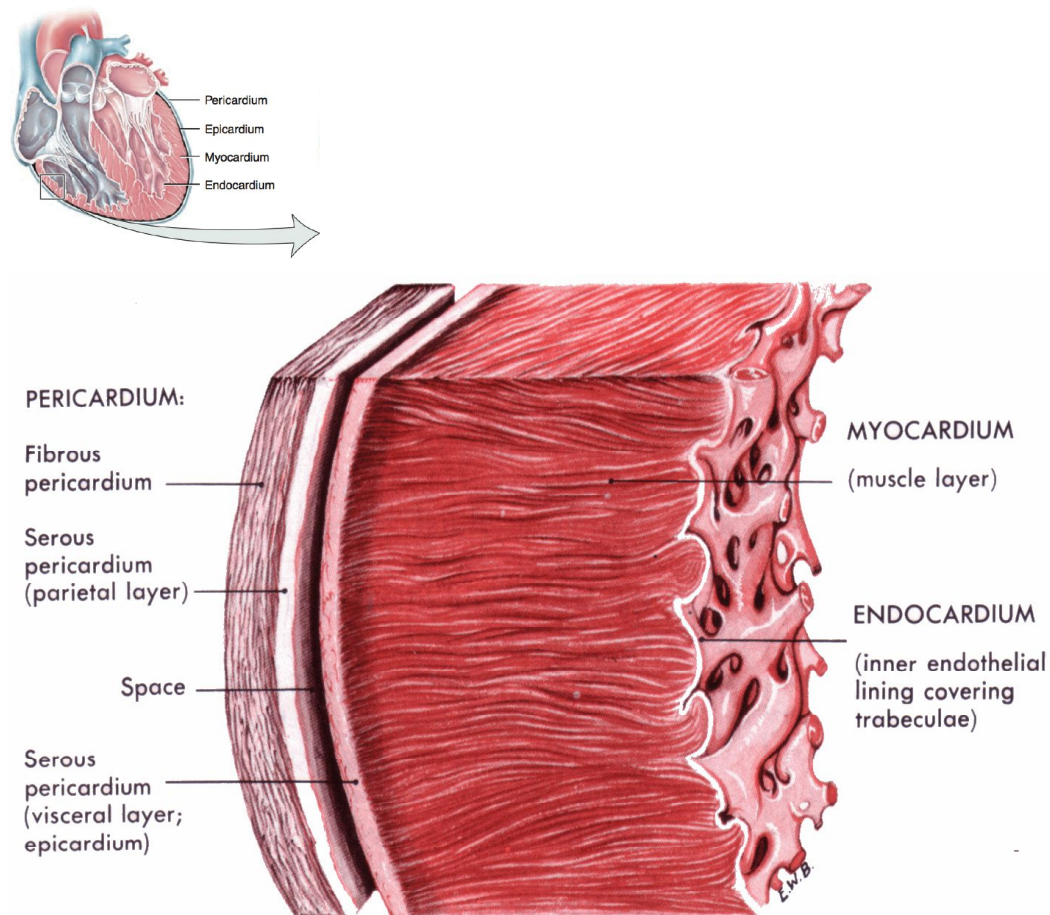


Fig. 3
Section of pericardium and right ventricular heart wall showing the divisions of the pericardium and layers of the heart wall. (Histology OLM, Steve Gallic.org)

The heart wall consists in 3 well-defined layers: the epicardium (external layer), the myocardium (middle layer), and the endocardium (internal layer). As noted before, the outer layer of the epicardium is also called the visceral layer of the serous pericardium. Which is composed of mesothelium and delicate connective tissue that imparts a smooth, slippery texture to the outermost surface of the heart. The myocardium, which is cardiac muscle tissue, constitutes about 95% of the heart volume and is responsible for its pumping action. Although it is striated like skeletal muscle, cardiac muscle is involuntary like smooth muscle. The cardiac muscle fibers swirl diagonally around the heart in bundles, and this particular distribution of the muscle layers, is the reason why the heart contracts concentrically in every direction (multiaxial) (Fig.4) (Marcé-Nogué et al., 2013) (Van Dalen et al., 2010) (Konstantopoulou et al., 2013) (Wang et al., 2012) instead of only in a single direction (uniaxial), like skeletal muscle.

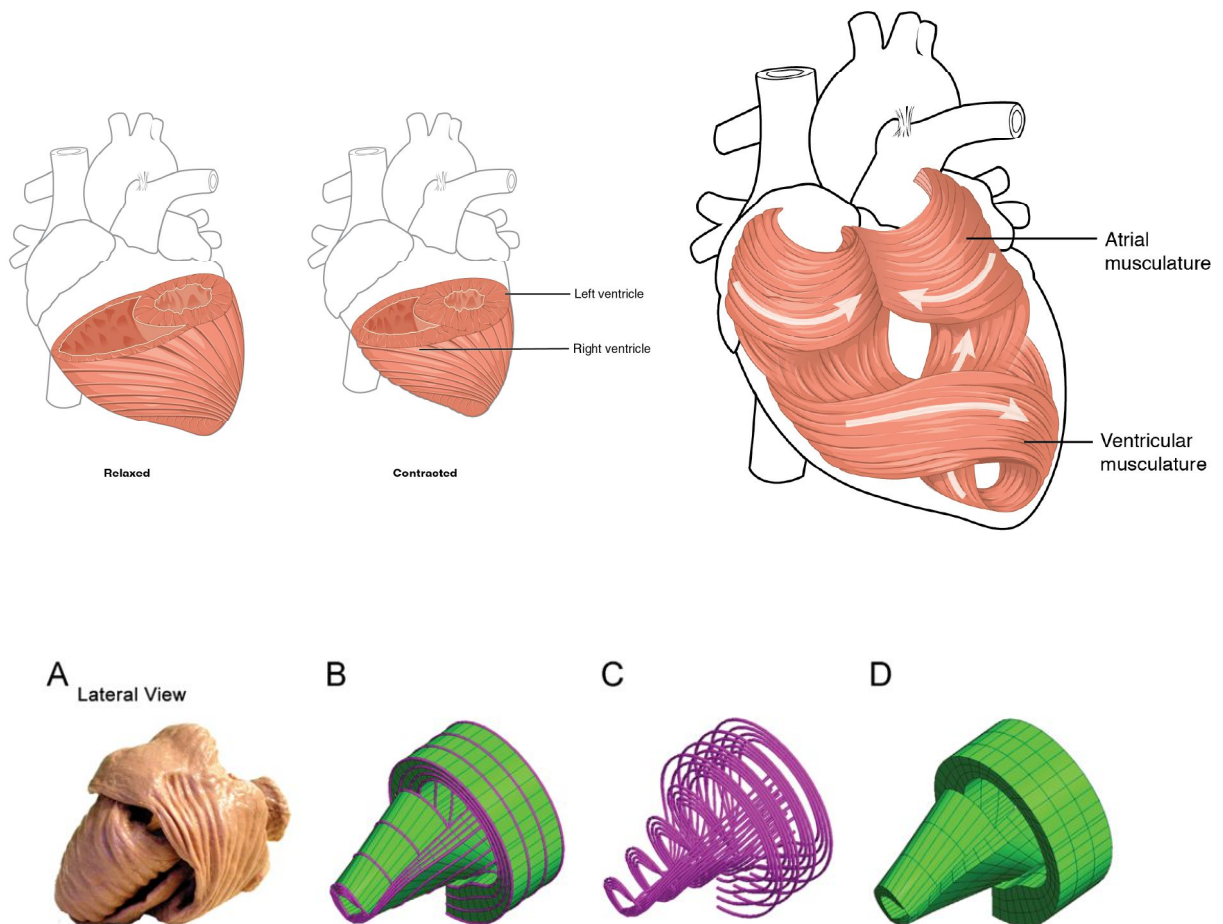


Fig. 4

Top left: Section of the heart showing variation of ventricle volume in the relaxed state, and contracted state. Top right: anterior view of the heart showing cardiac muscle bundles of the myocardium and predominant muscle fiber directions in atria and ventricles (Vietnam Open Educational Resources, VOER, www.voer.edu.vn). Bottom: Silicone (A) and finite-element (B-D) models for myocardium, (B) the whole model, (C) only the fibers and (D) only the tissue. Image modified from (Marcé-Nogué et al., 2013).

The innermost endocardium is a thin layer of endothelium overlying a thin layer of connective tissue. It provides a smooth lining for the chambers of the heart and covers the valves of the heart. The endocardium is continuous with the endothelial lining of the large blood vessels attached to the heart, and it minimizes surface friction as blood passes through the heart and blood vessels.

There is an important issue about the variable thickness of the myocardium, it varies according to each chamber's function. The thin-walled atria deliver blood under less pressure into the adjacent ventricles. Because the ventricles pump blood under higher pressure over greater distances, their walls are thicker (Fig.5). Although the right and left ventricles act as two separate pumps that simultaneously eject equal volumes of blood, the right side has a much smaller workload. It pumps blood a short distance to the lungs at lower pressure, and the resistance to blood flow is small. The left ventricle pumps blood great distances to all other parts of the body at higher pressure, and the resistance to blood flow is larger. Therefore, the left ventricle works much harder than the right ventricle to maintain the same rate of blood flow. The anatomy of the two ventricles confirms this functional difference, the muscular wall of the left ventricle is considerably thicker than the wall of the right ventricle. Note also that the perimeter of the lumen (space) of the left ventricle is roughly circular in contrast to that of the right ventricle (Nakano et al., 1990).

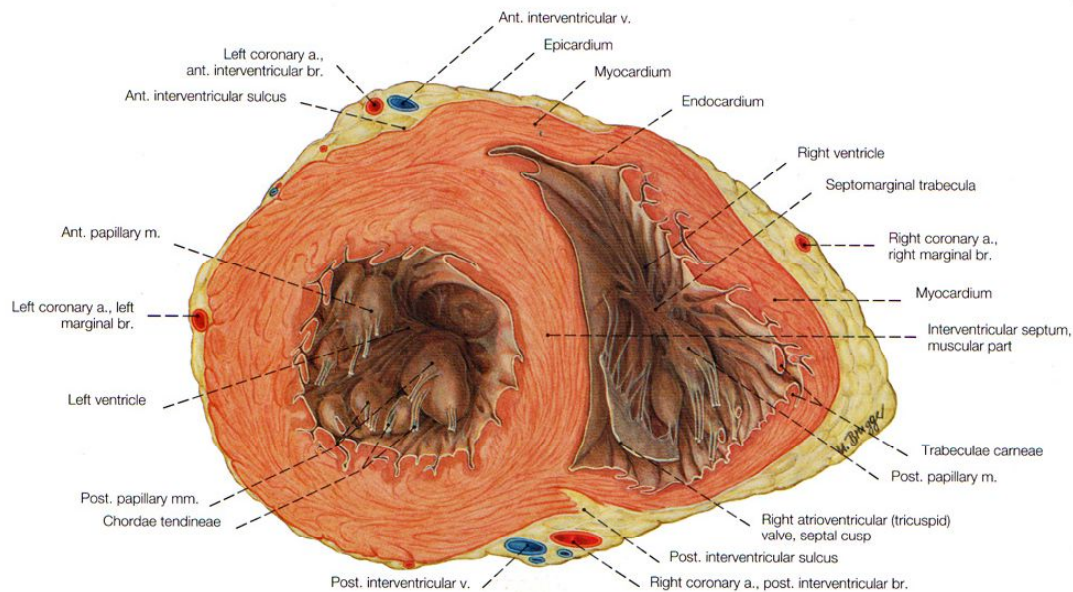


Fig. 5
Posterior inferior view of transverse section showing differences in thickness of ventricular walls (www.corpshuman.ca)

1.1.3 Histology of cardiac muscle

The human heart is composed of a variety of cells, all of them contributing to the structural, biochemical or electro-mechanical properties of the functional heart. Atrial and ventricular cardiomyocytes form the muscular walls of the myocardium. About 50% of the cells of the heart are cardiac fibroblasts (Xin et al., 2013). Endothelial cells form the endocardium, the interior of the blood vessels and cardiac valves. Smooth muscle cells contribute to the coronary arteries and the in- or outflow vasculature. Epicardium mainly gives rise to the precursors of the coronary vasculature and cardiac fibroblasts. Lastly, pacemaker cells and Purkinje fibers in the conduction system are specialized cardiomyocytes that generate and conduct electrical pulses.

More particularly, the heart muscle is compound of muscle fibers or cardiomyocytes (Fig.6). These fibers are shorter in length than the skeletal ones, and less circular in transverse section. They also exhibit branching, which gives them an appearance of Y-shaped cells interconnected between them. A typical cardiac muscle cell is 50-100µm long and has a diameter of 14µm. They are usually mononucleated, although sometimes an occasional cell may have 2 nuclei. The ends of each muscle fiber connect to the neighboring ones by irregular transverse thickenings of the sarcolemma called intercalated discs. The discs contain desmosomes, which are specialized cell structures necessary for the establishment of intercellular connections, and gap junctions, which allow electrical coupling between cardiomyocytes, and therefore allow the entire myocardium to contract as a single, coordinated unit (Zhuang et al., 2000) (Kostin et al., 1999).

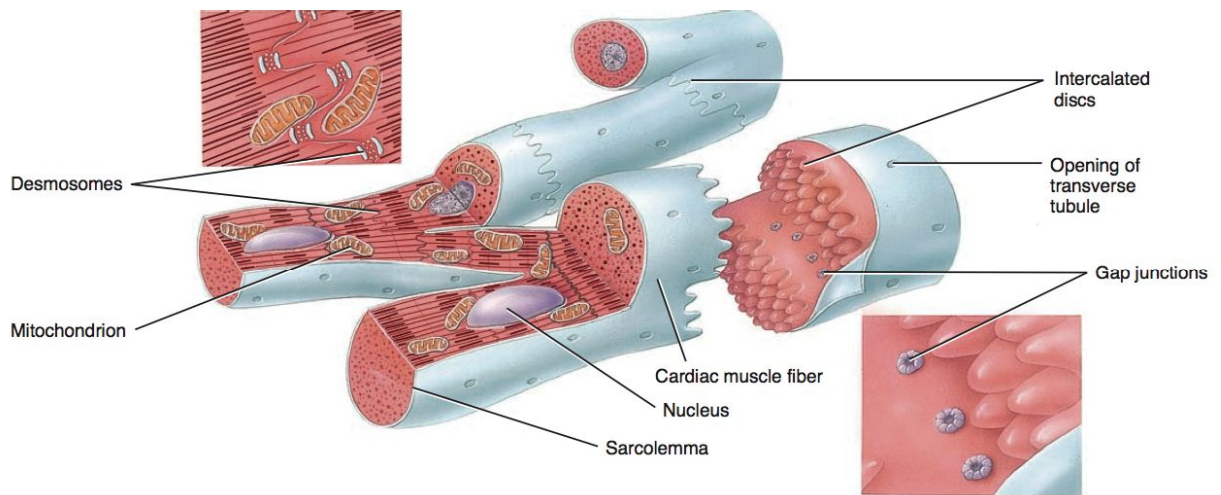


Fig. 6
Cardiac muscle fiber exhibiting its organelle (Principles of Anatomy and Physiology, Tortora & Derrickson, 12th Ed., 2008).

1.1.4 Electrical conduction

Every cardiomyocyte is involved in the conduction of the depolarization wave of the heart, in which an electrical current (an actual ion current) (Fig.7) goes over from side to side of the heart creating a synchronous contraction of the whole organ. But only a few of these cells generate it automatically. These are called the autorhythmic fibers, because they are self-excitable, a network of specialized cardiac muscle fibers that are the source of the electrical activity. These cells generate action potentials that trigger heart contractions. They continue stimulating the heart even after it is removed, such as in a heart transplant, where all its nerves have been cut (Xin et al., 2013). Only about 1% of the cardiomyocytes become autorhythmic fibers during embryo development, which have two fundamental functions:

- 1) To act as a pacemaker, setting the rhythm of electrical excitation that causes the contraction of each muscle cell, and therefore the whole organ.
- 2) They form the conduction system, a network of specialized cardiac muscle cells that provide a path for each cycle of cardiac excitation to progress through the heart. This network ensures that cardiac chambers become stimulated to contract in a coordinated manner, which makes the heart an effective pump.

Cardiac excitation begins in the sinoatrial (SA) node, located in the atrial wall. The spontaneous depolarization is a pacemaker potential. When the pacemaker potential reaches threshold, it triggers an action potential (AP). Each action potential from the SA node propagates throughout both atria via gap junctions in the intercalated discs of atrial cardiomyocytes. Following this potential, the atria contract. Then the AP reaches the atrioventricular (AV) node located in the interatrial septum. From the AV node the AP enters the AV bundle (also known as the bundle of His); this is the place where the APs can conduct from the atria to the ventricles. After propagating along the AV bundle, the AP enters both the right and left bundle branches, which extend through the interventricular septum toward the apex of the heart. Finally, the large diameter Purkinje fibers rapidly conduct the AP beginning at the apex of the heart upward the remainder of the ventricular myocardium. Then the ventricles contract, pushing the blood upward toward the valves.

During the first phase of the depolarization wave, a muscle fiber is brought to threshold (at about +20 mV) by an AP from the neighboring cells, and its voltage-gated Na^+ channels open (Fig.7). This is called the fast depolarization, because they open very rapidly in response to a threshold-level depolarization. The next phase, the plateau, is characterized by a period of maintained depolarization. It is due to the opening of voltage-gated slow Ca^{2+} channels in the sarcolemma. When these channels open, calcium ions move from the interstitial fluid (which has a higher Ca^{2+} concentration) into the cytosol. This increase in Ca^{2+} concentration ultimately triggers the contraction of the cell, even though different types of voltage-gated K^+ channels are also found in the sarcolemma to be involved in the process. In the final phase, the repolarization, the recovery of the resting membrane potential (about -90 mV) occurs, mainly when additional voltage-gated K^+ channels open and allow the outflow. At the same time, the Ca^{2+} channels in the sarcolemma and sarcoplasmic reticulum are closing, which also contributes to repolarization. (Marcé-Nogué et al., 2013)

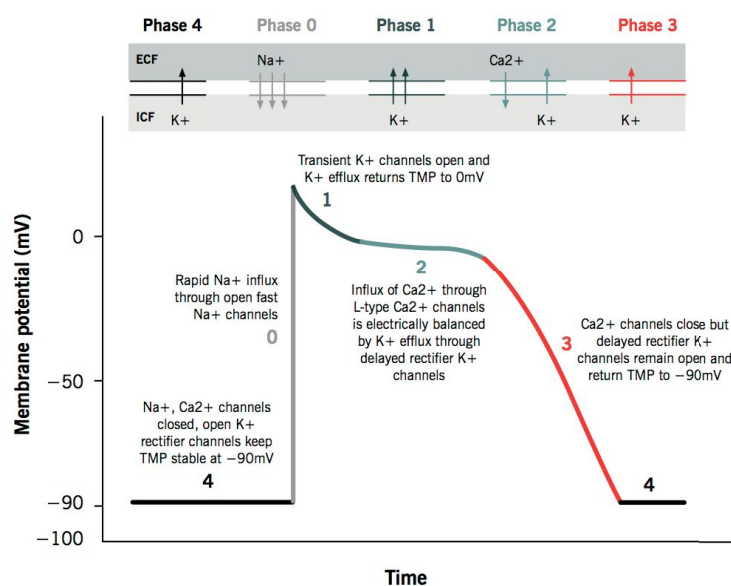


Fig. 7
Scheme of an action potential in a ventricular cardiomyocyte (www.pathophys.org).

1.1.5 Mechanical contraction

As it was previously shown, the electrical current that runs across the heart, cell to cell, is the cause of the contraction of the heart. Muscle cells achieve their single contraction by the massive shortening of an intracellular structure called myofibril. Myofibrils are the contractile organelles of any muscular cell. They are about 2 μm in diameter, and extend the entire length of the adult muscle fiber. Within myofibrils are smaller structures called filaments. There are 2 types: thin filaments, with 8 nm in diameter and about 1-2 μm long; and thick filaments, with 16 nm in diameter and also 1-2 μm long.

Regarding the composition of the myofibrils, there are 3 kinds of proteins regarding their function: (1) *contractile proteins*, which generate force during contraction; (2) *regulatory proteins*, which help switch the contraction process on and off; and (3) *structural proteins*, which keep the thick and thin filaments in the proper alignment, give the myofibril elasticity and extensibility, and link the myofibrils to the sarcolemma and

extracellular matrix. The two contractile proteins in muscle are: myosin (golf stick-shaped) and actin (bubble chain-shaped) see Fig.9, which are the main components of thick and thin filaments, respectively. Besides contractile and regulatory proteins, cardiac muscle contains about a dozen structural proteins, which contribute to the alignment, stability, elasticity, and extensibility of myofibrils, such as titin, α -actinin, or myomesin.

- Thick filaments contain several myosin molecules (Fig.8). Myosin functions as a motor protein that pulls cellular structures to achieve movement by converting the chemical energy to the mechanical energy of motion. A group of myosin molecules lie parallel to one another, forming the shaft of the thick filament. The myosin tails point toward the M lines, the center of the sarcomere, and their heads project outward toward the surrounding thin filaments.
- Thin filaments contain actin, troponin and tropomyosin, actin being their main protein component. Individual actin molecules join to form an actin filament that is twisted into a helix. On each molecule, there is myosin-binding site, where a myosin “head” can attach. Thin filaments are anchored to Z discs, at both ends of each sarcomere (Fig.8).

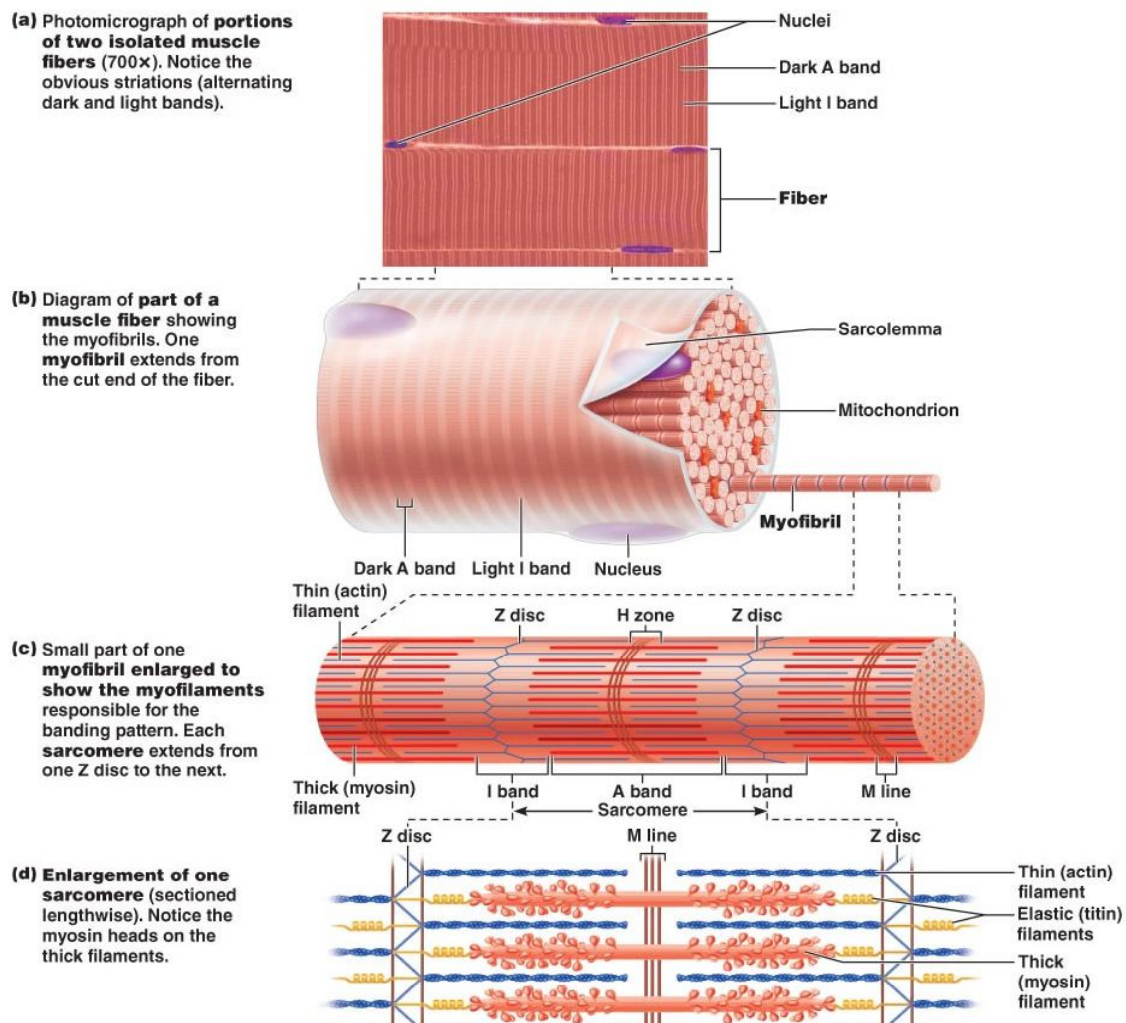


Fig. 8
(a) Photomicrograph of portions of two isolated muscle fibers. (b) Part of a muscle fiber showing the myofibrils. (c) Detail of a myofibril. (d) Enlargement of one sarcomere (Pearson Education Inc., 2008).

Muscle contraction occurs because myosin heads attach to and “walk” along the thin filaments at both ends of a sarcomere, progressively pulling the thin filaments toward the M line (Fig.8). As a result, the thin filaments slide inward and meet at the center of a sarcomere. They may even move so far inward that their ends overlap. As the thin filaments slide inward, the Z discs come closer together, and the sarcomere shortens. However, the lengths of the individual thick and thin filaments do not change. Shortening of the sarcomeres causes shortening of the whole muscle fiber, which in turn leads to shortening of the entire muscle. (Lafortune & Aris, 2012)

More specifically, at the onset of contraction, the sarcoplasmic reticulum releases calcium ions (Ca^{2+}) into the cytosol, where they bind to troponin. Troponin then moves tropomyosin away from the myosin-binding sites on actin (see Fig.9). Once the binding sites are “free,” the contraction cycle begins. The energized myosin head attaches to the myosin-binding site on actin. This attachment is referred to as crossbridge. After the crossbridges form, the power stroke occurs. During the power stroke, the site on the crossbridge where a molecule of ADP is still bound opens. As a result, the crossbridge rotates and releases the ADP. The crossbridge generates force as it rotates toward the center of the sarcomere, sliding the thin filament past the thick filament toward the M line. At the end of the power stroke, the crossbridge remains firmly attached to actin until it binds another molecule of ATP. As ATP binds to the ATP-binding site on the myosin head, the myosin head detaches from actin. This contraction cycle is repeated as the Ca^{2+} level near the filament is sufficiently high. During a maximal muscle contraction, the distance between two Z discs can decrease to half the resting length. In cardiac muscle, contraction of muscle fibers is about 12 - 15 % from its original length (Biomaterials, A Basic Introduction, Q. Chen & G. Thouas, CRC Press, 2015) (Katz et al., 2001) (Pinnell et al., 2007) (Zhang et al., 2015).

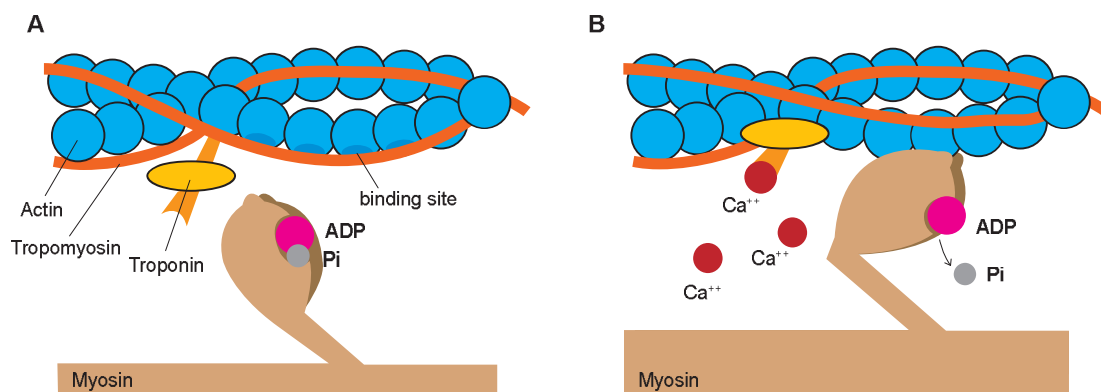


Fig. 9
(A) Relaxed state of an actin-myosin protein complex, where tropomyosin blocks the binding site. (B) Contracted state of an actin-myosin protein complex, where myosin head binds to actin (Modified from Molecular biology, The Mac-Graw Hill Companies, 2007).

1.1.6 The cardiac cycle

In each cardiac cycle, the atria and ventricles alternately contract and relax, forcing blood from areas of higher pressure to areas of lower pressure. When a chamber of the heart contracts, blood pressure increases within. Each ventricle, however, expels the same volume of blood per beat, and the same pattern exists for both pumping chambers. When the heart rate is 75 beats/min, a cardiac cycle lasts about 0,8 seconds (Fig.10).

In the first state, the atria contract (around 0,1 seconds) and at the same point, the ventricles are relaxed. The end of the atrial systole is also the end of the ventricular diastole (relaxation). The volume of blood the ventricle contains at this point is about 130 ml. This volume is called the end-diastolic volume (EDV). Right after reaching the end of atrial systole, and therefore the ventricular EDV, the ventricular depolarization begins, and so the ventricular systole (around 0,3 seconds). The pressure inside the ventricles increases and pushes blood up against the atrioventricular valves (AV). When left ventricular pressure surpasses aortic pressure at about 80 millimeters of mercury (mmHg) and right ventricular pressure rises above the pressure in the pulmonary trunk (about 20 mmHg), both semilunar (SL) valves open. At this point, ejection of blood from the heart begins. The period when the SL valves are open is the ventricular ejection and lasts for about 0,25 seconds. The pressure in the left ventricle continues to rise to about 120 mmHg, whereas the pressure in the right ventricle climbs to about 25 - 30 mmHg. The left ventricle ejects about 70ml of blood into the aorta and the right ventricle ejects the same volume of blood into the pulmonary trunk. The volume remaining in each ventricle at the end of the systole, about 60ml, is the end-systolic volume (ESV). The relaxation period (about 0,4 seconds) is when the atria and ventricles are both relaxed. As the heart beats faster and faster, the relaxation period becomes shorter and shorter, whereas the atrial and ventricular systole only shorten slightly. Ventricular repolarization causes ventricular diastole. As the ventricles relax, pressure within the chambers falls, and blood in the aorta and pulmonary trunk begins to flow backward towards the regions of lower pressure in the ventricles. As the ventricles continue to relax, the pressure falls quickly. When ventricular pressure drops below atrial pressure, the AV valves open, and ventricular filling begins.

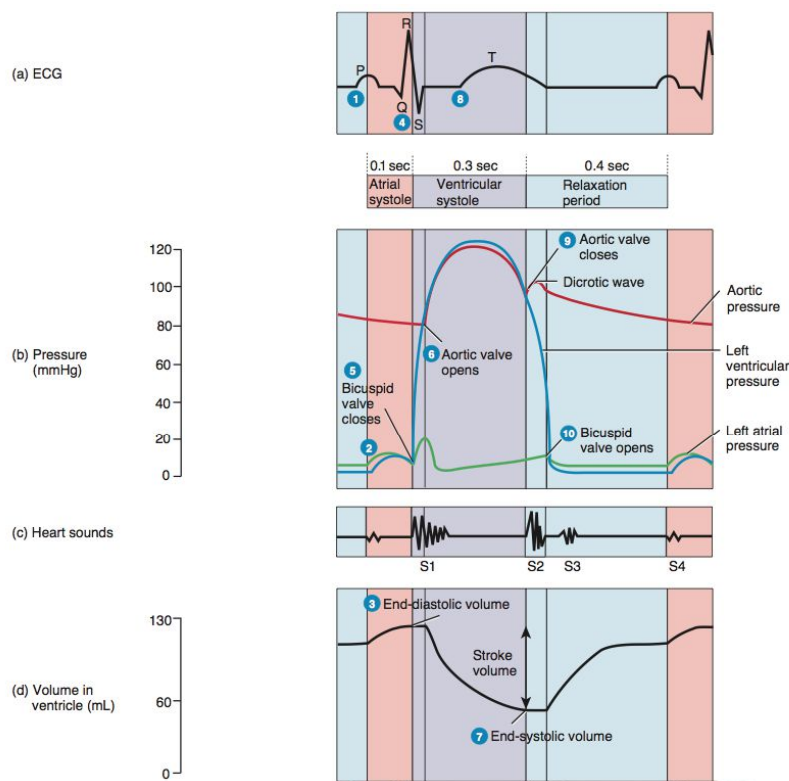


Fig. 10
 (a) Electrocardiogram (ECG) of one heart beat showing time lapse (b) Pressure (mmHg) graphic (c) heart sounds representation (d) volume (mL) in ventricle graphic (Tortora & Derrickson, 2008).

1.2 Heart development

1.2.1 Embryonic development

During embryogenesis, the heart is the first organ to show a mechanical function, and this function is initiated before structural organogenesis is complete.

Eight days after fertilization, cells of the inner cell mass in the blastocyst differentiate into two layers: hypoblast (primitive endoderm) and epiblast (primitive ectoderm) (Fig.11). Shortly afterwards, a small cavity appears within the epiblast and eventually enlarges to form the amniotic cavity. By the third week of development, the two-layered embryonic disc, consisting of epiblast and hypoblast, transforms into a three-layered (trilaminar) embryonic disc, consisting of three primary germ layers: the ectoderm, mesoderm, and endoderm. The primary germ layers are the major embryonic tissues from which the various tissues and organs of the body develop.

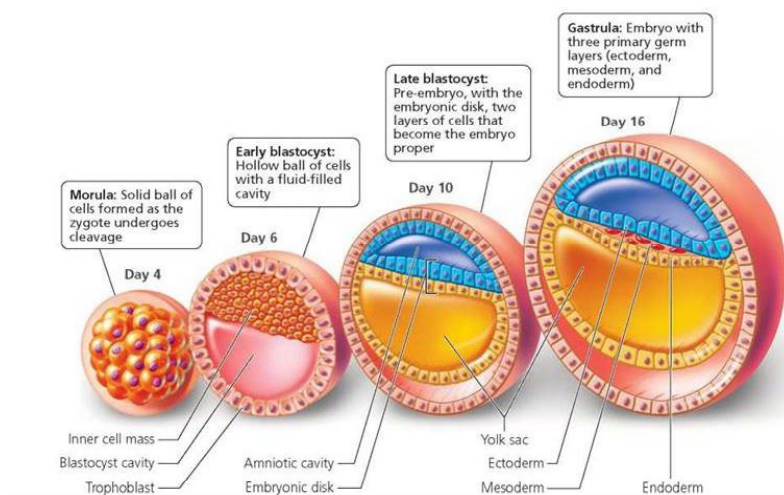


Fig. 11

Frontal section of the endometrium of uterus showing embryo at different stages of maturation (Biology.Kenyon.edu)

The heart forms from the mesoderm layer in the head end of the embryo on days 18 and 19 after fertilization. This region of mesodermal cells is called the cardiogenic area. In response to induction signals from the underlying endoderm, these mesodermal cells form the cardiac crescent, which fuses into the embryo midline to form the primitive heart tube. By the end of the third week, the primitive heart tube bends on itself, becoming S-shaped, and begins to beat. It then joins blood vessels in other parts of the embryo, connecting stalk, chorion, and yolk sac to form a primitive cardiovascular system (Fig.12) (Buckingham et al., 2005).

At this development stage, both biochemical and biophysical cues lead to the formation of the early heart tube (Bartman & Hove, 2005). The looping, or the bending and twisting actions that occur to transform the linear heart tube into an asymmetrical heart loop, and trabeculation, which are the ridge-like incursions of endocardium implicated on enhancing contractility, together with valvulogenesis and septation, which are formed by the role of blood flow-induced biophysical forces, and even cardiomyocyte specification and fate, just to mention a few of the processes that take place, are examples of the great complexity that heart morphogenesis and development comprehends.

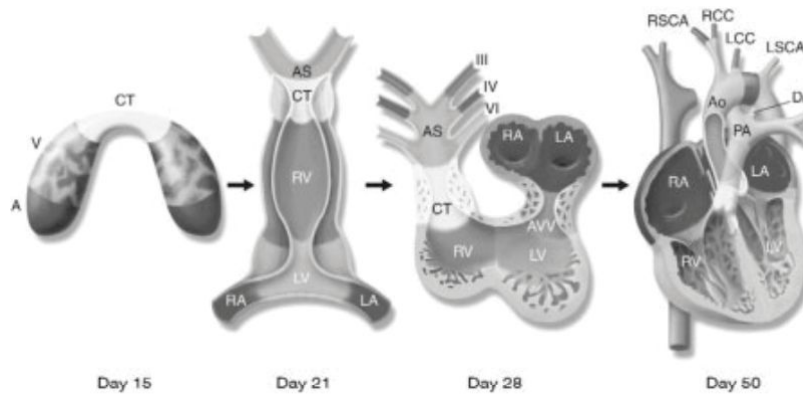


Fig. 12

Steps of heart morphogenesis. Illustrations depict cardiac development with conserved shading of morphologically related regions, seen from a ventral view. Cardiogenic precursors form a crescent (far-left panel) that is specified to form specific segments of the linear heart tube, which is patterned along the anteroposterior axis to form the various regions and chambers of the looped and mature heart. Each cardiac chamber balloons out from the outer curvature of the looped heart tube in a segmental manner. Neural crest cells populate the bilaterally symmetric aortic arch arteries (III, IV, and VI) and aortic sac (AS) that together contribute to specific segments of the mature aortic arch. Mesenchymal cells form the cardiac valves from the contruncal (CT) and atrioventricular valve (AVV) segments. Corresponding days of human embryonic development are indicated. A, atrium; V, ventricle; RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; PA, pulmonary artery; Ao, aorta; DA, ductus arteriosus; RSCA, right subclavian artery; LSCA, left subclavian artery; RCC, right common carotid; LCC, left common carotid. (Image taken from (Bartman & Hove, 2005).

1.2.2 Cardiomyocyte maturation: fetal VS adult

From the embryonic heart until the adult organ, the heart goes through several different growth stages where important changes in its physiology and morphology occur. Neonatal cardiac physiology differs considerably from the adult's in several ways. At birth, neonatal CMs are not fully developed and are not able to respond to volume loading with an increased cardiac output as adult CMs. This is due to the higher proportion of fibrous tissue. Moreover, due to the immature T-tubules and sarcoplasmic reticulum, neonatal physiology relies heavily on Ca^{2+} flux through the sarcolemma for myocyte function. On the other hand, neonates possess a limited ability to increase cardiac output by increasing stroke volume. Instead, neonatal cardiac output is significantly dependent on heart rate, indicating they have less preload reserve. Neonatal oxygen requirements are also much greater than the adult ones, which requires the neonatal heart to function at near maximal capacity (Smith et al., 2010). In addition, the mechanisms of calcium handling are altered in mature CMs. Extracellular calcium current through membrane channels accounts for most of the calcium transient in embryonic and neonatal CMs, whereas calcium release from internal sarcomeric stores accounts for the calcium transient of adult CMs (Husse & Wussling, 1996). The signals and mechanisms inducing maturation of CMs are poorly understood but are of major scientific interest (Baumgartner et al., 2015). There is a great difficulty and obvious limitation to study human cardiac tissue *in-vivo*, due to the impossibility to access samples. However, remarkable studies have been developed since the 1980's when the first cultures of adult and post-natal human CMs were established.

There is general agreement in how fetal, child and adult cardiac phenotype and physiology differ in each stage, but there is still no clear consensus in the field as to when a cardiomyocyte is considered mature. Despite there are few studies defining the differences in cardiomyocyte maturation at every stage of growth (embryonic, fetal, prenatal, neonatal, infant, teen and adult), relevant differences have been demonstrated in numerous studies by the scientific community in cardiac phenotype as

well as in protein organization and expression. Some of these characteristics can be observed in:

- *Cardiomyocyte morphology*

Adult CMs are known for having a rod-shaped body with striations (sarcomeres) along them (Fig.13). Adult CMs are larger than fetal or neonatal CMs and the network that they create is more compact and uniformly distributed in later stages of maturation than in fetal stages. Adult CMs have striations, as it has been previously seen, called Z-lines, distributed throughout the cytoplasm. They can be observed organized and parallel to one another in adult tissue. On the contrary, a poorly organized sarcomere arrangement is found in immature CMs at earlier stages of development (Pasqualini et al., 2015). Interestingly, when cultured *in-vitro*, CMs present a random configuration in the Petri dish and also exhibit a progressive loss of the rod-shape, that finally ends to change into a geometrical flat shape. Still, in spite they have lost the initial rod-shape morphology, adult CMs are larger than the post-natal ones in a 4 to 1 rate when cultured (Li et al., 1996). Adult CMs are bigger because they have achieved a hypertrophic state in response to growth stimuli, which includes growth hormones, various neuroendocrine factors, and importantly, an increase on the mechanical load. This mechanical loading is due to the hemodynamic conditions that increase wall tension of the myocardium lead to myocardial hypertrophy (Schlüter & Piper, 1999).

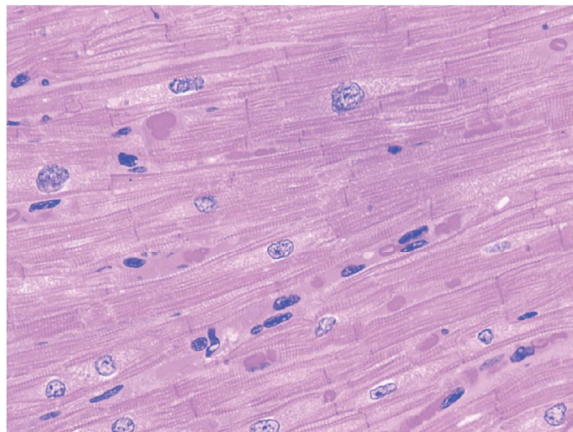


Fig. 13

Section of human ventricle stained with H&E where a network of CMs can be distinguished (in red) with cross-striations along them (http://medcell.med.yale.edu/histology/muscle_lab/cardiac_muscle_cells.php).

- *Cardiomyocyte proliferation*

CMs proliferate during prenatal development (Pasumarthi, 2002). However, soon after birth, CMs become binucleated (Paradis, Gay, & Zhang, 2014) and withdraw from the cell cycle, giving rise to the notion that adult CMs are incapable of proliferating, that is, they are terminally differentiated (Ahuja et al., 2007). When post-natal CMs were maintained in culture became larger and lost their capacity to proliferate, in contrast with fetal CMs that showed a high proliferation rate (Li et al., 1996). Interestingly, there are studies that demonstrate how differentiated CMs can be induced to proliferate and regenerate. This was found to be possible by the manipulation of an underlying molecular mechanism for controlling this process that involves the growth factor

neuregulin1 (NRG1) and its tyrosine kinase receptor, ErbB4 (Bersell et al., 2009).

- *Sarcomere number and organization*

Z-bands or Z-lines are more abundant, structured and organized in adult cardiac tissue than in fetal or post-natal tissue (Fig.14) (Lundy et al., 2013). This fact is directly correlated with cardiomyocyte's exerted force and size. Adult CMs show a high density of organized and aligned sarcomeres (visually as cross striations) (Bedada et al., 2015) and occupy almost the entire area of the cytoplasm. In contrast, fetal and neonatal CMs show lower densities of sarcomeres that only appear in certain parts of the cytoplasm (Fig.15). This is due to the degree of sarcomere organization inside the cell, which correlates with the force exerted. In most of the studies, sarcomere morphology and organization is analyzed by immunohistochemistry and electron microscopy (TEM) (Radisic et al., 2004) (Hirt et al., 2014) (Tandon et al., 2009).

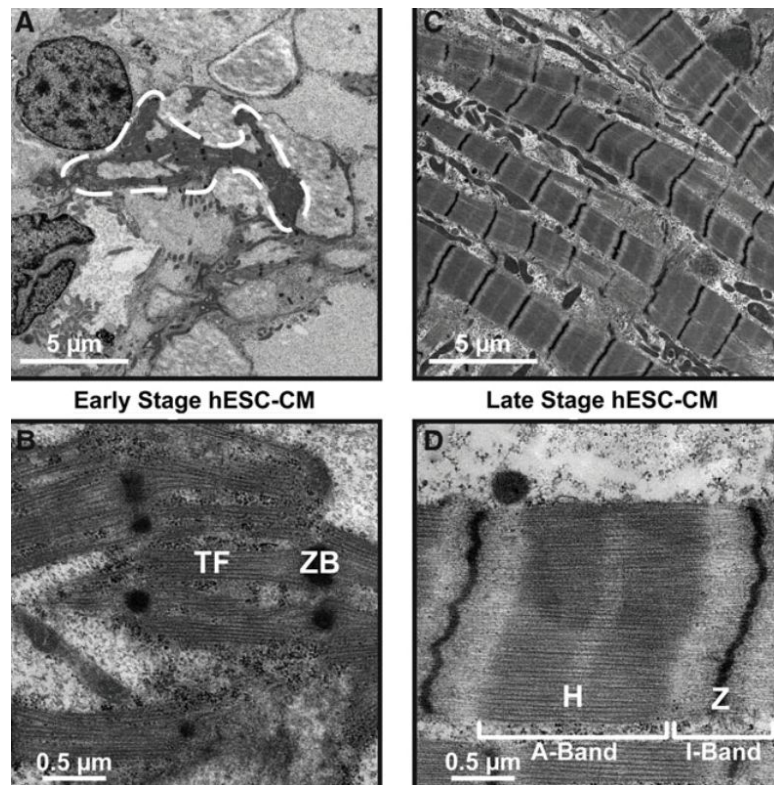


Fig. 14

Late-stage hESC-CMs demonstrate dramatically increased ultrastructural organization. (A) Transmission electron microscopy images of early-stage hESC-CMs at low magnification show fragmented myofibrils at low density (outlined by the white dashed line). (B) Higher-magnification images reveal fragmented Z-bands called Z-bodies (ZB) and irregularly spaced thick filaments (TF). (C) In contrast, late-stage hESC-CMs at a low magnification show a high density of organized and aligned myofibrils. (D) Sarcomeres under high magnification show clear aligned Z-bands (Z) and organized A- and I-bands with a clear H-zone (H). Image taken from (Lundy et al., 2013).

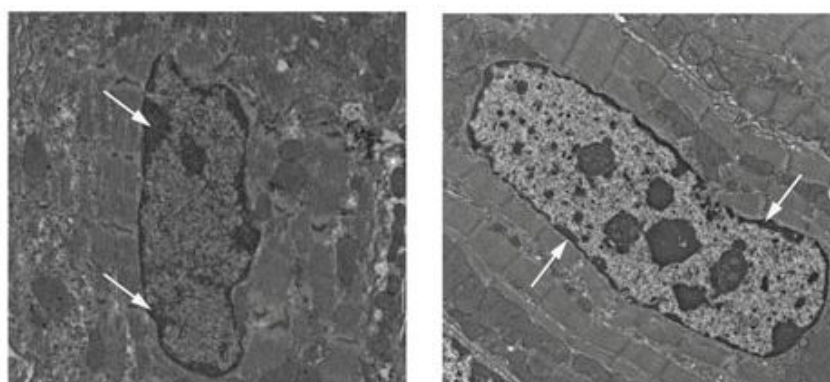


Fig. 15

Comparison between newborn (left) and adult (right) transgenic CMs. Left: poor ultrastructural level of sarcomere alignment and organization together with a low abundance in comparison with; Right: which shows a highly-organized and thickened sarcomeres, more abundant and well-defined Z-bands than in newborn. Image taken from (Furusawa et al., 2015), scale bar=2 μ m.

- *Cardiac protein expression*

Numerous proteins expressed in the neonatal heart are also expressed in the adult heart, whereas few specific cardiac markers are only expressed in a certain state. What it has been found to be significant is the difference in the relative expression between some of them (Lundy et al., 2013). It has been studied how some proteins vary relative protein expression isoforms from an early-like state of cardiac development to an adult-like state. Some of these proteins have been recently studied, such as:

Integrins

Are transmembrane proteins which function as connectors in cell-to-cell and cell-to-ECM interactions. For instance, $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins are expressed in fetal and neonatal CMs and mediate the attachment of these cells to fibronectin, laminin and type IV collagen proteins (Lam, Bartoli, & Claycomb, 2002). On the other hand, adult CMs, which express no detectable levels of $\alpha_1\beta_1$ and $\alpha_5\beta_1$ integrins, attach only to laminin and type IV collagen, and weakly to fibronectin.

Myosin proteins

Cardiac myosin consists of two cardiac myosin light chain-2 (MLC2) isoforms, MLC2a and MLC2v. In the developing human heart, MLC2a expression is detected in all chambers (atria and ventricles). In the postnatal heart, MLC2v is confined to the ventricle, and this chamber specificity that persists to adulthood. MLC2v expression is also considered to be a marker of maturity. (Bedada et al., 2015) (Reiser et al., 2001). There is also evidence with the finding of the switch in relative expression from the faster β -isoform in early stages of cell development to the slower α -isoform of myosin heavy chain (MHC), in later stages of cell maturity (Lundy et al., 2013). This finding can be explained by a shift down of the force-velocity relationship, as these late-state cells are more adherent and must contract against a greater perceived load than the comparatively unrestrained early-stage cells.

Connexin proteins

Connexins are a family of integral membrane proteins that oligomerize to form intercellular channels that are clustered at gap junctions. These channels are specialized sites of cell-to-cell contact that allow the passage of ions, intracellular metabolites and messenger molecules from the cytoplasm of one cell to its opposing neighbours. In the heart they permit the rapid cell-to-cell transfer of action potentials, ensuring

coordinated contraction of the CMs (Baumgartner et al., 2015) (Kostin et al., 1999). Ventricular CMs are extensively interconnected by clusters of Cx43-containing gap junctions, therefore the concentration and organization of these Cx43 clusters in the membrane of the cells is normally taken as a sign of cell maturity. These channels get fully functional and become more numerous in the later states of cardiac development, due to higher degree of the cell-to-cell interconnection (Kostin, 2004) (Camelliti et al., 2005). Moreover, the lateralization of the protein in both extremes of the CM has also been found to be significant in adult cells in comparison with neonatal CMs (Salameh et al., 2014).

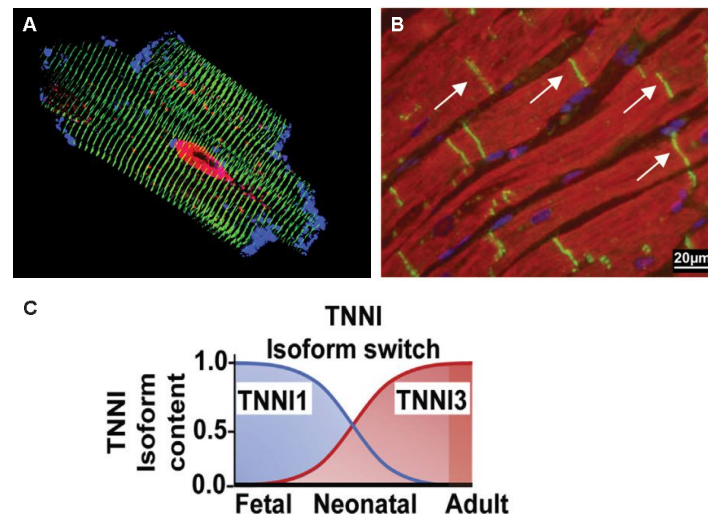


Fig. 16

Connexins are mainly located in the longitudinal ends of the CM besides other clusters throughout the cell body. (A) 3D confocal reconstruction of an adult mouse ventricular cardiomyocyte immunolabeled for connexin 43 (blue), α-actinin (green), and filamentous actin stained with phalloidin (red). Original magnification: X100. Image taken from (Smyth et al., 2012). (B) Representative specimen (~8 years old) immunostained for Cx43 (green) and troponin I (red), nuclei are counterstained in blue. The specific Cx43 staining is indicated by white arrows. Image taken from (Salameh et al., 2014). (C) Schematic of TNNI isoform switching time course during development of mammalian heart. Image taken from (Bedada et al., 2014).

Troponin proteins

Troponin T (cTnT) and troponin I (cTnI) cardiac proteins are cardiac regulatory proteins that control the calcium-mediated interaction between actin and myosin (Sharma et al., 2004). Both proteins are often used as cardiac markers of the Z-bands. These Z-bands, as it has been previously reported, appear to be more abundant and structured in adult cardiac tissue than in fetal. Moreover, in the rodent heart, the inactivation of the fetal troponin I gene (TNNI1), together with temporal exchange with its stoichiometric replacement by the adult TNNI gene product (TNNI3), has been considered as a quantifiable maturation parameter (Fig.16) (Bedada et al., 2014) (Epstein, 2010). Therefore, temporal changes in protein expression have been used to demonstrate cell maturation.

- *Mechanical properties of CMs*

Mechanical contraction is frequently evaluated to determine cardiac functionality and maturation of CMs. Adult myocardium typically exhibits a

positive force-frequency relation, primarily as a consequence of an increasing sarcoplasmic reticulum (SR) calcium load and subsequent release. Adult heart also exhibits a positive slope in its physiological contractile force-length Frank-Starling relationship (Tulloch et al., 2011). The beating rates of adult CMs are higher than neonatal or fetal CMs and their beating is more coupled and synchronized (Fig.17) (Kamakura et al., 2013). Twitch and resting tension are other parameters commonly used to measure force contraction (Zimmermann et al., 2002) (Engler et al., 2008) in CMs. For such measurements, most frequent techniques employed are: force transducers in the range of the mili- or micro-Newtons (mN or μ N) for small tissues and atomic force microscopy (AFM) for unicellular contractions and stiffness. However, indirect force measurements are also utilized by measuring displacements (Casares et al., 2015). These displacements are studied by image analysis techniques, normally with the help of fluorescent markers, in which spatial contraction of a tissue is measured and then force calculated considering the stiffness of the substrate (Jeon et al., 2011).

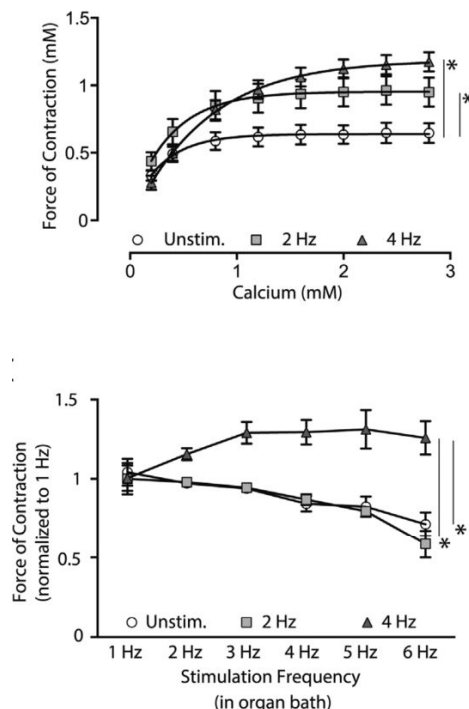


Fig. 17

Maturation of contractile parameters and force-frequency relationship by electro-mechanical stimulation. Top: Concentration response curve for extracellular calcium (0,2 - 2,8mmol/L) to assess the maximal inotropic capacity of the tissue. Bottom: Summary of isometric force of contraction data measured at increasing stimulation frequencies (1-6Hz). Image taken from (Godier-Furnémont et al., 2015).

- *Electrophysiological properties of CMs*

Electrical properties of the heart have been studied from fetal to neonatal and adult states. The phenotype and degree of maturation of CMs has been determined by electrophysiology, commonly using the patch clamp technique. This technique has been widely used as it is considered the gold standard to determine the functional properties and the level of maturation of excitable cells like CMs. Intracellular action potential (AP) parameters including action potential duration at (i.e.: 50 % or else) repolarization (APD50), maximal diastolic potential (MDP), beating rate, maximal upstroke velocity (V_{max}) are

studied in order to evaluate the level of maturation of CMs. Moreover, calcium (Ca^{++}) and other ionic currents (Na^+ , K^+ , etc.), conductivity, electrical coupling and other electrical parameters, are also measured to study CM maturity (Halbach et al., 2013) (Baumgartner et al., 2015) (Blazeski et al., 2012). These features can be measured by microelectrodes and microelectrode arrays (MEAs), which are devices that consist of multiple shanks or plates which can register (or emit) electrical signals. Cardiac optical mapping has also proven to be a powerful technology for studying electrical signaling, in which specific intracellular molecules are loaded with fluorescent labels, and then recorded and evaluated to obtain information about depolarization waves (Fig. 18) (Herron et al., 2012).

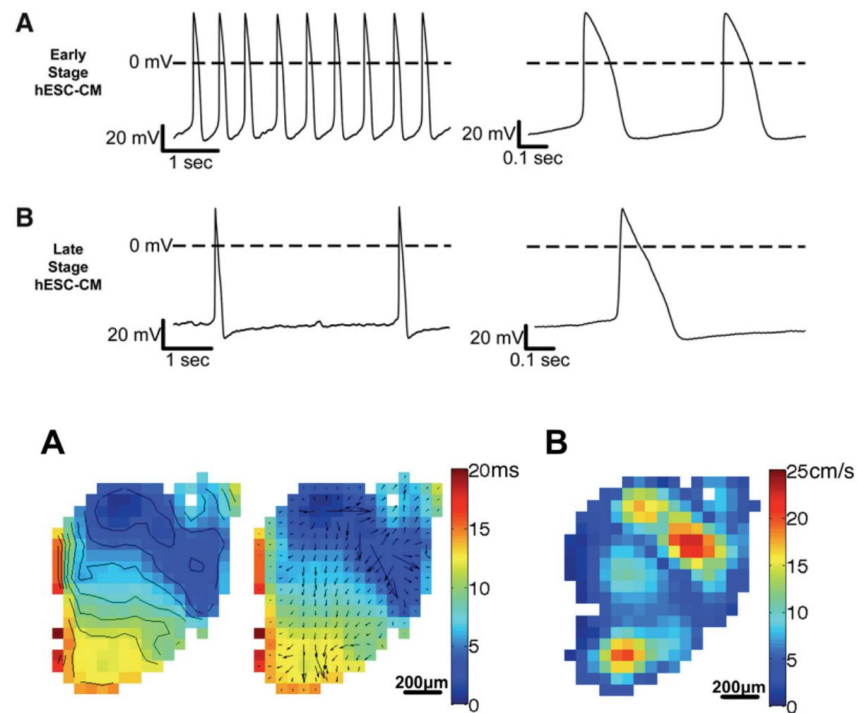


Fig. 18

Electrical recordings of action potentials (AP) in host mouse myocardium with microelectrodes. Top (A) Representative recording of early-stage hESC-CM action potentials exhibiting a relatively high spontaneous beating rate. Top (B) Compared to early-stage cells, late-stage hESC-CM exhibit a slower rate and higher maximum upstroke velocity (dV/dt). Image taken from (Lundy et al., 2013). Bottom: Optical mapping graphs of conduction properties of a beating cell cluster from a human embryonic stem cell-derived cardiomyocyte (hESC-CM). (A) Isochrones of 2 ms intervals and local conduction velocity vectors superimposed on activation map of the cluster subjected to 1 Hz electrical pacing. (B) Local conduction velocity magnitude map. Scale bars are 200 μm . Image taken from (Blazeski et al., 2012).

1.3 Epidemiology and myocardial infarction

Cardiovascular disease (CVD) constitutes the main cause of morbidity and mortality worldwide, with myocardial infarction (MI) being directly responsible of a majority of the cases (Fig. 19). In the European Union, 40 % of all deaths are caused by CVD, prevailing as well as the major cause of mortality (Table1) (Escardio.org).

THE 10 LEADING CAUSES OF DEATH IN THE WORLD BY PERCENTAGE

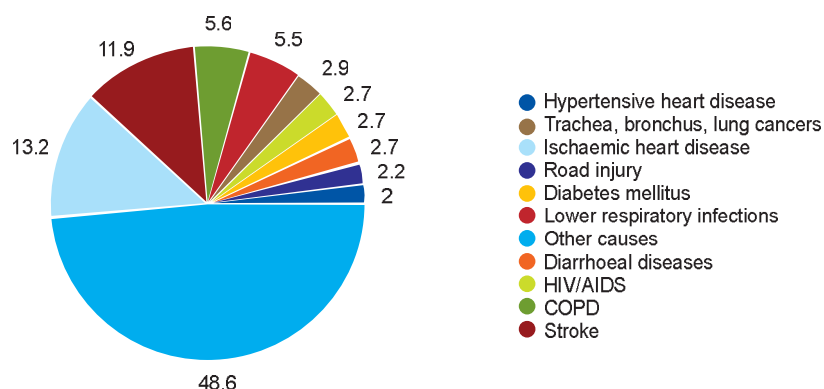


Fig. 19

The 10 leading causes of death in the world by percentage (<http://www.who.int/mediacentre/factsheets/fs310/en/#>).

Fortunately, the rate of mortality has decreased in most European countries, but CVDs will be the principal cause of death in the next decade. Smoking remains as the major public health issue together with alcohol consumption, hypertension, high cholesterol obesity, diabetes and physical inactivity are the major risk factors associated with ischemic heart disease (IHD) (Mozaffarian et al., 2016). In spite of these factors, disease is also associated with non-modifiable factors like aging, gender or familial history.

Table 1

Predominant causes of death according to European countries (http://ec.europa.eu/eurostat/statistics-explained/index.php/Causes_of_death_statistics).

	Total										Females		
	Circulatory disease	Heart disease (%)	Cancer (%)	Lung cancer (%)	Colorectal cancer	Respiratory diseases	Diseases of the nervous system	Transport accidents	Suicide	Breast cancer	Cancer of the cervix	Cancer of the uterus	
EU-28	393.6	136.8	266.9	35.4	31.9	82.9	37.5	6.3	11.9	33.3	4.1	6.5	
Belgium	308.6	82.4	260.0	60.9	29.0	111.2	51.9	7.6	18.7	39.0	3.1	6.0	
Bulgaria	1168.0	223.5	248.7	47.2	35.7	61.9	17.5	7.9	12.1	33.6	9.5	9.0	
Czech Republic	704.2	359.9	298.3	58.2	41.5	72.7	29.7	8.2	16.0	31.4	6.7	8.8	
Denmark	286.8	94.9	315.4	75.3	39.3	124.4	40.2	3.6	12.2	41.0	3.5	6.1	
Germany	404.1	148.0	253.3	50.9	29.7	69.4	27.9	4.9	11.5	36.1	3.4	5.0	
Estonia	745.4	363.0	291.9	53.9	33.8	37.1	22.0	6.7	18.3	34.5	9.7	6.1	
Ireland	351.2	173.7	287.4	60.1	33.6	134.1	48.7	3.8	12.2	39.9	4.8	6.4	
Greece	448.3	103.3	247.3	59.6	22.3	102.4	16.8	10.1	4.4	31.9	2.6	5.4	
Spain	271.0	76.8	242.7	49.8	35.3	105.4	48.3	4.5	7.4	25.0	2.7	6.0	
France (*)	223.0	55.7	252.8	50.1	28.2	54.8	52.8	6.3	16.9	33.2	2.4	7.3	
Croatia	691.1	317.3	336.5	66.2	50.4	57.5	22.4	10.3	18.2	43.2	4.5	10.2	
Italy	343.6	112.2	258.1	51.7	29.1	64.6	37.1	6.4	6.7	32.5	1.2	6.7	
Cyprus	402.2	115.9	205.7	39.9	20.9	89.6	36.3	7.5	3.8	30.6	2.0	6.3	
Latvia	920.7	480.8	305.3	49.0	40.7	36.7	15.1	10.2	21.9	32.4	10.8	12.6	
Lithuania	900.6	592.0	278.1	47.0	31.9	45.0	18.1	12.9	30.7	31.0	11.6	7.2	
Luxembourg	332.8	83.3	266.9	57.3	29.9	75.6	43.7	6.5	10.6	33.7	2.2	4.7	
Hungary	779.4	400.1	361.1	93.0	56.9	78.8	20.9	8.4	24.1	38.2	7.9	7.8	
Malta	519.4	300.0	254.9	49.4	34.8	95.6	17.4	3.1	6.4	38.0	3.2	8.9	
Netherlands	288.6	71.4	297.5	69.4	37.4	108.7	41.0	4.7	10.7	38.6	2.6	5.6	
Austria	450.2	197.9	255.5	46.1	27.3	51.8	34.7	7.1	15.2	32.8	3.3	6.4	
Poland	652.4	157.8	300.0	69.7	36.9	71.7	19.0	11.3	16.7	29.6	8.7	7.5	
Portugal	323.7	67.8	244.6	34.9	36.3	138.7	32.6	6.9	10.0	29.1	3.6	6.6	
Romania	1039.2	345.3	268.5	53.1	32.9	81.1	19.9	12.8	12.7	30.5	15.6	6.0	
Slovenia	462.4	118.5	305.5	57.2	40.9	86.2	18.8	8.0	21.5	35.4	3.8	8.1	
Slovakia	712.2	427.6	319.6	52.8	51.6	87.8	40.3	8.9	11.1	36.7	8.9	11.6	
Finland	411.9	225.1	223.7	40.8	23.1	39.8	136.6	5.4	16.1	29.4	1.8	6.2	
Sweden	371.4	149.9	239.4	39.0	29.1	66.0	43.1	3.4	12.4	28.7	2.5	6.5	
United Kingdom	284.6	130.5	296.3	63.1	29.2	141.6	42.7	2.8	7.2	36.7	2.9	6.6	
Liechtenstein	325.8	119.6	191.4	21.5	17.2	40.4	48.8	2.7	9.6	28.4	5.4		
Norway	311.7	116.2	259.5	53.1	39.0	103.4	41.8	4.0	10.6	27.2	2.85	6.5	
Switzerland	303.9	112.2	225.2	42.4	23.8	54.5	47.9	4.5	13.2	32.7	2.2	5.0	
Serbia	1028.2	177.2	301.5	68.5	39.4	82.7	28.0	9.3	17.3	42.9	11.8	8.0	
Turkey	340.4	104.9	175.5	53.0	15.3	89.1	36.2	6.7	2.1	12.1	1.7	3.5	

(*) Ischaemic heart diseases.

(*) Malignant neoplasms.

(*) Malignant neoplasms of trachea, bronchus and lung

(*) 2011.

Source: Eurostat (online data code: hlth_cd_asd2)

Myocardial infarction (MI) is induced when a coronary thrombosis occludes an artery, partially or totally, limiting the blood supply to the heart and thereby impeding the required oxygen and nutrients to reach the heart tissue. This thrombosis is usually caused by cholesterol plaques that grow and accumulate in the inner arteries walls, blocking the blood flux (Fig. 20). Over time, the damage becomes irreversible and it is accompanied by cell death and tissue necrosis. A compensatory mechanism of tissue remodeling is activated and a fibrotic scar that avoids rupture of the wall substitutes the damaged area. This process becomes a chronic maladaptive response that finally leads to contractile dysfunction, arrhythmias and eventually heart failure (Reddy et al., 2015).

Regarding specific diseases, atherosclerosis represents the principal cause of vascular occlusion (Otsuka et al., 2015). This phenomenon occurs due to chronic inflammation of the arterial vessels in response to an irritative stimulus. Arterial endothelial cells express adhesion molecules that capture leukocytes on their surfaces that lead to the accumulation of certain lipoproteins, that begin to partially occlude the vessel and ischemia of the tissue supplied by that vessel.

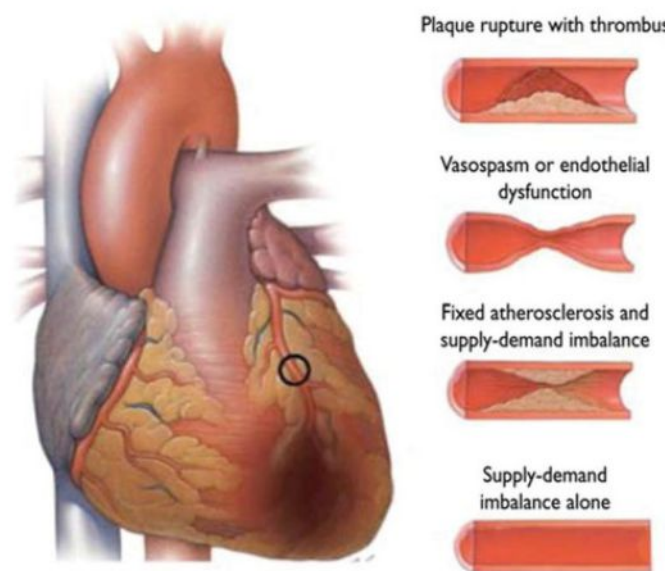


Fig. 20
Different causes of myocardial infarction (MI) according to the condition of the coronary arteries. Image taken from (Thygesen et al., 2012).

As the heart requires strict oxygen supply, an ischemic condition initiates a cascade of events within 10 seconds of occlusion that include: cessation of aerobic metabolism, anaerobic glycolysis onset and accumulation of anoxic metabolism. A minute after ischemia, the cardiomyocytes lose their contractile capacity and in few minutes, cell ultrastructural changes start. After less than an hour, the damage is irreversible with marked sarcolemmal damage. Within 4 to 6 hours, the necrosis of the cardiac muscle is already evidenced. The loss of myocardium results in the activation of remodeling mechanisms that modulate reparative changes, including hypertrophy and collagen scar formation. Ventricular remodeling may continue for weeks or months until the distending forces are counterbalanced by the tensile strength of the collagen fibrotic scar. These compensatory mechanisms, can be divided into 3 phases: 1) inflammatory phase, 2) granulation tissue formation, and 3) scar maturation phase (Matsui et al., 2010).

1. 3. 1 Stages in cardiac ischemia and extracellular matrix (ECM) remodeling

Myocardial ischemia, also called cardiac ischemia, is a pathology in which the blood flow of the heart is reduced, preventing it from receiving enough oxygen. This reduced blood flow is commonly the result of a partial or total blockage of the heart's arteries, called coronary arteries. Myocardial ischemia damages heart muscle, reducing as a consequence its ability to pump efficiently. When the blockage is severe and occurs suddenly, it can lead to a heart attack.

A series of events occur at various stages of cardiac ischemia with subsequent effects in the remodeling of the extracellular matrix (ECM):

1) Initiation or the inflammatory phase (0-7 days post infarction)

The hypoxic injury due to thrombotic occlusion of coronary artery creates coagulative necrosis in the cardiac tissue, characterized by hypereosinophilia and edema at day 1 followed by degradation of fibrillar collagen network between the cardiomyocytes (B. I. Jugdutt, 2003). The inflammatory cells; neutrophils, monocytes, macrophage cells migrate to the site of ischemia and mediate myocyte necrosis, which results in acute inflammatory response due to the secretion of matrix metalloproteinases (MMP) and cytokines. This outcome of excessive MMP and the degradation of the collagen network of the ECM lead to the myocyte slippage from the cardiac ECM, the inflammatory response has occurred.

After CMs death, their intracellular content is released, initiating an inflammatory response by activation of compensatory mechanisms of the innate immunity. The release of inflammatory cytokines induces activation of the MMPs and collagen deposition, which contribute to the structural changes and tissue repair of injured myocardium (Ertl & Frantz, 2005). The activated MMPs degrade the ECM, disrupting the fibrillar collagen network and allowing the inflammatory cells to migrate into the damaged tissue to remove the necrotic CMs. Infarct expansion occurs within hours of CMs injury and with time, results in wall thinning and abnormal ventricular dilation, causing the elevation of diastolic and systolic wall stress.

2) Progression or proliferative phase (1-3 weeks post infarction)

Early remodeling of the ventricle takes place in this phase. The edema and the acute inflammation are reduced with a significant decrease in MMP activity. A clearance of the necrotic cardiomyocytes takes place by the action of macrophages in the infarcted zone, and replaced by granulation tissue. The gradual transition from granulated tissue to scar (fibrotic) tissue is mediated by apoptotic cell death of vascular and fibroblast cells (Desmoulière et al., 1995). Activated myofibroblasts secrete modified ECM collagen that re-establishes the microvasculature in the scar tissue. During this phase, various revascularization mechanisms are activated to heal the damaged tissue. Cardiac endothelium and inflammatory cells activate the release of chemoattractant factors that are involved in the recruitment of vascular progenitors to the injured area (Jugdutt et al., 2002). Accumulation of fibroblasts and monocytes into the injured area promotes the formation of deposits of fibronectin allowing the creation of a second matrix and inducing the release of anti-inflammatory cytokines, like growth factor- β 1 and tissue inhibitors of MMPs (TIMPs).

3) Maturation or ECM remodeling phase (3 weeks to months after infarction)

In this phase the provisional matrix deposited during early remodeling phases is stabilized. Myofibroblasts play an important role in this stage inhibiting the degradation of the collagen of the ECM and also inducing deposition of collagen fibers that contribute to the scar formation and prevent ventricular dilatation (Sun et al., 2002). Moreover, circulatory hemodynamics are disturbed triggering the activation of the sympathetic adrenergic system, stimulating catecholamine synthesis that leads to the production of certain peptides, such as ANP and BNP. These peptides reduce intravascular volume, normalizing ventricular dilatation and finally improving pump function. During this phase a decreased collagenase activity and increased secretion of tissue inhibitor matrix metalloproteinase is observed, which in turn upregulates the secretion of collagen type I and III by myofibroblasts. The crosslinking ratio between the two collagens prevents the infarct scar from further dilation and gives considerable tensile strength to the ischemic tissue (Perea-Gil et al., 2015). There is a zone between the ischemic zone (IZ) and the non-ischemic zone (NIZ) formed by the disarrangement between the CMs in the IZ and the hypertrophic CMs in the NIZ. Generally two patterns of myocardial fibrosis take place in ischemic and non-ischemic heart. Reparative fibrosis repairs the myocardial damage due to necrotic and apoptotic death of myocytes, which later converts to scar tissue. Reactive fibrosis by hypertrophied cardiomyocytes occurs in response to inflammation with no cell loss in the NIZ, characterized by perivascular fibrosis, remote from ischemic scar (Bodh I Jugdutt et al., 2002). Late structural remodeling in this phase involves expansion of IZ with regional stretching and thinning, which accounts for the left ventricle dilation mediated cardiac dysfunction.

1. 3. 2 Cardiac function evaluation in myocardial infarction

Early expansion of the infarcted zone is associated with left ventricle (LV) dilation, which is caused by the increase of regional wall stress. Between one half to one third of the patients experience progressive post-infarction heart dilatation with distortion of ventricular geometry and secondary mitral regurgitation (Lang et al., 2005).

Quantitative bi-dimensional transthoracic echocardiography is routinely performed to characterize the heart remodeling associated with these cardiomyopathies, LV end-diastolic and end-systolic volumes and LV mass being estimated to predict adverse cardiovascular follow-up events, including recurrent infarction, ventricle arrhythmias, mitral regurgitation and heart failure.

For the evaluation of the heart function, echocardiography or magnetic resonance imaging (MRI) are the common techniques to study left ventricle (LV) function. This function can be measured through functional heart parameters such as volume in diastole (VD), volume in systole (VS), ejection fraction percentage (EF%), and fractional shortening (FS%); where fractional shortening corresponds to the fraction of any diastolic dimension that is lost in systole, and ejection fraction represents the volumetric fraction of blood pumped out of the ventricle. The damage provoked to the myocardium after MI, compromises the heart's ability to perform as an efficient pump and therefore, reduced values of EF% and FS% are observed which indicate a clear worsening of cardiac function (Borbely, 2005) (Mauritz et al., 2008) (Xin, Olson, & Bassel-Duby, 2013). On the other hand, the LV function can also be measured as the percentage of change in the left ventricular cross-sectional area between diastole and systole (fractional area change, FAC%), which has been found to correlate well with the EF% both in normal situations and when disease is present (Domanski et al., 2016).

Porcine and rodent MI models are commonly used to provide new insights into the physiology and treatment of cardiovascular diseases, and also study new therapy approaches. In these models, infarction is triggered by the temporary or permanent occlusion of the left anterior descending (LAD) coronary artery that leads to a necrosis of the affected tissue. (Soler-Botija et al., 2014) (Eschenhagen et al., 2002) (Wu et al., 2011) (Carpenter et al., 2012).

2. Cardiac tissue engineering and stem cells

2. CARDIAC TISSUE ENGINEERING AND STEM CELLS

Tissue engineering was born as an alternative or complementary technique whereby tissue and organ failure is addressed by implanting natural, synthetic (or semisynthetic) tissue or organ mimics that are functional, or that they grow into the required functionality (Lanza et al., 2000). Tissue engineering evolved from the fields of cell biology and biomaterials development, and refers to the study and the practice of combining scaffolds, cells, and biologically active molecules into functional tissues (Fig.21). The goal is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs (Meyer et al, 2009). Regenerative medicine is a broad field that incorporates tissue engineering but also incorporates research on self-healing, where the body uses its own systems, sometimes with help foreign biological material to recreate cells and rebuild tissues and organs. Both terms “tissue engineering” and “regenerative medicine” have become largely interchangeable, as the field hopes to focus on cures instead of treatments for complex, lot of them chronic, diseases. (National Institutes of Health, USA, <http://www.nibib.nih.gov/>).

Tissue engineering was initially referred as it at the beginning of the decade of the 90s, in which Dr. Robert Langer (Langer & Vacanti, 1993) published a relevant article about the birth of the new science, where he defined it as: “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.”

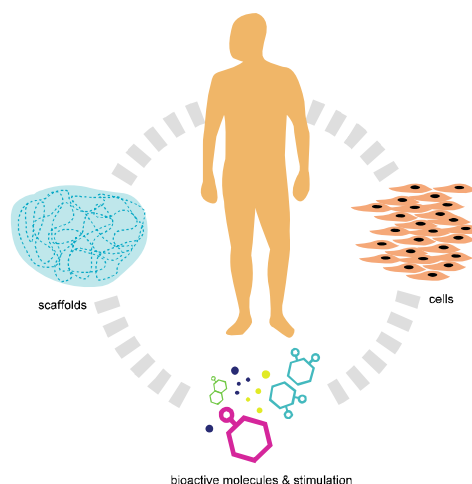


Fig. 21

Representative scheme of the fundamental elements of tissue engineering, where the combined use of cells, bioactive molecules, and biocompatible scaffolds are grafted to improve, repair or substitute a tissue or an organ of the patient.

Cardiac tissue engineering is the science that studies the creation of a living, beating, ideally autologous, and non-immunogenic myocardial patch or graft that can restore the contractile function of the failing heart (Radisic & D. Black III, 2014), besides other applications such as to study preclinical drug testing to discover new targets for cardiac therapy and eliminate drugs, for instance. It generally involves the same component triad previously explained for tissue engineering, which is based on the combination of suitable cell types, mainly cardiomyocytes in cardiac tissue engineering, and supporting cells, with an appropriate biomaterial made out of either synthetic or natural components and cultivation in an environment that reproduces some of the complexity of the native cardiac environment, as electrical pulses, suitable mechanical stimulation, or micro-topographical cues among many others.

In late 1990s, when the first publications appeared, neonatal rat cardiomyocytes (CMs) (Eschenhagen et al., 1997) or embryonic chick CMs were utilized as a cell source. Since then, the field has evolved significantly to include a range of approaches that have generated *in-vitro* cardiac tissues capable of developing contractile forces and propagating electrical impulses. Advances in human embryonic stem cell research and induced pluripotent stem cell technology now provide the possibility of generating millions of bona fide human cardiomyocytes (Zhu, Hauch, Xu, & Laflamme, 2009) (Zimmermann & Eschenhagen, 2007), as large numbers of CMs are needed to generate a functional biomimetic tissue (Engler et al., 2008), and no-optimal cell source is available for such purpose.

2.1 Components of a cardiac construct

The major challenge in cardiac tissue engineering is the generation of a functional in vitro-created cardiac tissue, suitable to regenerate or substitute the injured myocardium after infarction. This cardiac patch or graft engineering, is focused on the development of an optimal combination of the three main components of tissue engineering, as previously stated.

2.1.1 Cellular components

The approach consists in the injection or implantation of cells alone in the damaged site. The procedure avoids the complications of surgery, and allows replacement of only those cells that supply the needed function, and permits manipulation of cells before infusion. Its main potential limitations include failure of the infused cells to maintain their function in the recipient, and immunological rejection. Failure of the infused cells is associated with a highly compromised cell viability upon transplantation, as the majority of injected cells die due to a lack of an external matrix that give mechanical and adequate biochemical support to the injected population (Shimizu et al., 2002).

As previously seen, native myocardium is composed of different cell types. *CMs* are the active cellular component, occupying 70-75 % of the volume (Lakshmanan, Krishnan, & Sethuraman, 2012), but only 25-30 % in number. These cells have a limited proliferative capacity once they reach their adult state, as previously seen, fact that limits their use as an appropriate cell source for cardiac tissue engineering. *Cardiac fibroblasts* are the next vital cellular component in the cardiac tissue. Though CMs are functionally essential, cardiac fibroblasts maintain the proper functioning of the heart through cell-to-cell and cell-to-extracellular matrix interactions. Intercellular electrical communications between CMs and cardiac fibroblasts is mediated by gap junctions (connexin-43 and connexin-40 respectively), which we have seen are essential for the uniform propagation of the action potential and electrical synchronization by cell-to-cell coupling of the whole heart. *Cardiac endothelial cells and vascular smooth muscle cells (VSMCs)* are the last cellular components. A constant supply of oxygen and nutrients to myocardial cells would be impossible without the blood vessels that are lined with endothelial cells that are distributed throughout cardiac tissue along with VSMCs.

Considering regeneration, and taking into account that cardiomyocytes do not readily proliferate, a number of candidate cell types have been considered for such therapies (Table 4), including skeletal myoblasts, bone marrow-derived hematopoietic stem cells, mesenchymal stem cells (MSC) (Piryaei et al., 2015) (Li et al., 2012), intrinsic cardiac stem cells, and embryonic stem cells (ESCs) (Zhu et al., 2009) (Zimmermann & Eschenhagen, 2007) (Liang & Zhang, 2013) and more recently, human induced pluripotent stem cells (hiPSC). To date, no ideal adult autologous cell type has been identified for complete cardiac regeneration. This has directed the development of diverse strategies to aid in the repair of injured myocardium, including increasing angiogenesis (Huang et al., 2006), limiting cardiomyocyte loss in stunned, hibernating, or scarred myocardium, and modulating the extracellular matrix to limit left ventricular (LV) dilatation and heart failure.

Regarding stem cells as an ideal cell source in cardiac tissue engineering due to their differentiating potential and scalable use, considerable debate has focused on the contrasting merits of embryonic stem cells (ESC) and adult stem cells. Fetal stem cells represent an intermediate cell type in this controversy (Meyer et al., 2009). Adult stem cells have limited capacity to differentiate into fully functioning mature cell types of their tissue of origin (multipotent), whereas ESC have the advantageous capacity to develop into all tissue types (pluripotent). However, ESC research has been hampered by both safety concerns and ethical reservations due to requisite destruction of the blastocyst during harvesting. Transplantation of ESC is frequently followed by the development of embryonic teratomas, precluding cell transplantation. On the other hand, adult stem cells have the advantage of greater accessibility, but the disadvantage of more limited proliferative capacity and restricted plasticity. In contrast induced pluripotent stem cells (iPSC) represent an alternative to these cell sources due to their plasticity and relatively easy and massive production but it is still in an early experimental stage considering *in-vivo* approaches (Rane & Christman, 2011) (Yamakawa & Ieda, 2015).

Table 4

Different cell types used in cardiac tissue engineering in transplantation studies. Table from (Passier, van Laake, & Mummery, 2008).

Cell type*	Number (×10 ⁶)	Recipient species	Time after infarction	Measurement of function	Result	Longest follow-up	Control	Reference
BMCs	0.03–0.5	Mouse	3–5 hours	Pressure–volume loop	↓ LVEDP, ↑ LVSP, ↑ dP/dt	1–2 weeks	No injection or Kit ⁺ BMCs (control groups pooled)	9
	1	Macaque	Directly	Echocardiography	↑ FS	2 weeks	Saline	24
Mesenchymal stem cells	2	Rat	7 days	Echocardiography	↑ FS, ↓ EDV	4 weeks	Saline	27
	50	Pig	Directly	MRI, nuclear imaging	↑ EF, ↓ ESV	4 weeks	No injection	26
Multipotent adult progenitor cells	0.5	Mouse	Directly	Echocardiography	↑ EF, ↑ FS	8 weeks	BMCs, fibroblasts or medium	31
	50	Pig	Directly	MRI, nuclear imaging	↑ EF, ↑ bioenergetics	4 weeks	Saline	77
EPDCs (human)	0.4	Mouse	Directly	MRI	↑ EF, ↓ EDV	6 weeks	Medium	33
Skeletal myoblasts (50% pure)	10	Rat	7 days	Treadmill, ex vivo pressure–volume loop	↓ EDV, ↑ LVSP, ↑ exercise tolerance	6 weeks	Saline	78
Skeletal myoblasts	150–750	Sheep	14 days	Echocardiography	↑ EF, ↓ EDV	16 weeks	Medium	79
ES cells committed to cardiac lineage (mouse)	50	Sheep	14 days	Echocardiography	↑ EF (relative change over time)	4 weeks	Medium	80
ES-cell-derived cardiomyocytes	0.03–0.1	Mouse	Directly	Pressure–volume loop	↑ EF, ↓ EDV	3–4 weeks	BMCs, fibroblasts or medium	81
ES-cell-derived cardiomyocytes (human, 25% pure)	1	Mouse	Directly	MRI	↑ EF (4 weeks), ↔ EF (12 weeks)	12 weeks	ES-cell-derived non-cardiomyocytes (human)	22
ES-cell-derived cardiomyocytes (human, 71–95% pure)	10	Rat	4 days	Echocardiography, MRI	↑ FS, ↓ EDV, ↑ wall thickening	4 weeks	ES-cell-derived non-cardiomyocytes (human) or saline	54
ES-cell-derived cardiomyocytes (human, <10% pure)	1.5	Rat	7–10 days	Echocardiography	↑ FS, ↓ EDV	4 weeks	ES-cell-derived non-cardiomyocytes (human) or saline	82

BMCs, bone-marrow cells; dP/dt, change in systolic pressure over time; EDV, end diastolic volume; EF, ejection fraction; ESV, end systolic volume; EPDCs, epicardium-derived stem cells; FS, fractional shortening (shortening of the ventricular diameter during systole; it indicates the degree of contraction); LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; medium, cell-culture medium; MRI, magnetic resonance imaging; nuclear imaging modality; ³¹P magnetic resonance spectroscopy; wall thickening, thickening of the ventricular wall. * Unless otherwise specified, transplanted cells are from the same species. If the purity is not listed, then it is almost pure.

2.1.1.1 Human induced pluripotent stem cells (hiPSC) in cardiac tissue engineering

Pluripotent stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth (Fig.29). All stem cells, regardless of their source, have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types, that is they can be induced to become tissue- or organ-specific cells with special functions (<http://stemcells.nih.gov>, National Institutes of Health). Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells (forbidden by law to experiment with for certain purposes in many countries) and non-embryonic somatic (or adult) stem cells. Despite cell differentiation process was believed to be non-reversible, in 2006 the laboratory of Nobel Prize Dr. Yamanaka reported the generation of embryonic stem-like cells from somatic cells, calling them for the first time "induced pluripotent stem cells" (iPSC) (Takahashi & Yamanaka, 2006). It was the first time that some specialized adult cells were literally "reprogrammed" genetically to assume a stem cell-like state.

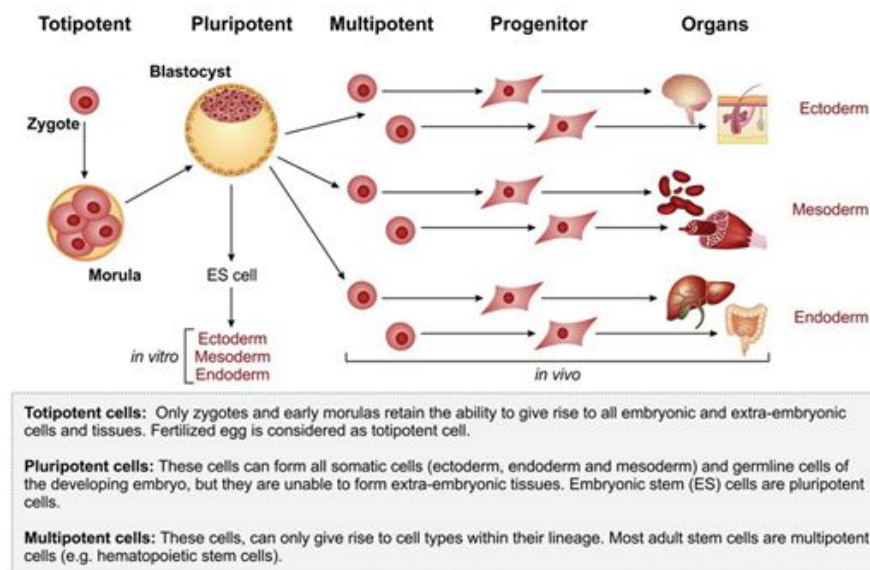


Fig. 29

Stem cells, which can give rise to any sort of tissue, can be classified into toti-, pluri-, and multipotent stem cells (and progenitor cells) before they reach a fully differentiated state (Molecular therapies, Balajthy et al., 2011. <http://www.tankonyvtar.hu>)

Cell de-differentiation was initially induced by retroviral transduction of several factors involved in pluripotency and self-renewal of embryonic stem cells (ESC). Initially, a total of 24 genes were selected and overexpressed in different combinations, being finally restricted to only four of them, Oct3/4, Klf4, Sox2 and c-Myc (OSKM) as those necessary for reprogramming the mouse adult fibroblast into iPS cells. One year later, human iPS generation was also reported by using the same combination of factors (Kwon et al., 2007) (Yamanaka et al., 2007) (Yamanaka et al., 2007) or a different one (Oct3/4, Sox2, Nanog and Lin-28) (Willems et al., 2012). The generated iPS cells

presented similar morphological and genetic properties than ESC, including the capacity to differentiate *in vitro* and *in vivo* towards cell types derived from any of the three germ layers. Despite the similarity of iPS cells to ESC, differences in gene expression signatures, DNA methylation patterns and efficiency degree in differentiating to specific lineages have been also evidenced (Takeuchi & Bruneau, 2009). Remarkably, the protocol developed for iPS derivation has been proved to be quite reproducible (with minor modifications) iPS have been already generated from many other species apart from human, such as mouse, primate (Liu et al., 2008), pig (Esteban et al., 2009), rat (Liao et al., 2009), sheep (Bao et al., 2011), between others. Besides, many cell types have been assayed with success for iPS cells, including pancreatic β cells (Pellegrini et al., 2015), neural stem cells (NSC) (Eminli, Utikal, Arnold, Jaenisch, & Hochedlinger, 2008), mature B lymphocytes, keratinocytes (Streckfuss-Bomeke et al., 2013), hepatocytes, hematopoietic stem cells, adipose stem cells, cord-blood derived cells, and several types more.

In general terms, this consistent method for deriving pluripotent cells from almost every tissue has demonstrated the striking plasticity of the cells, independently of their origin and differentiation stage, and in addition, it has also offered a new pluripotent stem cell source that could be autologously applied, bypassing the immune response and the ethical issues derived from the use of human embryos.

2.1.1.2 Human iPSC-derived cardiomyocytes

The generation of cardiac cells represents a key issue for therapeutic applications in cardiovascular diseases. Despite proven differentiation capacity of many stem cell populations towards different lineages, a robust differentiation towards functional cardiac cells has not been shown for many of them. The discovery of iPS cells as a new source for cardiac cells has opened new perspectives for the establishment of future autologous myocardial cell-replacement therapies. However, as it could be expected, due to their embryonic characteristics, cells also exert a tumorigenic potential (Nelson et al., 2009). In order to bypass this serious limitation and also, to enrich the cardiac population, cardiac cells have been *in-vitro* derived from hiPSC to be then injected into the heart (Carpenter et al., 2012) (Iglesias-García et al., 2015). Abundant retention of the transplanted cells within the infarcted heart and an induction of positive remodeling after ischemic damage has been shown. Importantly, no tumor formation has been reported yet, although carefully driven long-term experiments are still required to confirm this. In spite of the fact that cardiac cells can be successfully derived and selected from iPS cells, an optimization of the existing protocols is still needed. Rendered differentiated CMs present poor levels of maturation (Xi et al., 2010) with high interline variability (Kaichi et al., 2010).

Numerous protocols, most of them based on previous ESC differentiation methods, have been followed to guide the *in-vitro* differentiation of mouse iPS (miPS) and human iPS (hiPS) cells towards cardiomyocytes. Moreover, their *in-vivo* differentiation potential has been confirmed in various animal models. To give an example, undifferentiated iPS cells differentiated towards cardiac and vascular cells after transplantation into an ischemic rodent heart (Nelson et al., 2009) (Carpenter et al., 2012). However, despite the proven pluripotency of the iPS cells, differentiation towards specific cell types can be affected by the origin and epigenetic characteristics (Liang & Zhang, 2013) (Kim et al., 2010) of the

derived iPS, which can be more prone to differentiate towards specific cell types.

Many protocols have been tested with ESC to specifically induce cardiac differentiation (reviewed in (Burridge et al., 2012)), that have been also later applied for iPS specification. Sequential combination of different growth factors (FGF2, bFGF, VEGF, etc.) and cytokines (Activin A, BMP2, BMP4, etc.) have shown positive results for cardiac differentiation (Burridge et al., 2011). Particularly, manipulation of Wnt/ β -catenin signaling pathway, which is necessary for mesoderm formation and heart development can induce cardiac specification (Gessert & Kühl, 2010). One of the most effective protocols published also mediated through the activation of the Wnt/ β -catenin signaling by treatment with the GSK3 inhibitors such as CHIR99021, has been reported to promote robust cardiac differentiation of both hiPSC and hESC (Lian et al., 2012). Other groups have induced cardiac differentiation of hiPSC and hESC by treatment with the Wnt signaling inhibitors IWR1 or IWP4 (Willems et al., 2011) (Ren et al., 2011). As research in the stem cell field continues to move forward, new discoveries are made, and new combinations and molecules are being explored for the generation and induction of human cardiomyocytes from human pluripotent stem cells, which currently appears the most promising cell source for regenerative medicine therapies.

2.1.2 Biomaterials and strategies for cardiac scaffolds

In the case of scaffolds alone, the procedure would consist on the grafting or patching (depending on the therapy) of an acellular scaffold in the damaged site. The scaffold itself acts as a temporal extracellular matrix (ECM) that gives mechanical support to the damaged tissue, in order to allow cell migration and proliferation through itself. The nature of the transplanted biomaterial has a critical influence on the healing capacity, due to biomaterial's inherent properties, besides its physical state (solid, hydrogel) and topography (micro- or nano-scaled). As a temporal substitutive of the ECM, biomaterials utilized to fabricate it should mimic the ones found in the ECM besides its mechanical properties (Fig. 30).

ECM COMPLEX

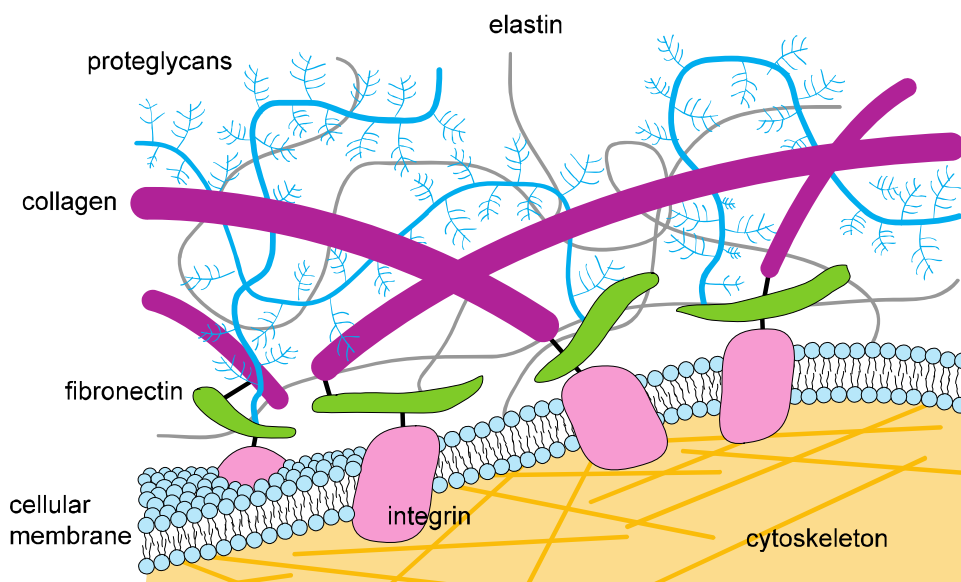


Fig. 30
Representation of the ECM protein complex. Many of the proteins found, such as collagen, elastin, and fibronectin are commonly used to fabricate scaffolds for tissue engineering in order to mimic cell's natural environment.

The criteria for selecting an optimal material to heal a myocardial infarction may vary considerably depending on the requirements selected, such as mechanical performance, electrical conductance, biocompatibility, and degradation time, among many others. In any case, the concept remains the same: to design a provisional housing for the cells that helps to recover a myocardial damage. The idea is not to develop a permanent prosthesis but a temporal, bioactive shelter for the cells that should be degraded within time as is replaced by the new tissue.

Both, synthetic and naturally-derived scaffolds have been developed for cardiac tissue engineering therapies, in which implantation strategy may condition the sort of biomaterial chosen. Synthetic scaffolds are generally made of polymers (Table 6), whereas naturally derived scaffolds often consist of ECM components including collagen (Eschenhagen & Zimmermann, 2005), fibrin

(Noailly et al., 2008), elastin (Planell et al., 2013), chitosan, etc. or from synthetic biomaterials (polymers), such as polylactic acid (PLA) (Lee et al., 2006), polyethylene glycol (PEG), poly (N-isopropylacrylamide) (pNIPAM), among many others (Radhakrishnan et al., 2014). In all of the cases, immunological rejection may be prevented by immunosuppressive drugs or by using autologous cells. In addition, scaffolds can be prepared by various techniques to display multiple gradients of immobilized chemical signals. The type of scaffold should be selected based on its ultimate application (for instance, self-renewal, differentiation or transplantation), its capacity to replicate the chemical, mechanical, 3D architectural or degradative environment of a specific extracellular matrix, to the extent that this is known, and its availability. Natural and synthetic biomaterials (Table 6) can be obtained commercially and easily processed to prepare 3D scaffolds, with an adequate geometry, such as porous, mesh, fibrous, gel, film, etc. Whereas natural biomaterials have the advantage of providing biological signals to the cells cultured on them, they have the disadvantages of batch-to-batch variability and limited ability for controlled modification (Kraehenbuehl et al., 2011). The tunable geometries of polymers (foams, gels, fibrous, etc.) offer different strategies for the treatment of myocardial infarction (MI), which can be classified as A) Injectable approaches, B) cardiac patches, and C) LV restraints.

A) *Injectable approaches*

Over the last 5 years there has been an extensive growth in the field of injectable biomaterials for treating MI (Table 6). Although injectable scaffolds do not allow for control over the cell and biomaterial organization afforded by *in-vitro* engineered and patch-based approaches, research in this area has rapidly expanded. These materials have been injected alone or used as delivery vehicles for cells and biological moieties and have shown positive results such as improvement in cardiac function, reduction of infarct size, and increase in neovascularization, as well as improve cellular retention and viability (Singelyn & Christman, 2010) (Li et al., 2009). One of the main benefits of injectable materials is the potential to be delivered via catheter without the need for a surgical-based procedure and general anesthesia. Injectability also allows the material to be delivered directly inside the infarct wall to the site of damage. Materials are typically hydrogels, which are water-swollen, cross-linked polymer networks derived from either natural or synthetic sources.

Hydrogels are liquid outside the body and then are able to gel into a semisolid form on injection *in vivo* (Fig. 31). Depending on how the hydrogel is cross-linked, for example with covalent bonds, ionic interactions, or physical entanglements, and also by its chemical makeup, hydrogels can respond to different environmental triggers, such as temperature, ion contents or pH (Wu et al., 2009) (McGann et al., 2013) (Shachaf et al., 2010) (Sun et al., 2012). Hydrogels have advanced considerably in the last decade, natural hydrogels like collagen, hyaluronic acid (HA), alginate, keratin, Matrigel®, chitosan are known to be extensively used in the field, whereas synthetic hydrogels can offer totally new characteristics to perform as injectable heart grafts, as their properties can be tuned in the laboratory to match desired characteristics. These biomaterials maintain their sol-to-gel transition besides other new properties. Elastomeric hydrogels (like resilin-like

polypeptides RLP, and its combinations with poly-glycolic acid, PLG-RLP), conductive hydrogels (polypyrrole incorporated to polyethylene glycol PEG, gelatin-based hydrogels with carbon nanotubes (Samchenko et al, 2011)), and oxygen-releasing hydrogels, particularly useful to bypass oxygen deficiency due to the lack of vasculature within the injected gel, are remarkable examples of the latest advances in hydrogel engineering (Li & Guan, 2011).

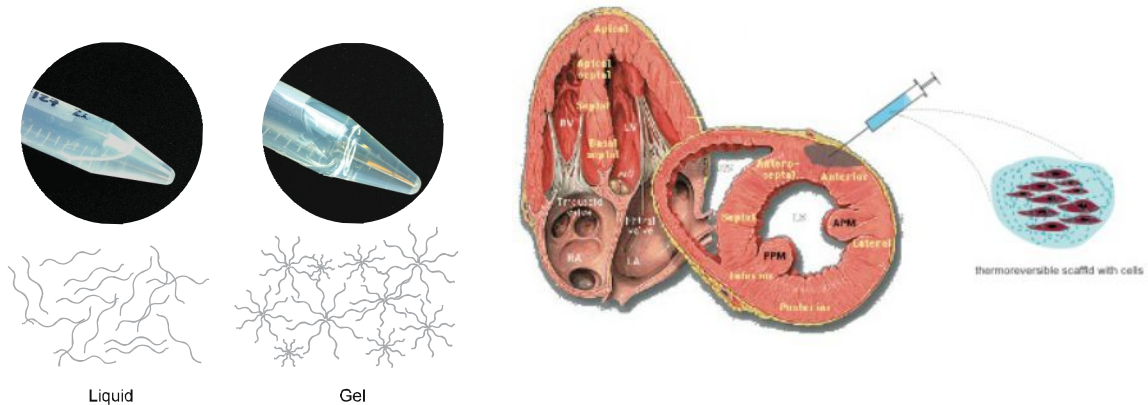


Fig. 31

A) Example of a thermoreversible hydrogel is Pluronic F127, which present a relative low temperature sol-to-gel transition depending on polymer concentration. The mechanism of gelification is due to an aggregation to micelles of the hydrophilic poly-ethylene oxide (PEO) blocks (Bercea et al., 2011). B) Representative scheme of a direct injection in the infarcted site of a thermoreversible hydrogel loaded with cells.

Regarding cardiac regeneration, numerous injectable materials have been found to preserve or improve various measures of cardiac function in animal models, including alginate, collagen, decellularized tissues, hyaluronic acid (HA), Matrigel®, chitosan, alginate, keratin, and synthetic materials, such as self-assembling peptides and polymers. Numerous *in-vivo* studies in rodent and pig models have proved the benefits of this approach (Wu et al., 2011) (Landa et al., 2008) (Radisic & Christman, 2013), more studies are needed to confirm results and better understand the exact mechanisms of how cardiac function is improved with injection, issue still under debate. Overall, considerable progress has been made during the past decade and a half with various approaches to cardiac tissue engineering. It is likely that biomaterial-only based approaches will reach the clinic faster; however, with continued advances in stem cell biology, achieving a functional contractile patch or graft may be possible in a short time.

B) Cardiac patches

Cardiac patches consist on cellular or acellular constructs sewed or glued to the heart wall to help the wall remodeling and cell integration or migration through the infarcted site (Fig. 32B). Nonetheless, a major drawback with this method remains the inability to generate patches with sizable thickness due to diffusion limitations of oxygen and nutrients (Radisic et al., 2006) (Radisic & Christman, 2013). Functionality should be assessed by the ability of the patch to generate active force during contraction and propagate electrical impulses. The ideal cardiac patch

may consist of autologous cardiomyocytes that allow generation of a minimal immune response on implantation. Synthetic and natural biomaterials either in porous or fibrous form or as hydrogels have been used in scaffolds. Microfabrication approaches, soft lithography, and patterning of synthetic materials are being specifically advanced in conjunction with other techniques to develop *in-vitro* tissues that better adapt to the native niche. Last-generation scaffolds permit to tune mechanical properties as well as shape and structure anisotropy of the material itself, managing to control cell orientation and partial performance inside the scaffold (Table 6).

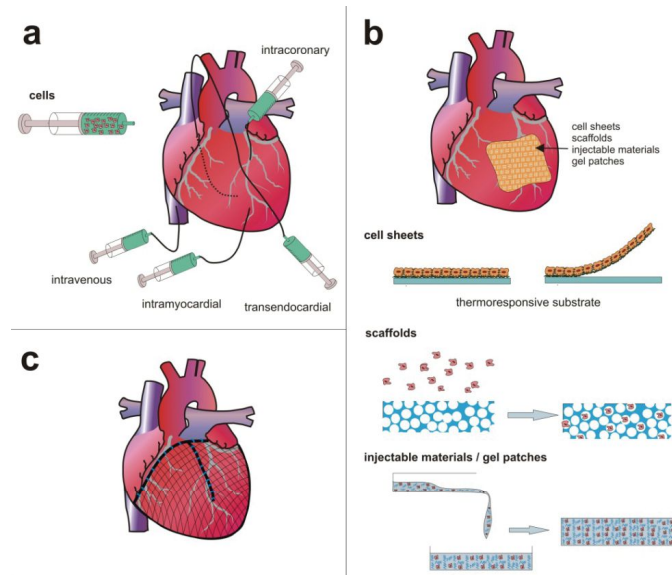


Fig. 32

The 3 major strategies in cardiac tissue engineering. (a) Cell therapy directly injected in the damaged part. (b) tissue engineered approaches to implant a graft: cell sheets, cell-scaffold construct and injectable materials (such as hydrogels) with cells (c) example of ventricular restrain device (Arnal-Pastor et al., 2013).

Importantly, stiffness of the scaffold is a particular feature that directly influences CM biomechanical behavior, therefore it should be considered as an important issue when designing an ideal cardiac patch (Engler et al., 2008). Human myocardium ranges stiffness 20 kPa at the end of diastole to 500 kPa at the end of the systole, whereas in the rat myocardium stiffness ranges from 0.1 to 140 kPa respectively (Table 5) (Chen et al., 2008) (Nagueh, 2004) (Watanabe et al., 2006), hence mechanical stiffness of the patch may directly affect cell survival in the inside, besides the electro-mechanical global coupling with host myocardium.

Table 5

Comparative table showing different values of elasticity (Pa) for various animal tissues. Bottom: values of YM (Pa) for synthetic and natural polymers. Table taken from (Lakshmanan et al., 2012).

Source	Mechanical properties
Post-mortem human left ventricle (passive)	Bulk modulus 0.25 GPa Tangent shear modulus 60 – 148 kPa
Rat left ventricle (non infarcted)	Elastic modulus 18 ± 2 kPa
Rat left ventricle (infarcted)	Elastic modulus 55 ± 15 kPa
Bovine pericardium	Storage modulus 7.3 MPa High modulus 14 MPa
Rat left ventricle epicardium (passive)	Elastic modulus 10 kPa
Rabbit cardiomyocytes	Elastic modulus 100 ± 10.7 kPa
Skeletal muscle cells (C2C12)	Elastic modulus 24.7 ± 3.5 kPa
Human umbilical vein endothelial cells	Elastic modulus 1.4 – 6.8 kPa

The engraftment and consequent survival of transplanted engineered cardiac tissues (grafts of patches) is principally limited by the lack of vasculature in the ischemic area where the patch is transplanted, and thus, a limited oxygen diffusion (Radisic et al., 2006). To overcome this hurdle, it has been shown how the combination of different cell lines has contributed to enhance performance and survival of stem-cell populations in cardiac tissue engineering therapies for heart failure (P W Burridge et al., 2014). Moreover, sophisticated structures created during *in-vitro* electromechanical conditioning can rapidly disappear on implantation. In the context of cardiac tissue engineering, angiogenic scaffolds containing angiogenic growth factors or precisely designed porous structures are being used to enhance cell survival and angiogenesis on implantation, together with the culture of different cell lines may contribute to the success of these approaches. Still, a lack of angiogenesis in the implanted construct represents a major drawback within this technique (Roy et al., 2010) (Matsuo et al., 2015)

Table 6

General classification table for biomaterials and transplantation strategies for cardiac constructs. Transplantation strategies can be distributed into 3 different groups: injectable grafts, cardiac patches and LV restraints. Biomaterials can be classified in 2 main groups, natural or synthetic. Table taken from (Rane & Christman, 2011).

2006–2011		
Material	Transplantation	Model
Chitosan	Alone w/ESCs w/bFGF	Rat
Gelatin	Alone w/bFGF	Rat, dog
Collagen	—	—
Matrigel	Alone w/hESC derived CMs w/pro-survival factors	Rat
Collagen type I and Matrigel	—	—
Small Intestinal matrix	Alone	Mouse, rat
Myocardial matrix	Alone	Rat, pig
Pericardial matrix	Alone	Rat
Hyaluronic acid	Alone	Rat, sheep
Self-assembling peptides	Alone w/SkM + PDGF-BB, BMMNCs w/IGF-1, PDGF-BB, FGF-2	Rat, pig
Calcium hydroxyapatite	Alone	Sheep
PEG based	Alone w/BMSC, hESC + thymosin β 4 w/VEGF, thymosin β 4, erythropoietin	Rat, rabbit
PNIPAAm based	Alone w/BMMNCs, BMSC w/bFBF	Rat, rabbit
Material	Transplantation	Model
LV restraints		
Polypropylene	—	—
Polyester	Alone	Sheep
Nitinol	Alone	Dog, sheep, human
Cardiac patch		
Gelatin	—	—
Alginate	—	—
Poly(glycolide)/poly(lactide)	—	—
PTFE, PLA mesh, collagen type I and matrigel	—	—
PTFE	—	—
Collagen type I and Matrigel	w/SkM	Rat
Collagen type I	Alone w/BMC, HUCBCs, hBMC CD133+, hMSC, hSkM	Rat, human, mouse
Alginate and Matrigel	w/SDF-1, IGF-1 and VEGF w/Neonatal CM + SDF-1, IGF-1 and VEGF	Rat
Chitosan	—	—
Fibrin	Alone w/hESC-derived ECs and hESC-derived SMCs	Pig
Polyurethane	Alone w/SkM	Rat
Polyester urethane urea	Alone	Rat
Poly(glycolide-co-caprolactone)	Alone w/BMMNC	Rat
Poly(lactide-co- ϵ -caprolactone)	Alone w/MSCs	Rat
Decellularized myocardium/fibrin	w/Mesenchymal progenitor cells	Rat
Small Intestinal submucosa	Alone w/MSCs	Rabbit
Pericardium	w/MSCs	Rat
Urinary bladder matrix	—	—
Injectable biomaterials		
Fibrin	Alone w/BMCs, ASCs, MCSCs, BMMNCs w/bFGF, HGF, TGF β -1	Mouse, rat, dog
Alginate	Alone, RGD modified, RGD+ YIGSR modified, RGE modified w/VEGF, PDGF-BB, IGF-1, HGF w/hMSCs w/Polypyrrole	Rat, pig, human
Fibrin -Alginate	Alone	Pig

Regarding scaffold's architecture, an important example of a cardiac patch approach would be the use of mammalian decellularized tissue as a scaffold (Fig. 33). The question of how to mimic the unique network of the ECM may be successfully answered by using tissue-derived cellular or acellular scaffolds (Wang et al., 2010) (Crapo, Gilbert, & Badylak, 2011). The main advantages of the decellularization approach would be the preservation of ECM composition, biologic activity, and mechanical integrity. Furthermore, a remarkable outcome in the field was the creation of a complete heart decellularization for various animal models (Badylak, Taylor, & Uygun, 2011) (Wainwright et al., 2010) (Wang et al., 2012)) to later on reseed it with suitable cell types. The next ambitious step has been to apply such approach to a larger xenogenous heart, like a porcine heart, as Kitahara et al. have demonstrated to be possible to decellularize, re-cellularize and then transplant in a porcine model (Kitahara et al., 2016). Important attempts are also being developed with decellularized human myocardium (Perea-Gil, Prat-Vidal, & Bayes-Genis, 2015) but still the whole-organ approach (or neorganogenesis) remains a challenge to accomplish.

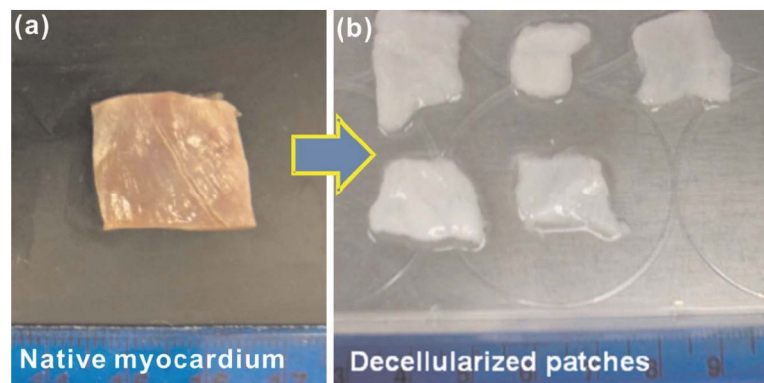


Fig. 33
Piece of (a) native myocardium and (b) various decellularized myocardial scaffolds (Wang et al., 2010).

Another outstanding example of cardiac patch would be the cell-sheet engineering approach, developed by Shimizu et al. (Shimizu et al., 2002). In which a pile of cardiac-cell monolayers were detached from the culture plates by the use of a thermoreversible biopolymer that allowed to collect the intact monolayer, and stack several together in order to create a 3D patch. The results of this approach demonstrated the feasible efficacy of generating a patch with highly functional iPSC-CMs and implanting it in a damaged myocardium, with a remarked increase in neovascularization, besides cardiac function, in a porcine model of MI (Kawamura et al., 2013).

C) *Left ventricle restraints*

This strategy is based on a polymeric mesh that covers the left ventricle (LV), which basically act as a mechanical support to constrain it (Fig.26C). In this approach the aim is not to regenerate the ischemic tissue, but to avoid the progress of the adverse remodeling following a myocardial infarction (Arnal-Pastor et al., 2013). Although the outcomes

of this trial are currently unclear, the few studies on LV restraints indicate declining interest, possibly due to the invasive procedure required for this approach (Table 6) (Lee et al., 2010) (Rane & Christman, 2011).

2.1.3 Signaling molecules

The rationale for the use of growth factors and signaling molecules is based on the necessity to control proliferation and proper differentiation of cells in the affected tissue. *In-vivo*, exogenous growth factors can enhance or modulate the migration, proliferation and differentiation of cells from the edges of the treated tissue. Thus, such factors can serve first to optimize tissue-engineered cell-scaffold constructs *in-vitro*, and second to improve their performance and integration in vivo (Brochhausen et al., 2009). The success of this approach depends on the purification and large-scale production of appropriate signal molecules, and in many cases, the development of methods to accurately deliver these molecules to their targets (Table 7) (Meyer et al., 2009).

One of the great remaining challenges in cardiac tissue engineering is the vasculogenesis and angiogenesis effect within the scaffold as previously seen. Several growth factors and chemokine molecules have been loaded into scaffolds in an attempt to promote cellular infiltration and blood vessel formation (Roy et al., 2010). Several peptide molecules have been conjugated within scaffolds ranging from the simple tripeptide RGD (arginine-glycine-aspartate) to complex hepatocyte growth factor (HGF) and leukemia inhibitory factor (LIF) for the purpose of adhesion, migration, proliferation, differentiation and vascularization (Table 7). Among all the polypeptides, the 43-aminoacid thymosin beta-4 (Tβ4) is a potent molecule which by itself reduces the scar thickness and initiates the fetal wound healing process in adult ischemic region (Nicola et al., 2011). It promotes neovascularization and repairs the infarct tissue, with the help of progenitor cells present in the epicardium and inhibits the chemotaxis of inflammatory cells. For example, cardiac progenitors are a pool of undifferentiated multipotent cells residing in the cardiac tissue with c-kit cell surface markers. They are sheltered in a specific niche where they interact structurally and functionally with ECM components and neighboring fibroblast, cardiomyocytes and transit amplifying cells (Goldstein et al., 2012). Since the field of regenerative cardiology is in its infancy, potential strategies have to be developed to regenerate the cardiac ischemic tissue with effective growth factors. This strategy holds immense potential in recruitment of cardiac progenitors to ischemic zone for cardiac repair and regeneration.

Table 7

Table with the most frequent used bioactive molecules for enhancing tissue regeneration. Table from (Lakshmanan et al., 2012).

Bioactive molecules	No of aminoacid residues	Physiological functions	Mode of delivery	Ref.
VEGF	165	Increases blood vessel density Promotes cell proliferation within scaffold Promotes the paracrine release of SDF1	Engineered Cardiac patch	[120]
bFGF	114	Increased density of neo microvessels, vital for long term survival of transplanted cells	Bovine pericardial patch	[121]
EPO	192	Prevents apoptosis, Reduces inflammation, Recruits endothelial progenitor cells (EPC) from circulation, participates in repair, neoangiogenesis	EPO injection	[122]
G-CSF	138	Inhibits apoptosis, Reduce remodeling of diseased hearts	Electrospun PLA cardiac patch	[123]
HGF	679	Endogenous antifibrotic factor Inhibits TGF β mediated ECM accumulation Induces mobilization of mesoangioblast for cardiac repair	Gelatin hydrogel sheets	[124]
IGF-1	71	speeds up ischemic muscle regeneration, guard cells from apoptosis, Increases angiogenesis	Alginate gel	[125]
LIF	202	stimulates cardiomyocyte hypertrophy, Promotes neovascularization via VEGF upregulation, enhances the mobilization of bone marrow cells into myocardium	LIF cDNA plasmid injection	[126]
SDF-1	93	Promotes survival of cardiomyocytes, mesenchymal stem cells, Prevents progressive scar formation, suppression of apoptosis, Induce angiogenesis and myogenesis	hSDF -1 transduced MSC injection	[127]
PDGF	109	Regulates extra vascular tissue signaling of cardiac endothelial cells Induce angiogenesis,	hPDGF transduced endothelial cells injection	[128]
Thymosin β 4	43	Reduce scar volume, reduces myocardial cell death after AMI, Induce angiogenesis, initiates embryonic myocardial gene expression, activates epicardial progenitors	T β 4 injection	[129]

bFGF: Basic fibroblast growth factor; EPO: Erythropoietin; G-CSF: Granulocyte colony stimulating factor; HGF: Hepatocyte growth factor; IGF: Insulin growth factor; LIF: Leukemia inhibitory factor; PDGF: Platelet derived growth factor; SDF: Stromal cell derived factor; TGF β : Transforming growth factor- β ; VEGF: Vascular endothelial growth factor.

2.1.4 Last generation cardiac constructs

The latest strategy in cardiac tissue engineering is the use of the combined 3 previous approaches, implying not only an adequate cell source (autologous or not) in a suitable biomimetic scaffold with bioactive or tissue-inducing molecules loaded in the scaffold. In this strategy, the construct in addition of delivering adequate mechanical and biochemical support and stimulation, is also subject to suitable biomechanical and/or bioelectrical stimulation that enhances cell performance (CM maturity) and survival in the injured tissue (Li & Guan, 2011).

One of the key challenges in tissue engineering is the ability to grow 3D tissue structures of relevant clinical sizes. In human tissues there are considerable mechanical and biochemical interactions between cells of the same type, between different cells and also with the extracellular matrix and its components. It has been documented that the behavior of cells in 2D varies considerably from the one in a 3D culture and this has important implications for the *in-vitro* generation of functional human tissues. A second challenge will be the growth and 3D assembly of multiple cell types that are required for more complex functional tissues. In addition to the 3D requirements for tissue structure it must also be considered that tissue growth is a dynamic, non-steady state process where biological parameters, physical rate processes and the local mechanical environment are continually changing along time (Chaudhuri & Al-Rubeai, 2005). In essence, current cardiac tissue engineering is a 4D challenge. The alternative to static growth is to use dynamic culture systems (dynamic growing devices and bioreactors) where mixing gives rise to homogeneous concentrations of nutrients, toxins and other components thus

removing the problems associated with gradients and establishing the culture environment.

The future graft will be entirely electromechanically functional since grafting, providing adequate electromechanical support, optimal vascularity and molecular pathways to enhance coupling with host damaged myocardium, and will be degraded by the gradual action of native and implanted cells as host and implant merge in a unique regenerated muscle.

2.2 Bioengineering scaffold stimulation

We have seen in previous sections how cardiac tissue engineering is the emergent field that provides alternative therapies to myocardial infarction. How cells, scaffolds, and molecular signaling constitute the fundamental 3 elements for creating an *in-vitro* piece of living tissue. Considering signaling, cardiac cells do not only receive molecular signaling but also physical signaling, more particularly, mechanical and electrical. Therefore pre-conditioning of the cells in the constructs constitutes one of the latest advances in the design of cardiac patches or grafts for myocardial infarction. This fact has driven the development of the biomimetic paradigm of cardiac tissue engineering, which involves the application of physiologically relevant physical stimuli (Baumgartner et al., 2015) (Xia, Buja, Scarpulla, & McMillin, 1997) to cultured cells, and optimizing in this way their final coupling and engraftment to the host myocardium. For such achievement, 3 basic techniques have been studied, alone or in combination, in order to enhance cell maturity and therefore increase electromechanical coupling to host tissue: mechanical stimulation, electrical stimulation, and bioreactors..

2.2.1 Electrical stimulation

The heart is the body's largest bioelectrical source. Applying physiological electrical signals to cardiomyocyte cultures in scaffolds, constitutes a way of mimicking the electrical activity that actually occurs in native myocardium. This signaling results in a progressive development of conductive and contractile properties characteristic of cardiac tissue, including cell alignment and coupling, increased amplitude of synchronous construct contractions and a remarkable level of ultrastructural organization. In general terms, a more adult-like performance and phenotype, considering the fact embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) must be differentiated to cardiomyocytes, increasing with this stimulation their degree of maturity (Tandon et al., 2009). For cardiac tissue engineering, the signals of interest are those found both in the developing and in the adult heart. The main types of electrical currents in embryonic heart are the direct current (DC) signals thought to direct cell migration during the development of the cardiac primitive streak and left-right asymmetry, and the pulsatile signals implicated in the development of the cardiac syncytium (Feld et al., 2002).

The effects of electrical stimulation strongly depend on the time of its initiation. If applied too early, electrical stimulation inhibits the accumulation of cardiac proteins and yields poor contractile behavior. If applied too late, electrical stimulation can no longer aid the functional assembly of for the double layers on stimulation electrodes to dissipate between the cells (Tandon et al., 2009).

For vertebrates the physiologically significant range of endogenous gradients of electrical fields is on the order of 0.1 to 10V/cm (Song et al., 2007). In terms of frequency, the heart's beating rate can range from 3-4 beats per minute (for blue whales during deep dives), up to 360 beats per minute for a rat heart and 1,200 beats per minute for the hummingbird during flight. Corresponding to 0.05 to 20Hz. In terms of pulse duration, 1-2ms is sufficiently long to excite heart tissue and cells, and moreover, this signal duration is sufficient also for the double layers on stimulation electrodes to dissipate between subsequent pulses.

The most efficient devices to stimulate cells cultures and scaffolds work with

carbon rods (Fig.30), situated at a certain distance in the culture plate (Barash et al., 2010) (Sarró et al., 2012) (Xia et al., 1997), or platinum or gold slides (Wang et al., 2014) (Baumgartner et al., 2015) connected to function generators that permit the electrification parameters previously described. It also has been stated that biphasic electrical stimulation enhances maturation in CM cultures in comparison with monophasic stimulation (Chiu, Iyer, King, & Radisic, 2011).

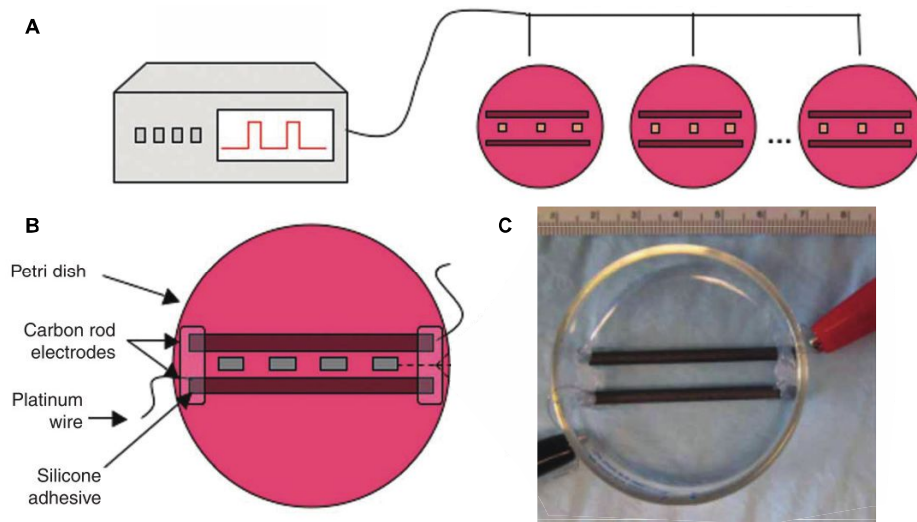


Fig. 30
Experimental setup for applying pulsatile electrical field stimuli to cardiac cells. (A) overview of the setup. An electrical stimulator connected to 3 modified Petri dishes. (B) Detail of each electrical device. (C) Photograph of an assembled electrical stimulation chamber. Image taken from (Tandon et al., 2009).

2.2.2 Mechanical stimulation

Myocardium is highly organized in structure and possesses unique electrophysiological and mechanical properties. Myocardial fibers have a complex multilayered helical architecture that is essential for well coordinated heart contraction (Wang et al., 2012). As previously described, among the individual myocardial fibers there exists a 3D network (the ECM) that results critical to maintain the structural integrity of myocardium, which passively supports CM contractile forces, as well as prevents excessive stretch of muscle fibers (Wang et al., 2014). Hence, when we develop an engineered contractile construct, challenges not only fall into the generation of large numbers of functional CMs, but also the to integrate of the cells within a scaffold that perform synchronously (Wang et al., 2010) (Nenadic et al., 2009) (Yang et al., 2006).

To further improve the effectiveness and efficiency of cell differentiation and tissue remodeling of the stem cell approaches, it has been explored the effects of physical stimulations on the outcomes of the tissue constructs. Multiple studies suggest that physical stimulations can affect the cell proliferation, differentiation, and migration, and consequently the biological, mechanical, and other properties of the cardiac construct (Fig.31) (Lux et al., 2015) (Zimmermann et al., 2006) (Eschenhagen et al., 1997). Mechanical stretching, thus, can lead the cardiomyocytes to a more advanced stage of maturation that can better guaranty host integration when transplanted. Considering CMs contract in a range of 10 - 15 % as previously seen, the majority of studies focus on achieving such rate when stretching cell constructs. For example, in

an *in-vitro* study Zhuang et al. (Zhuang et al., 2000) reported that a 10% cyclic uniaxial stretch produced upregulation of connexin-43 and N-cadherin in the intercellular junctions and increased the propagation velocity, as well as Zimmermann and Eschenhagen et al. did (Zimmermann et al., 2002) reporting structural and functional remarkable changes in the CMs, whereas Yamada et al. (Yamada et al., 2005) also reported upregulated expression of electrical (connexin-43) and mechanical (plakoglobin, n-cadherin) junction proteins.

Despite notorious examples with scaffolds in 3D, such as Eschenhagen and Zimmermann's engineered heart tissues strained by uniaxial torsion springs (Zimmermann & Eschenhagen, 2003) or Shimizu and Okano's cell-sheet approach (Shimizu et al., 2002), the majority of mechanical stimulation studies (not only in cardiac tissue engineering) are exerted to 2D cell cultures, with strainable membranes (Casares et al., 2015) (Navajas et al., 2008) (Fig. 31), custom or commercialized like in the case of the stretchable membranes of Flex-Cell®. Despite this fact, the synergistic effects of mechanical (and electrical) stimulations on cardiac tissue engineering suggest positive but limited results. It has not yet been fully investigated, despite remarkable examples like Wang's et al. previously cited, Gordana's et al. (Vunjak-Novakovic et al., 2014) or (Radisic & D. Black III, 2014).

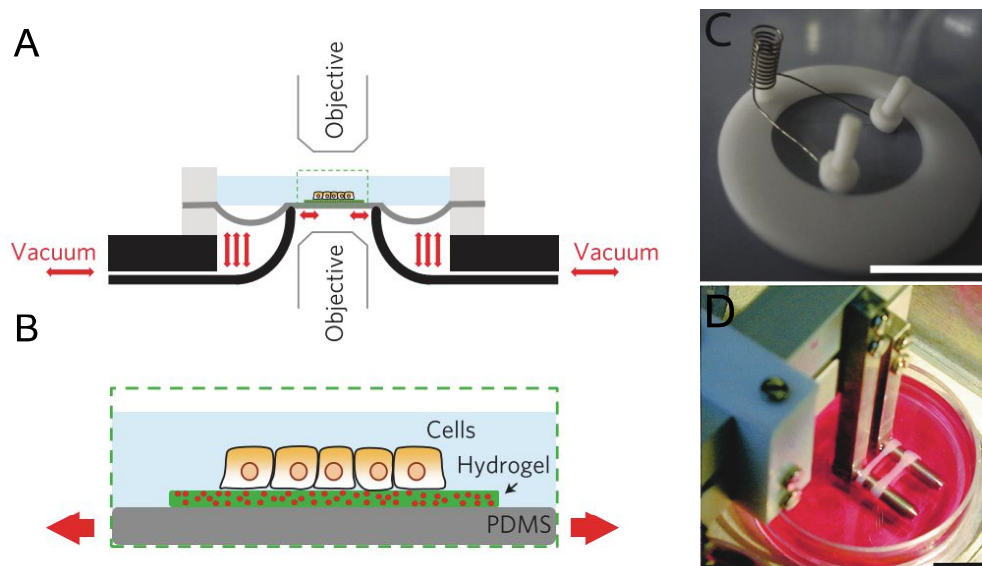


Fig. 31 Mechanical stimulation devices. (A) scheme of a vacuum-working device, based on FlexCell® technology, in which an elastomeric membrane is strained in every direction of the plane (biaxial). (B) detail of cells attached to an PAA hydrogel covalently bonded to a PDMS membrane (Casares et al., 2015). (C) torsion spring to stimulate with a constant force and among one axis (uniaxial) a ring-shaped collagen and cells scaffold (Godier-Furnémont et al., 2015) (D) cyclic stimulation machine for ring-shaped collagen and cells scaffolds (Zimmermann et al., 2002). Scale bars 10mm.

In spite of these and others great advances in the field, new inventions and technologies are still needed to better assess mechanical stimulation to constructs and cell cultures, in order to better mimic native stimulating conditions and thus study behavior in a biomimetical approach.

2.2.3 Bioreactors

A bioreactor is the general term applied to a closed culture environment that enables control of one or more environmental or operating variables that affect biological processes (Chaudhuri & Al-Rubeai, 2005). In tissue engineering, bioreactors are used to provide controlled and reproducible cell and tissue culture. The variables that are controlled include temperature, pH, gas concentration, media flow-rate, shear stress, hydrodynamics and mechanical forces. In tissue engineering the desired product is 3D functional tissue and the bioreactors used here differ from conventional cell culture bioreactors in that they must provide an environment that prompts the cells into proliferating and differentiating as they would in vivo.

In early studies, cells were seeded in spinner flasks or rotating wheels in order to provide them of a dynamical flow of culture medium that may supply more effectively oxygen (dissolved in the culture medium and transported to cells by molecular diffusion) and nutrients to the cells. In human cardiac muscle, from 1-3 cm thick, oxygen can only diffuse about 100 to 300 μm deep (Radisic et al., 2006), that is the reason why a vast mesh of vascular vessels is spread all through the cardiac muscle. When speaking about scaffolds, same phenomenon occurs, and therefore scaffolds cannot be designed in a large scale due to this oxygen limitation. To overcome this oxygen lack during tissue culture in 3D, perfusion bioreactors were developed (Fig.32). According to tissue engineering 3 fundamental elements, cells, scaffold and signaling molecules, bioreactors would be an extension of the third element; considering bioreactors as implemented systems to control cellular microenvironment, facilitate mass transport to the cells and provide necessary biochemical and physical regulatory signals (Radisic et al., 2008).

Although the use of bioreactor systems for tissue engineering is in its infancy we can consider some general features to allow us to rationally design reactors. A bioreactor should perform at least one of the following: create an environment that enables cells to proliferate and differentiate as in vivo; establish spatially uniform cell distributions on 3D scaffolds; maintain desired concentrations of nutrients; provide efficient mass transfer to tissue; and expose tissues to physical stimuli (Freed and Vunjak-Novakovic, 1997).



Fig. 32

Perfusion culture bioreactors used in tissue engineering. (A) Perfusion loop for cell seeding consisting of a multichannel peristaltic pump, (1) filters, (2, 3) U-shaped tubing (Radisic et al., 2008). (B) Perfusion culture setup with a peristaltic pump, tubes and (detail on the right) the perfusion container where the scaffold is placed (Chaudhuri & Al-Rubeai, 2005).

The existing bioreactor system that is most relevant to fixed, adherent human tissue is the perfusion bioreactor. A perfusion bioreactor is based on interstitial medium that flows through a porous scaffold or flows through a channel array (Miklas et al., 2014) (Radisic et al., 2008) in which cells have a constant medium flow during culture. Some can provide pulsatile electrical or mechanical stimulation as well as gradients of biochemical factors that enhance cell survival, expansion and maturation. Despite the relative novelty of the approach and the continuous improvement of the techniques, remarkable results published so far suggest bioreactors as a potential platform to study and fabricate cardiac functional tissue for regenerative medicine therapies (Cimetta et al., 2013).

3. Clinical trials

3. CLINICAL TRIALS

Due to obvious limited supply of donor hearts for transplantation, cell-based therapies have become the alternative source of treatment for the past decade. Limited success of stem cell therapy is due to lack of optimal cell source for transplantation, route of cell delivery to infarcted myocardium, paracrine signaling and homing (Behfar et al., 2014) (Passier et al., 2008). Survival of cells in the inflammatory milieu poses a great challenge, since the majority of supplemented cells die within a week. There exists no optimized technique to inject stem cells to the heart, as a result of which 90 % of injected cells delivered via needle escapes into circulation (Breckwoldt et al., 2015). Also, the scar tissue prevents the electromechanical coupling of grafted cells to the host myocardium resulting in arrhythmia (Table 8).

Table 8

Top: Ongoing human clinical trials with different cell types for the treatment of myocardial infarction (US National Institutes of Health, www.clinicaltrials.gov). Bottom: Available commercial products for the treatment of myocardial infarction. Tables obtained from (Lakshmanan et al., 2012).

Clinical trial identifier	Source of stem cells	Mode of delivery	Status	Phase
NCT00474461	Cardiac stem cell infusion in patients with ischemic cardiomyopathy (SCPIO)	Intracoronary infusion	Ongoing	Phase-I
NCT00877903	ex vivo cultured adult human mesenchymal stem cells	Intravenous Infusion	Ongoing	Phase-II
NCT00442806	Adipose derived stem cells – Apollo trial	intracoronary infusion	Ongoing	Phase-I
NCT00426868	Adipose derived stem cells – Precise Trial	Direct injection in left ventricle	Ongoing	Phase-I
NCT00684060	Adult bone marrow mononucleated cells (Late TIME Study)	Intracoronary infusion	Ongoing	Phase-II
NCT01167751	Autologous bone marrow derived stem cells	Intracoronary infusion	Ongoing	Phase-III
NCT00316381	Autologous bone marrow derived stem cells	Intracoronary infusion	Completed	-
NCT00313339	Autologous bone marrow derived stem cells	Intracoronary infusion	Ongoing	Phase-I
NCT00279175	REPAIR-AMI Autologous bone marrow derived stem cells	Intracoronary infusion	Completed	Phase-III
NCT00363324	Autologous bone marrow derived stem cells	Intracoronary infusion	Completed	Phase-II
NCT00400959	Autologous hematopoietic cells	Intracoronary infusion	Completed	Phase-III
NCT00384982	Myocardial Stem cell administration after acute myocardial infarction	Intracoronary, percutaneous and intramyocardial	Completed	Phase-II
NCT00135928	Neupogen® (G-CSF) Bone marrow derived stem cells	Intracoronary infusion	Completed	Phase-II
NCT01392105	Mesenchymal stem cell	Intracoronary infusion	Completed	Phase II
NCT00200707	BONAMI (BOne Marrow in AMI)	Intracoronary infusion	Completed	Phase III
NCT01291329	Human Wharton's Jelly derived mesenchymal stem cells	Intracoronary infusion	Ongoing	Phase-II
NCT00268307	Autologous Bone Marrow Derived Stem Cells	Intracoronary infusion	Ongoing	Phase-I
NCT00883727	Ex Vivo Cultured Bone Marrow Derived Allogenic MSCs	Intravenous	Ongoing	Phase-I
NCT00893360	CADUCEUS- CArdiosphere-Derived aUtologous Stem Cells to Reverse ventricUlar dysfunction	Intracoronary infusion	Ongoing	Phase-III
NCT00874354	REVITALIZE- Randomized Evaluation of Intracoronary Transplantation of Bone Marrow Stem Cells in AMI	Intracoronary infusion	Ongoing	Phase-I
NCT00199823	ASTAMI (Autologous Stem Cell Transplantation in AMI)	Intracoronary infusion	Completed	Phase-II

Commercial products	Biomaterial	Features
Gore-Tex® (W. L. Gore & Associates, Inc.) http://www.goremedical.com/resources/dam/assets/AK0363EN4.PVGS.BRO-FNL%20MR.pdf .	ePTFE	High strength resistance to aneurysmal dilatation Proven clinical safety, efficacy, multiple thicknesses for optimal tissue approximation Good biocompatible
Neoveil (Gunze Ltd®.) http://www.gunze.co.jp/e/medical/product/product_02.html Hemashield platinum finesse knitted cardiovascular patch (Maquet GmbH & Co. KG®.) http://www.maquet.com/productPage.aspx?m1=112599774495&m2=0&productGroupID=121025933185&languageID=1&productConfigID=127972186837	PGA felt patch	
Vascutek Porcine Pericardial Patch (Vascutek Ltd, a TERUMO Company®.) (http://www.vascutek.com/vascutek/products/item/porcine-pericardial-patch) PeriPatch™ (NeoVasc, Inc.) http://www.neovasc.com/tissue-products/peripatch-sheet.php Peri-Guard® (Synovis™ Surgical Innovations.) http://www.pelegrinamedical.com/Brochures/Synovis/Periguard.pdf SJM® Pericardial Patch (St. Jude Medical, Inc.) http://www.sjmphprofessional.com/Products/Intl/Heart-Valve-Repair/Pericardial-Patch-with-EnCap-Technology.aspx Edwards Bovine Pericardial Patch (Edwards Lifesciences®) http://www.edwards.com/products/porcinevalves/pages/patch.aspx	Knitted impregnated polyester patch	Collagen impregnation Improved tissue ingrowth
	Porcine pericardial patch	Ultra-thin flexible patch
	Bovine pericardium	Glutaraldehyde cross linked Non-thrombogenic and non-carcinogenic
	Bovine pericardium	Strong multidirectional collagen fibers, biocompatible
	Bovine pericardium	EnCap AC Technology biologically stable, Promote endothelial covering, Minimize calcification
	Bovine pericardium	XenoLogiX treatment reduces calcification, Neutrolagic fixation for tissue stability and reduced antigenicity
Anginera™ (Theregen, Inc.) http://www.theregen.com/prod1.html	Epicardial patch + living human fibroblast cells + extracellular matrix	Derived from rat heart tissue. Stimulate new mature blood vessels
CardioRel™ (Reliance Life sciences) http://www.rellife.com/pdf/cardiorelinsert.pdf	Autologous MSCs 5 × 10 ⁶ – 300 × 10 ⁶ cells per patient	Prepared from bone marrow of suitable patients No abnormal tissue formations in the heart

ePTFE: Expanded poly(tetra fluoro ethylene); MSC: Mesenchymal stem cells; PGA: Poly-(glycolic acid).

Cardiac tissue engineering strategies along with suitable scaffolds and cells seems more suitable than the standalone cellular therapies to address the challenges in myocardial patch engineering. Some commercial products are available with proven efficacy in the treatment MI, but still more research is needed (Table 9).

Several therapeutic strategies have been proposed in the last decade to limit the adverse spread of the ischemic tissue and ventricle dilation or even to generate new myocardial tissue. These treatments consist in cellular therapy (so-called cellular cardiomyoplasty) (Arnal-Pastor et al., 2013) where cells of different origin are implanted by different techniques onto the infarcted ventricle with the hope that cells will contribute to the generation of new contractile tissue to replace the scar, electrically coupled with the host myocardium. But despite the intense efforts and work put in the field, numerous attempts so far have failed. Most of the implanted cells die soon after transplantation due to the fact that cells cannot withstand the mechanical forces they experience in the host tissue. Mechanisms underlying the slight improvements observed are still undetermined. New strategies already under study envision improving cell survival by pre-conditioning the cells, pre-treating the host tissue or combining cells with other elements.

Human iPSC seem to be the right platform to develop tissue engineering-based therapies but important genetic and epigenetic consequences complicate their definitive clinical implantation. Most studies so far had a rather short follow-up period that was certainly not long enough to properly estimate the teratoma risk (Breckwoldt et al., 2015).

4. Hypothesis & Objectives

4. HYPOTHESIS AND OBJECTIVES

Cardiac tissue engineering focused on the fabrication of cell-scaffold constructs represents a great promise for cardiac regenerative medicine research in general and particularly for the development of new approaches to improve and heal a myocardial infarction.

Interestingly, the use of human induced pluripotent stem cells-derived cardiomyocyte (iPSC-CM) for these engineered cell-scaffold constructs offers a new platform to develop functional in vitro created cardiac tissues.

It has been described the importance of the cardiomyocyte stage of maturity to perform an enhanced electromechanical behavior, as well as to guarantee a more efficient coupling when grafting it to native tissue in the in vivo trials. Certain degree of CM maturity can be achieved by applying mechanical stimulation to the cell culture. The majority of systems developed to mechanically stimulate scaffolds provide axial or biaxial stimulation, differing considerably from the 3-dimensional native heart's stimulation.

Thus, the hypothesis of this study is that biomimetic dynamic mechanical stimulation exerted to a 3-dimensional scaffold with human iPSC-CM may provide a higher degree of CM maturation in comparison with axial stimulation, and therefore, a suitable graft that could positively contribute to the regeneration of a damaged heart tissue after myocardial infarction in a mouse model.

Based on these premises, the present thesis attempts to fulfill the following objectives:

- I. To generate and characterize functional collagen-based scaffolds with human iPSC-CM.
- II. To design, implement, and build a biomimetic mechanical stimulation system that provides a 3-dimensional radial pulsed stimulation similar to native heart, and apply it to in vitro cultured iPSC-CM constructs to enhance cell maturation in comparison with the non-stimulated condition.
- III. To analyze cardiomyocyte maturity and establish an objective criterion to compare results.
- IV. To study the regenerative potential of the iPSC-CM constructs in a mouse model of myocardial infarction.

5. Materials & Methods

5. MATERIALS & METHODS

5.1 Porcine myocardium mechanical analysis

To evaluate the mechanical properties of porcine myocardium, strain-stress mechanical tests were carried out in collaboration with Dr. Estefanía Peña research group (*Structural mechanics and materials modeling group, Institute for Research in Engineering of Aragon (I3A), University of Zaragoza* (Calvo et al., 2014).

Five pig hearts ($n=5$) similar in weight (~ 400 g) were obtained at Mercazaragoza slaughterhouse, and maintained in PBS 1X (Sigma) at 4°C. Hearts were dissected and tissue strips of 4 mm thickness were sectioned for each heart. The samples were cut in 3 equal size blocks ($n=3$) of 3 cm long by using a slicer matrix (Fig. 39 A). First slice was cut following the alignment of the muscle fibers; the second slice was cut at 45° and the third, perpendicular to the fiber direction (Fig. 39 B & C).

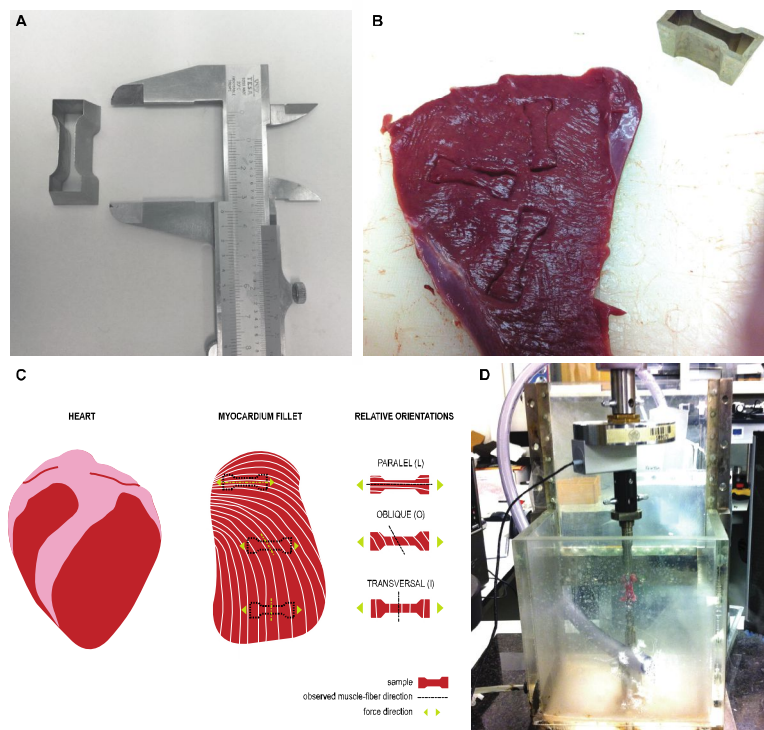


Fig. 39

(A) Image of the slicer matrix used for cutting the samples. (B) Samples cropped with the matrix in the 3 relative orientations on the myocardium fillet. (C) Representative scheme of the 3 relative orientations cut per sample. (D) Sample on the Instron Microtester at 37 °C and 100% HR.

Uniaxial tensile test were carried out to determine the mechanical properties of each sample of myocardium (Fig. 39 D). Tests consisted of 3 pre-warming stretch-relaxation cycles at $\epsilon = 10\%$ followed by 5 stretch-relaxation cycles with an Instron Microtester 5548 (Instron) with a 5 N full scale load cell and with a minimal resolution of 0,001 N. Samples were tested at 37 °C and in a humidified chamber, allowing a constant humidity during the tests.

5.2 Design of a hydrogel scaffold

Different hydrogels were selected as suitable candidates for the scaffold, due to their unique physical and biochemical properties. A requirement for our study was that hydrogel should be thermoreversible (Li et al., 2009) (Li et al., 2012), in order to allow possible delivering in a hypothetical in-vivo approach. A set of hydrogels was studied in order to study our ideal candidate.

5.2.1 Pluronic F127 preparation

Pluronic F127 (PF127, Sigma) was selected because of its thermoreversibility (Fig. 40) and biocompatibility (Almeida et al., 2012). Pluronic F127 was diluted with 1X phosphate buffered saline (PBS) (Sigma) to obtain a final concentration of 20% and 25%. Solutions were mixed and stirred for 2h and then warmed at 37 °C for 10min to allow solution-to-gel transition.

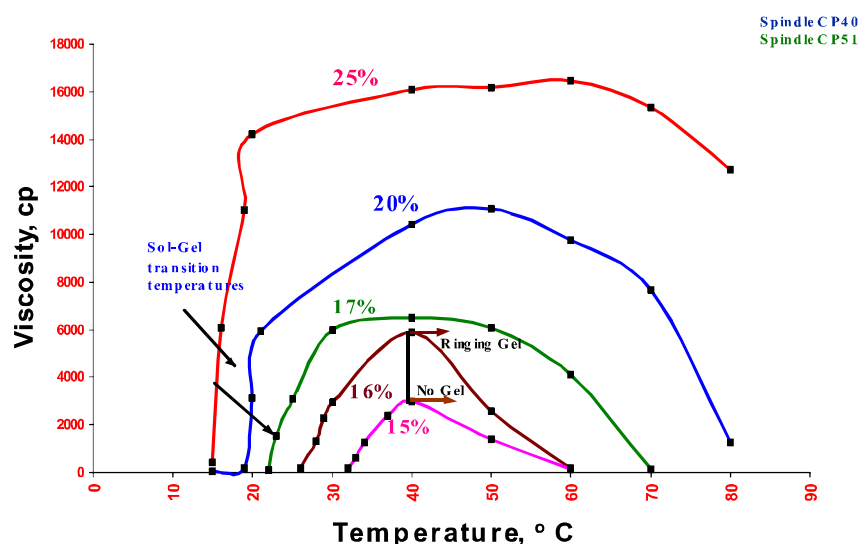


Fig. 40

Pluronic® F127 surfactant viscosity graph as a function of concentration and temperature. Considering the fact that cells must survive inside the hydrogel, concentrations >20% (w/v) may compromise the viability of cells due to the sol-gel transition temperature (Lippens et al., 2013).

5.2.2 Pluronic F127 rheological tests

Rheological shear stress tests were performed to study hydrogel's stiffness (YM) with a HAAKE RheoStress 1 rheometer (Thermo Scientific), in collaboration with Dr. Lyda Halbaut from the Pharmacological Technology department, University of Barcelona. Two types of tests were performed for every sample: (a) Pascal (Pa) sweep test from 0 to 500 Pa, at constant frequency (1 Hz), increasing torsion angle (δ); and (b) frequency (Hz) sweep test from 10 to 0.01 Hz (with variable force), and constant torsion angle. All samples were preconditioned at 37 °C. Tests show the elastic modulus (E) for each sample (Fig. 52): $E = G' + G''$, where G' is the elastic component of the modulus and G'' the viscoelastic component. In our study, only G' was considered as we only wanted to know hydrogel's elastic behavior.

5.2.3 Pluronic F127 culture with human foreskin fibroblasts (HFF)

To analyze cell biocompatibility into the scaffold, 20% and 25% solutions of PF127 were mixed with 1.5×10^6 of HFFs* and seeded into circular casting molds (donut-shaped). Samples were incubated at 37 °C, 5% CO₂ during 7 days in IMDM (Gibco) medium supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), 1% Glutamax (Gibco) and 1% penicillin-streptomycin (Lonza). Samples were analyzed every day.

5.2.4 Pluronic F127-gelatin hydrogel

To improve cell viability inside the scaffold we studied the crosslinking of PF127 with porcine gelatin (gel strength 300, Sigma) (Kim et al., 2010) (Fakirov et al., 1997). In collaboration with the group of Dr. Miguel A. Mateos-Timoneda from the Biomaterials for Regenerative Therapies research group at the IBEC, a PF127-gelatin hydrogel was synthesized following published protocols (Park et al., 2008) (Kim et al., 2010).

Briefly, 5 g of PF127 were dissolved with 5ml of 1,4-Dioxane (Sigma) and stirred at RT for 24 h in a glass Florence flask in Argon (Ar) atmosphere. 24 mg of succinic anhydride (Sigma) were added to solution, together with a 48 mg of 4-dimethylamino pyridine (DMPA, Sigma) mixed with 42 mg of trimethylamine (TEA, Sigma) in 5ml of 1,4-Dioxane, and stirred at RT for 24 h in a Florence glass flask in an Ar atmosphere. Ether was added to solution to precipitate it, and then was filtrated. The resultant polymer was let to dry for 48 h at RT in a Florence glass flask with vacuum (Carboxylated PF127, Fig. 41). In parallel, a 0,1 M solution of 2-(N-morpholino) ethanesulfonic acid (MES buffer, Sigma) was prepared. 1 g of the processed PF127 was mixed with 25 ml of MES, and 1,185 g of gelatin type B (Sigma) dissolved in 20ml of MES. 9,01 mg of N-hydroxysuccinimide (NHS) together with 27,1 µl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma) were added to the PF127 solution together with the gelatin solution. The resulting mixture was stirred for 24 h at RT.

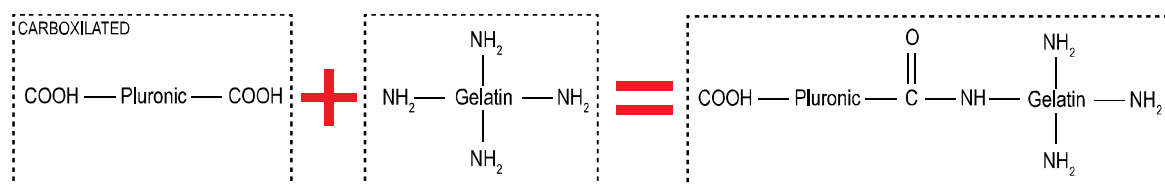


Fig. 41

Scheme of the synthesis of Pluronic-Gelatin thermoreversible hydrogel.

Solution was filtrated (dialysis) with a 24KDa membrane (Spectrum) at RT for 48 h, changing mili-Q water every 24 h. After dialysis, the resulting polymer (Fig. 41) was lyophilized, and a 15% (w/v) solution was prepared in mili-Q water.

5.2.5 *Hydrogel of Poly-DL-lactic-co-glycolic acid (PDLLGA) with polyethylene glycol (PEG)*

With the help of Dr. Miguel A. Mateos-Timoneda a new experiment was settled to achieve a new hydrogel that met previous stated requirements. 2,98 mg of PDLLGA (Purasorb[®]) were dissolved in 64,5 ml of dichloromethane (Sigma) at RT stirring overnight (ON). Solution was placed in a glass Petri dish and let solvent to evaporate for 24 h. The created film of PDLLGA was detached carefully from the Petri dish bottom and cut into pieces (~0,5 cm²). Pieces were hydrolyzed with a 0,5 N sodium hydroxide solution (NaOH) for 1 h at RT in a 24 well-plate and then mixed with a 0,1 M EDC and 0,02 M NHS solution in MES buffer (pH= 6,4) for 1 h at RT. Parallel to this, a 5 ml PEG (Sigma) solution in PBS 1X 100 mg/ml was added to the treated polymer and mixed for 2 h at RT, after this time supernatant was collected. Finally, samples were washed with mili-Q water and tried to dissolve in PBS.

5.3 Fabrication of engineered heart tissue (EHT)

5.3.1 Isolation of neonatal rat heart cells

All animal procedures were approved by the University of Barcelona Institutional Committee on Care and Use of Laboratory Animals. A total of 25 - 30 Sprague-Dawley neonates of 2-3 days were sacrificed by decapitation, their chests opened and hearts immediately extracted with tweezers and submerged in a calcium and bicarbonate-free Hank's Balanced salt solution (ThermoFisher) with HEPES solution (Sigma) (CBFHH) at 4 °C. Once all hearts were collected, ventricles were isolated and cut into small pieces (~1 mm³) and submerged in a dissociation buffer (trypsin 0,25% (Sigma) with 2 µg/ml DNAase I (ThermoFisher), in order to obtain a homogeneous cell suspension. Finally, cells were centrifuged 5 min 1200 rpm and pellet re-suspended in rat DMEM (Gibco) medium. Rat DMEM medium was prepared with DMEM Low-glucose volume (Gibco), adding 10% Horse Serum (Sigma), 2% Chicken embryo extract (US Biological), 1% Penicilin/Streptomycin (P/S) (Lonza).

5.3.2 Fabrication of EHT scaffolds

Neonatal rat cardiac cells were seeded onto the scaffolds following Dr. Zimmermann's protocol (Zimmermann et al., 2000) (Zimmermann et al., 2006). EHTs were prepared by mixing 210 µl of Rat tail collagen type-I at 2,3 mg/ml (Millipore) with 210 µl of fresh rat DMEM medium (Gibco), 160 µl of 0,1 N NaOH solution (Sigma) and 100 µl of a basement membrane protein mixture (Matrigel®, Corning). In parallel, rat cardiac cells (2,5 M to 3 M per scaffold) were mixed with 430 µl of rat DMEM medium and added to the mixture. A total volume of 1110 µl was poured in circular casting molds (Fig. 41 A-D) for 1 h at 37 °C 5% CO₂ in order to allow collagen and Matrigel to gel. After this time, fresh media was added to the molds until covering the scaffolds. Medium was changed every 2-3 days. EHTs were cultured for 7 in the casting molds, after this period, they were transferred to torsion spring stretchers (Fig. 41 B-C), in order to assess uniaxial auxotonic stretch.

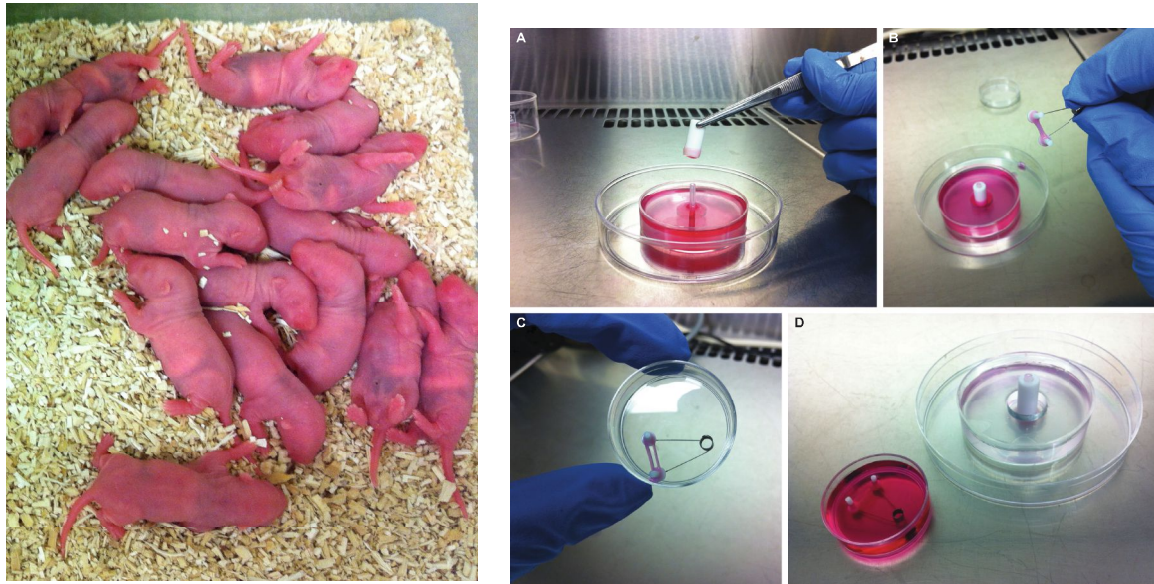


Fig. 42

Left: neonatal P2-P3 rat pups. Right: (A) PTFE cylinder with the EHT ring attached on the lower part and casting mold. (B) EHT transferred to the torsion spring. (C) Detail of the EHT ring on the torsion spring. (D) Left: Torsion spring cultured on a 6cm Petri with medium, and Right: casting mold. Re-escribe este pie de figura, mira los papers de Zimmerman

5.4 Fabrication of ring-shaped human engineered keratinocyte iPSC-derived cardiomyocyte (iPSC-CM) cardiac tissue

5.4.1 In-vitro differentiation of Keratinocyte induced pluripotent stem cells (KiPSC)

KiPSC were thawed and seeded on a 10 cm Petri dish previously coated with 1:40 Matrigel® in DMEM, and maintained with conditioned HES medium (CHES). CHES medium is the supernatant of irradiated mouse embryonic fibroblasts (MEFs) collected each 24 h. MEF medium was prepared by mixing 385 ml of KO-DMEM (Gibco) with 100 ml of KO-Serum (Gibco), 5 ml of Glutamax 100X (Gibco), 5 ml of P/S 100X (Lonza), 0.5 ml of 2-mercaptoethanol 50mM (Gibco), and 50 µl of Basic recombinant human fibroblast growth factor (FGF) 100 µg/ml (Peprotech).

KiPS were then cultured until a confluent state was observed in the Petri dishes, and then detached with Accutase® (ThermoFisher), counted and seeded (day -3) in a concentration ranging from 350K to 600K cells/well, into 6-well plates previously treated with 1:40 Matrigel®. Cells were maintained for 3 days in the wells with mTeSR medium (Stemcell) with 5X supplement (Fig. 43).

At day 0, cells medium was changed for RPMI-1640 (Life Technologies) with B-27 supplement without insulin (Life Technologies) (RPMI/B27-insulin) and CHIR-99021 (Selleckchem) at 12 µM. After 24 h medium was changed for RPMI/B27-insulin. At day 3 medium was changed for RPMI/B27-insulin with IWP4 (Stemgent) at 5 µM. At day 5 medium was changed for RPMI/B27-insulin. Cells were maintained in RPMI/B27 starting from day 7, with the medium changed every 3 days. On days 9-10 of differentiation spontaneous beating clusters may appear, which were the first KiPSC-derived cardiomyocytes (KiPSC-CM).

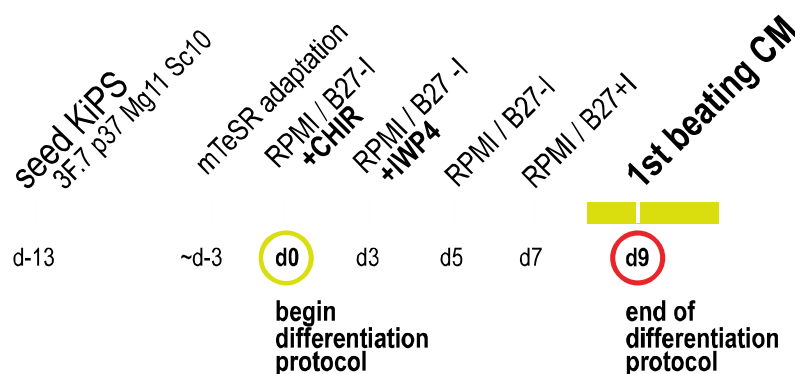


Fig. 43
Experimental cardiac differentiation protocol timeline for generating iPSC-CM.

5.4.2 Fabrication of iPSC-CM ring-scaffolds

Ring-shaped KiPSC-scaffolds were fabricated following same procedure as with rat cardiac cells. Briefly, 210 µl of Rat tail collagen type-I at 2,3

mg/ml were mixed with 210 µl of fresh RPMI/B-27, 160 µl of NaOH 0,1 N solution, 100 µl of Matrigel[®], and 430 µl of RPMI/B27 medium with the KiPSC-CM suspension, from 2,5 to 6M cells per scaffold (depending on protocol efficiency), with a 10% of human foreskin fibroblasts (HFF). The resulting blend was then incubated in the molds for 1 h at 37 °C 5% CO₂ in order to allow collagen and Matrigel to gel, and then fresh RPMI/B27 medium was added until covering the scaffolds. Medium was changed every 2-3 days. Scaffolds were cultured for 7 days in the molds, and then transferred to the spring stretchers for another 7 days.

5.5 Mechanical stimulation of rat engineered heart tissues (EHTs) and ring-shaped scaffolds with iPSC-CM

Rat engineered heart tissues were subjected to mechanical stimulation 7 days after their culture in the casting molds. Two torsion springs were used to stimulate the EHTs, the original torsion spring designed by Dr. Zimmermann (Godier-Furnémont et al., 2015) (generously donated by Dr. Galiñanes, Vall d'Hebrón Institut, de Recerca (VHIR), Barcelona) and a torsion spring fabricated in our laboratory with a similar force (N) than the original one (Fig. 44 and 45). Human iPSC-CM rings were stimulated with auxotonic stretch on the springs during 7 days. Finally, EHTs were fixed in 4% paraformaldehyde (PFA) solution overnight (O/N) at 4°C.

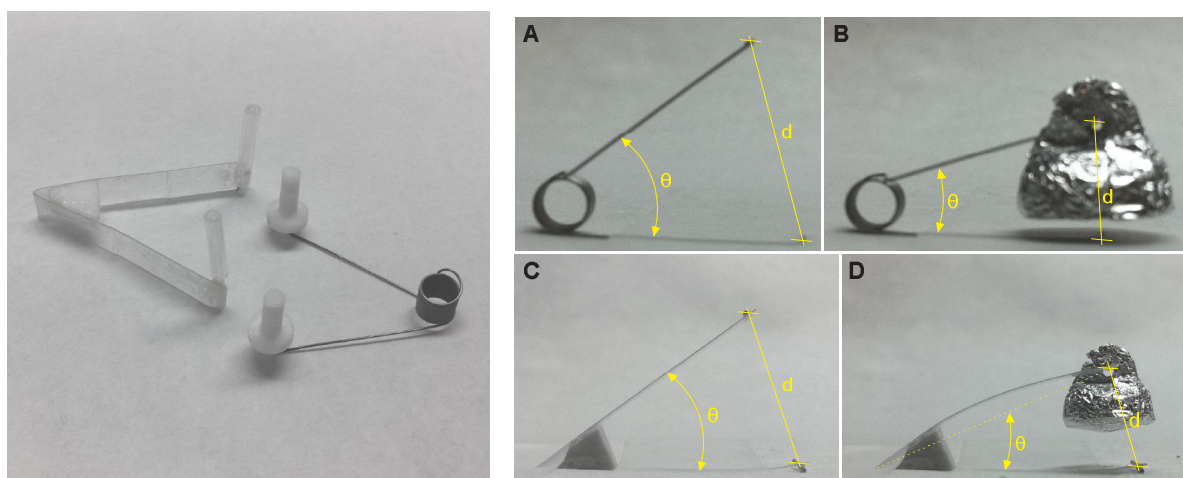


Fig. 44

Left: Torsion springs. Spring on the left: plastic spring designed and fabricated in our laboratory. Spring on the right: spring generously donated by Dr. Galiñanes, Vall d'Hebrón Research Institute. Right: Force estimation study for our custom made spring (A) Original spring relaxed: $\theta = 40^\circ$, $d = 18$ mm. (B) Original spring bended with $m = 0.5$ g ($F = 5$ mN), $\theta = 18^\circ$, $d = 9$ mm. (C) Custom-spring relaxed: $\theta = 38^\circ$, $d = 18$ mm. (D) Custom-made bended $m = 0.5$ g ($F = 5$ mN), $\theta = 21.5^\circ$, $d = 11$ mm.

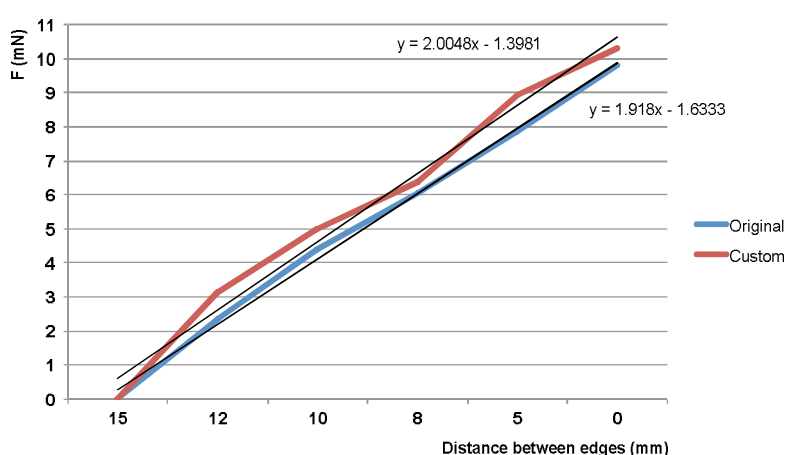


Fig. 45

Force - deformation graphic plotting experimental data comparing both torsion springs, where force is correlated with the distance between the edges of each stretcher (deformation = distance). In blue, original spring, in red, custom-made spring. Experiment

5.6 Mechanical stimulation of the biomimetic stretching device (BSD) with iPSC-CM cardiac tissues and rat cardiac tissues

To improve the mechanical stimulation of cardiac tissues, a 3-dimensional (3D) membrane-based biomimetic stretching device (BSD) was designed in order to reproduce the cyclic pumped stimulation of the native heart, and hence, apply a more biomimetic stimulation to the scaffolds. BSDs were fabricated with polydimethylsiloxane (PDMS) (Sylgard® 184, Dow Corning) in circular molds (~18 mm) all-in-one piece. The resulting silicon piece (basically a toroid with a top membrane) was then integrated by attaching the inferior surface of the toroid on a well plate. Each BSD was connected to an electronically-controlled pneumatic pump through a silicone tubing allowing air flow into the device (Fig. 46). Plates were sterilized with 70 % ethanol during 30 minutes before their use.

BSD scaffolds were prepared as previously described for EHTs, 210 µl of Rat tail collagen type I at 2,3mg/ml (Millipore) were mixed at 4 °C with 210 µl of fresh medium, 160µl of NaOH 0,1 N solution (Sigma), 100µl of Matrigel® (Corning), and 430µl of medium with approx. 2,5 to 5 M cells (iPSC-CM or neonatal rat cardiac cells in previous experiments). The mix was pipetted on top of the membrane device and incubated 1 hour at 37 °C, 5% CO₂, in order to induce gelification.

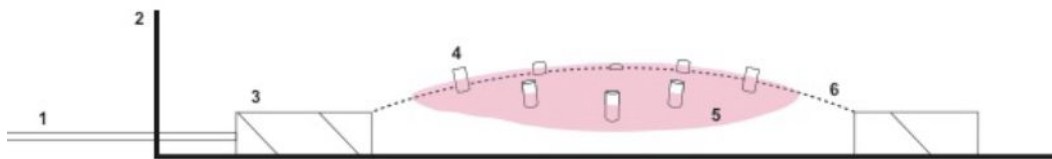


Fig. 46

Schematic representation of the definitive BSD components: 1. Air-flow tube, 2. Petri dish, 3. Silicone-made toroidal base of the BSD, 4. Anchoring posts, 5. Scaffold, and 6. Stretchable silicone membrane.

The pneumatic system (all components supplied by Festo) (Fig. 46.1) is composed of a filter unit at the beginning, in order to keep the air clean, then an electro-valve connected to an electric timer, which opens/closes at the desired time periods (ms), a flow valve, which regulates the final flow rate, and a distributor, to pump various devices at the same time. The system was directly connected to the airflow tap (P= 3 Bar).

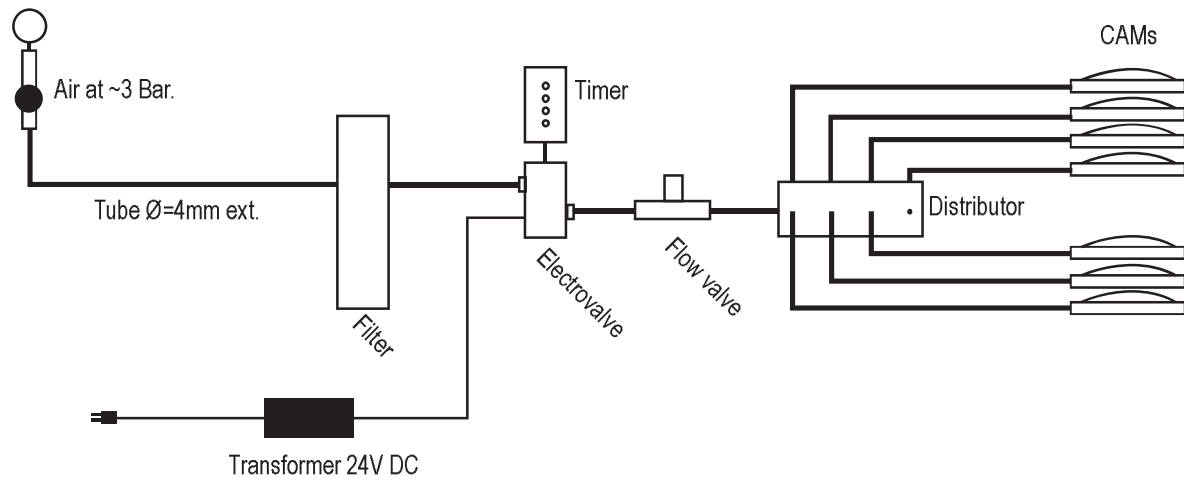


Fig. 46.1
Scheme of pneumatic circuit to pump the BSDs.

The frequency (1 Hz, 350 ms inflow, 650 ms outflow) was controlled by the timer connected to the electro-valve, and the strain* of the membrane ($\epsilon=6-10\%$) was controlled through the flow-valve opening/closing (Fig. 47).

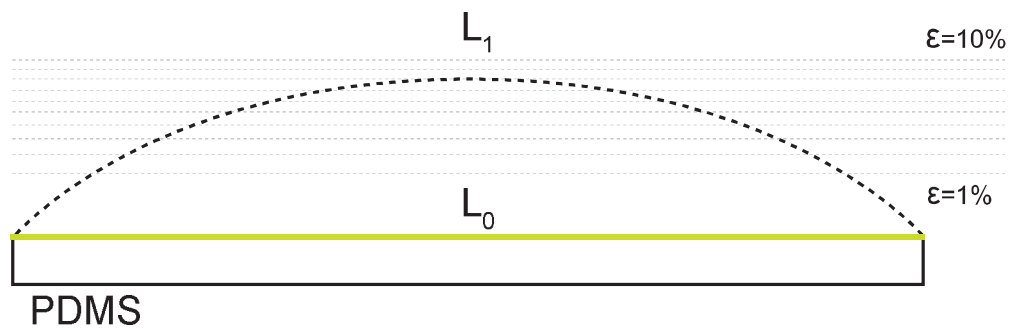


Fig. 47
Scheme of membrane deformation, from 1% to 10% (grey lines) of its original diameter length, $L_0=13\text{ mm}$.

5.7 Characterization of engineered iPSC-CM cardiac tissues and rat EHTs

5.7.1 Immunohistochemistry

Ring-scaffolds were fixed with 4% PFA at 4 °C O/N. Then were cryoprotected in 30% sucrose (Sigma) (4 °C, 6 h), embedded in OCT compound (Tissue-Tek®) and frozen at -80 °C. For histological analysis 50 µm sections were performed. Ring-shaped scaffolds were oriented in the freezing boxes in the desired position (Fig. 48). In contrast, BSD scaffolds were treated *in-toto*.

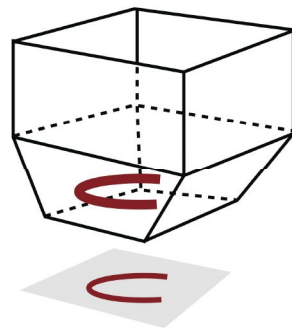


Fig. 48

Left: Representative scheme of a ring-scaffold processing. Right: image of the frozen block with the scaffold being cut in sections of 50 µm. in the cryostat.

Sections were washed with 1X TBS (Sigma) for 15 minutes at R/T and blocked with 0.3% Triton-X100 (Sigma) and 3% donkey serum for 2 hours at R/T, scaffolds were incubated at 4°C O/N with the primary antibodies diluted in TBS.

Table 1

Antibodies used for immunofluorescence techniques for characterization of CMs.

Type	Antibody	Host	Manufacturer	Dilution
Primary	Troponin T	Mouse	Sigma-Aldrich	1:100
Primary	Connexin-43	Rabbit	Sigma-Aldrich	1:250
Secondary	Anti-mouse IgG Cy3	Donkey	Invitrogen	1:200
Secondary	Anti-rabbit IgG Alexa Fluor 488	Donkey	Invitrogen	1:200

After three washes of 30 minutes with 0.3% Triton-X100 (Sigma) and 3% donkey serum, Alexa Fluor-488 donkey anti-rabbit and Cy3 donkey anti-mouse (Invitrogen) secondary antibodies were diluted in the same solution as primary antibodies and incubated at 4°C o/n. Scaffolds, were washed thrice with 1X TBS for 15 minutes and incubated with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, 1:5000) (Sigma) 2h at RT to stain. Finally, samples were washed thrice with 1X TBS and mounted in glass-slides with Mowiol 4-88 (Sigma). All photographs were taken on a Leica SP5 laser confocal microscope (Leica) and analyzed with a computerized system (Image-J). For determining cardiac maturation, ten random pictures were taken with a 40X magnification objective.

5.7.2 Morphological evaluation of the scaffolds

Immunofluorescence images were acquired for each scaffold of representative regions. For each image, cell length, cell alignment and abundance of Z-bands were analyzed following this procedure:

Cell length

CMs were measured with Image-J software, setting a known scale and drawing a line from one extreme to the another of the cytoplasm following the longitudinal axis of the cell. Arithmetic media ($\bar{X}$) was calculated for each image and then for each stimulating condition, and plotted in a graph representing the average $\pm$ SEM (Fig. 49)

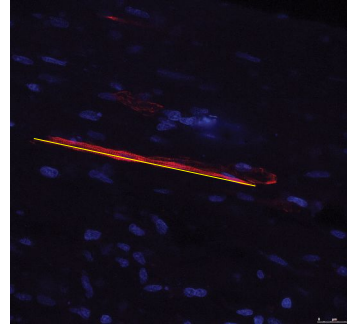


Fig. 49

Immunofluorescence image of a CM stained with cTnT (red) and DAPI (blue), representing the line used to measure each CM of every image to evaluate cell length.

Cell alignment

CM alignment was measured with Image-J software setting a known scale, as previously described for cell length. When drawing the line, the software also provided the value of the angle ($^{\circ}$) of the drawn line in relation to the horizontal axis. All values were statistically analyzed and plotted in a graph (Fig.57).

Presence of Z-bands

CMs in the scaffolds subjected to mechanical stimulation exhibited more numerous and well-defined Z-bands than CMs in the control condition scaffolds. For evaluating the presence of Z-bands, TnT was stained and CMs were separated into 3 groups for each immunofluorescence image:

NO Group: positive for TnT, but not exhibiting any Z-bands at all (Fig.50A).

SEMI Group: positive for TnT, and exhibiting Z-bands only in certain parts of the cytoplasm. CMs showed organized sarcomeres but only present in determined regions of the cell, not constituting a continuous structure along them (Fig.50B).

ALL Group: positive for TnT, Z-bands were shown throughout the entire cytoplasm of the CM. Organized and parallel sarcomeres could be easily observed along the cell, being a continuous structure from one end to another of each CM (Fig.50C).

Analyzed cells were plotted in a graph in 3 different groups, as a relative percentage of the counted population for each condition.

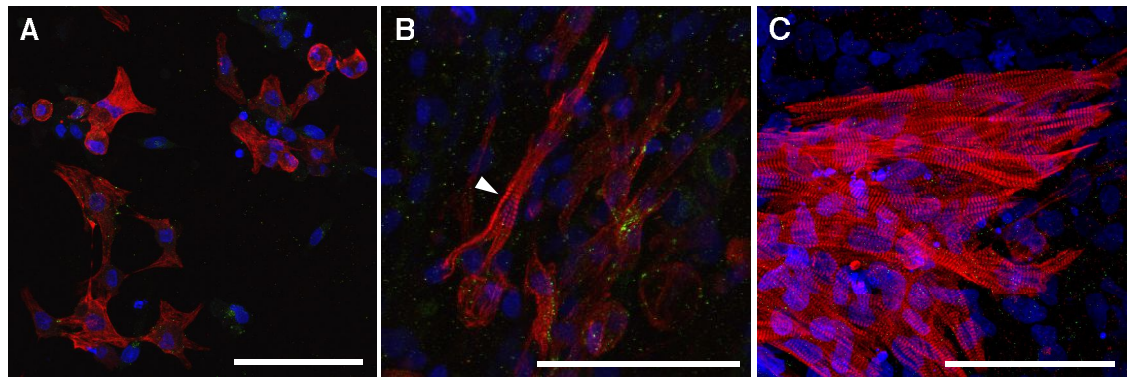


Fig. 50
 Immunoconfocal image of CMs stained with TnT in red, Cx43 in green, and DAPI in blue. (A) NO group, where CMs do not show any Z-bands. (B) SEMI group, where CMs are partly filled with Z-bands, and (C) ALL group, where CMs show Z-bands throughout the entire cytoplasm. Scale bar= 75 μ m.

5.7.3 *Transmission Electron Microscopy (TEM)*

Samples of scaffold of $\sim 1 \text{ mm}^3$ were fixed in 2.5% glutaraldehyde (GTA) solution (Electron Microscopy Sciences, EMS) and cacodylate 0.1 M for 2 hours at 4 °C. After 3 washes of 15 minutes in cacodylate, scaffolds were gradually dehydrated with ethanol, and embedded in epoxy resin (Ted Pella). Semi-thin sections (250 nm) were cut with an ultramicrotome (Leica UC6) and mounted onto slides and stained with 1% toluidine blue (Panreac). Finally, ultra-thin sections (80 nm) were prepared with the ultramicrotome and contrasted with uranyl acetate (EMS) and lead citrate (EMS) and studied in the electronic microscope (JEOL 1011).

5.7.4 Quantitative Polymerase Chain Reaction (qPCR)

A piece of each scaffold was cut (~1 mm³), total RNA was isolated using TRIZOL (Sigma) and 1 µg was used to synthesize cDNA using the SuperScript-III kit (Invitrogen). 5 µl of the reaction were mixed 1:5 with milli-Q water and 1 µl of this volume was used to quantify gene expression by qPCR.

Cardiovascular gene expression

RNA isolation was performed as described above. Cardiac specific markers were evaluated using the primers indicated in Table 2 and *Gapdh* (Sigma) was included as housekeeping gene.

Table 2

qPCR primers for cardiovascular markers. cTNT: cardiac Troponin T; RYR2: Ryanodine receptor 2; CX43: Connexin-43; MYH6: α -Myosin heavy chain; MYH7: β -Myosin heavy chain; ACTC1: α -Actinin cardiac muscle; CACNA1C: Calcium voltage-gated channel; MLC2V: Myosin light chain 2 ventricular.

Cardiac	Sense	Antisense
cTNT	TGCAGGAGAAGTTCAAGCAGCAGA	AGCGAGGAGCAGATCTTTGGTGAA
RYR2	ACAGCACAAGCCATTCTGCAAGAC	ATGTAATCCAGCCCACCCAGACAT
CX43	CAATCACTTGGCGTGACTTC	CCTCCAGCAGTTGAGTAGGC
MYH6	ATTGCTGAAACCGAGAATGG	CGCTCCTTGAGGTTGAAAAG
MYH7	GCATCATGGACCTGGAGAAT	ATCCTTGCCTTGAGAGCATT
ACTC1	GCTCTGGGCTGGTGAAGG	TTCTGACCCATACCCACCAT
CACNA1C	TTTGGTCCATGGTCAATGAG	GCATTGGCATTTCATGTTGG
MLC2V	CAACGTGTTCTCCATGTTCTG	GTCAATGAAGCCATCCCTGT

5.7.5 Electrical coupling of the scaffolds

It has been seen how CMs begin spontaneously their beating when they are cultured. In our scaffolds, CMs (rat or human) normally augmented the beating synchronization between them progressively, from the day we fabricated the scaffolds (d1) until the day we finished the experiment (d14).

These contractions were usually observed in certain zones of the scaffold rather than in the entire construct. Comparing 2D cultures with 3D cultures, cells in 2D normally exhibit great clusters of CMs beating coordinated at the same time, sometimes even a great monolayer of cells (almost covering the entire well) could be found to beat synchronously. In 3D, as our scaffolds were, this behavior was observed to be partly altered. As cells could establish more complex networks between them, it was rare to find great regions of synchronized beating.

Scaffolds were stimulated with electrical pulses at the end of the experiment in order to test external electrical coupling. For such duty, we designed custom-made electrical chambers with carbon rods and gold wire (Fig.49), based on electrical systems published in the literature (Radisic et al., 2009) (Tandon et al., 2009) (Llucià-Valldeperas et al., 2013). Electrical pulses were applied to the scaffolds using a custom made programmable biphasic electrical stimulator system designed by Dr. Ramon Bragós group (Electrical Engineering department of the Polytechnic University of Catalunya, UPC) and a function

generator (Agilent 33250A, Agilent Technologies) provided by María Valls (Institute for Bioengineering of Catalunya, IBEC).

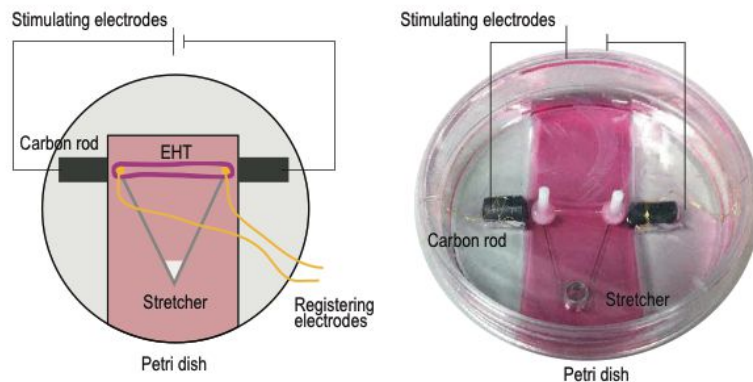


Fig. 49

Left: scheme of the electrical chamber for the torsion springs. Right: actual image of the electrical chamber designed to apply electrical pulses to the springs.

Electrical stimulation parameters (Fig.50) used for scaffolds were: square wave pulsed at a range of frequencies from 0.5 to 4Hz, with a voltage amplitude ranging from 3 V to 50 V (monophasic or biphasic), a pulse width (PW) duration of 2 ms to 100 ms, and a distance between stimulating electrodes of 2 cm.

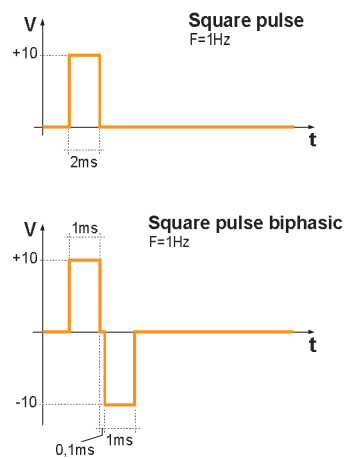


Fig. 50

Scheme of the square pulses applied to the scaffolds, monophasic (top) and biphasic (bottom) pulses respectively.

According to the literature, Drs. Eschenhagen & Zimmermann applied to their EHTs square pulses of 1 - 2 Hz, 5 ms, 80 - 100 mA (Zimmermann et al., 2002) (Eschenhagen et al., 1997). Nevertheless, other authors like V-N. Gordana *et al.* or M. Radisic *et al.* have interesting studies on the optimal electrical stimulation values (Radisic et al., 2009) (Tandon et al., 2009) (Miklas et al., 2014) (Nunes et al., 2013), where they suggest that an electric field of 5V/cm is an optimal parameter to apply to a cell culture. We decided to follow this guidance in order to try to establish our own suitable values for the scaffolds.

Studies of energy loss were also developed (Fig.51) with recording electrodes and an oscilloscope (DSO3062A Digital Storage Oscilloscope, Agilent technologies) in order to measure the amount of the potential (V) that actually

reached the scaffold, as the electric field decreases from electrode to electrode whilst culture media dissipates considerably part of the energy.

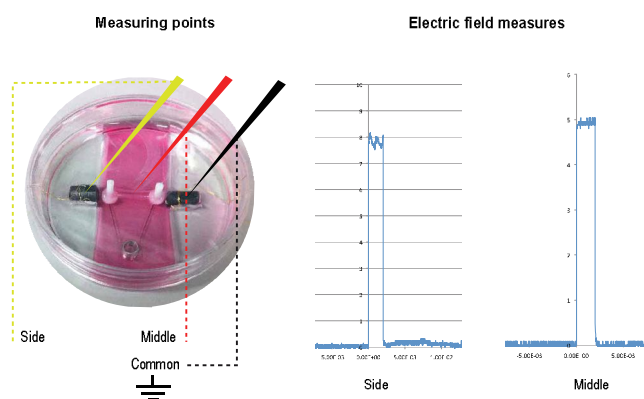


Fig. 51 Position and measurements of the recording electrodes in the electrical chamber. Left: Scheme of the position of the electrodes in the electrical chamber with a ring scaffold on the stretcher. Right: For a voltage of 10 V, when the recording electrode was placed at the other carbon rod (side), the electrodes registered actually 8V. When the recording electrode was placed in the center of the scaffold (middle), the electrode registered 5V. This is due to a loss of energy for the applied electric field.

5.7.6 Flow cytometry

To test the efficiency of cardiac differentiation process, 2.5×10^5 hiPS-derived cardiomyocytes at day 19 – 20 of the differentiation protocol, before fabricating the scaffolds, cells were detached with accutase, fixed with PFA 4% and stained with Troponin-T (TnT) and α -actinin sarcomeric (AAS) antibodies. After incubation for 30 minutes at R/T, cells were analyzed with FACS MoFlo (Beckman Coulter) and data acquisition and analysis performed by flow cytometry analysis software Kaluza (Beckman Coulter).

5.7.7 Calcium imaging

For optical measurements, scaffolds were incubated and stained for 2 hours (37 °C 5% CO₂) with a 2 μ M solution* of Cal-590 (AAT Bioquest) in HBSS. Then, tissue constructs were washed twice with PBS from dye working solution to remove excess probes, and replaced with RPMI/B27. Dye fluorescence was excited at 540 nm with the laser of a confocal microscope (Leica TCS SP5), and emitted fluorescence was measured at >590 nm at 37 °C 5% CO₂. Video sequences of ~10 seconds were recorded for each scaffold by capturing spontaneous contractions.

*Cal590-DMSO stock solution: 2 mM Cal-590 (AAT Bioquest) in DMSO (Sigma).

5.7.8 Fractional Area Change

Videos of the beating scaffolds were taken with 5X objective under a stereomicroscope (Leica MZ10 F) and a color CCD BSDera (Leica DFC 400C). Recordings were then imported in the software (Image-J) and ten random beating regions of interest (ROI) were analyzed by measuring the area at the beginning and at the end of the each contraction. Fractional area change was analyzed with Image-J software and variation of the areas obtained by calculating the percentage of contraction from the relaxed state and the contracted state.

Temporal deformation field (TDF) was also calculated for some samples in collaboration with Prof. M. A. González from Dept. of Information and Communication Technologies, University Pompeu Fabra (UPF), by analyzing original captured videos frame to frame, and compensating the global displacement of the scaffold with a custom made software (Matlab). The TDF was then applied to a grid to illustrate displacement, and a randomly selected point in the grid was plotted in X and Y axis to evaluate displacement.

5.8 Transplantation of BSDs with iPSC-CM in a mouse model of acute myocardial infarction (MI)

A total of 48 female severe combined immunodeficiency mice of 13 week-old (20-25 g; Charles River Laboratories, Inc.) were used for this study, performed in collaboration with Dr. C. Soler-Botija from *Institut d'Investigació Trias i Pujol*, Barcelona. The animals were randomly assigned to one of the following groups: (1) MI induction only (the negative control group, n= 12), (2) MI induction and stimulated scaffold implantation (the stimulated implant group, n= 12), (3) MI induction and non-stimulated scaffold implantation (the control implant group, n= 12), (4) MI induction and implantation of collagen-only scaffold (the negative control scaffold group, n= 12).

5.8.1 Surgical procedure for induction of MI and tissue grafting

For surgical procedure, animals were anesthetized with 2% isoflurane, placed on a heating table in supine position and endotracheally intubated for mechanical ventilation with supplementary oxygen. During surgery, anesthesia was maintained with a combination of 3% isoflurane and 0.01 mg/kg/ fentanyl (Fentanest, Kern Pharma). A thoracotomy was then performed at the left three intercostal space, the pericardium was opened and the left anterior descending (LAD) coronary artery ligated with a 7.0 absorbable suture. Myocardial ischemia was confirmed by color change of the left ventricular wall. Scaffolds were then implanted using synthetic surgical glue (Vetbond™) sticking one extreme on the healthy myocardium and covering with the rest the entire damaged area.

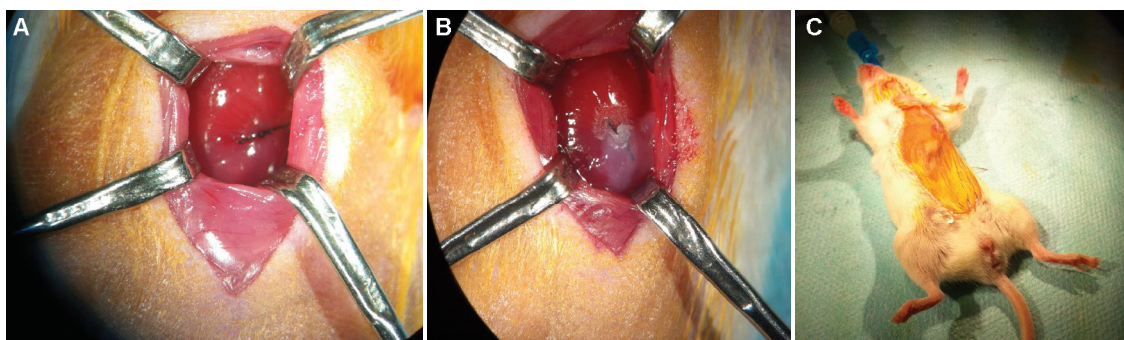


Fig. 52

(A) Ligation of the descending anterior coronary artery for the acute infarct model provocation. (B) Grafting of the scaffold over the infarcted site with surgical glue. (C) Post-surgery recovering mouse.

5.8.2 Histological evaluation

4 weeks post-implantation hearts were arrested in diastole with cardioplegic solution (68,4 mM NaCl, 59 mM KCl, 11,1 mM glucose, 1,9 mM NaHCO₃, 29,7 mM 2,3-butanedione monoxime, 1000U heparin), excised, fixed, and cryopreserved in 30% sucrose in phosphate-buffered saline (Sigma). Hearts were then cut in 3 pieces (Fig. 53) and embedded in Tissue-Tek® OCT (Sakura), and snap-frozen liquid nitrogen-cooled isopentane. Tissue blocks were stored at -80 °C until sectioning (10 μ m thick). All animal handle procedures were approved by the Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Research (NIH Pub. No. 86-23, Revised 1996).

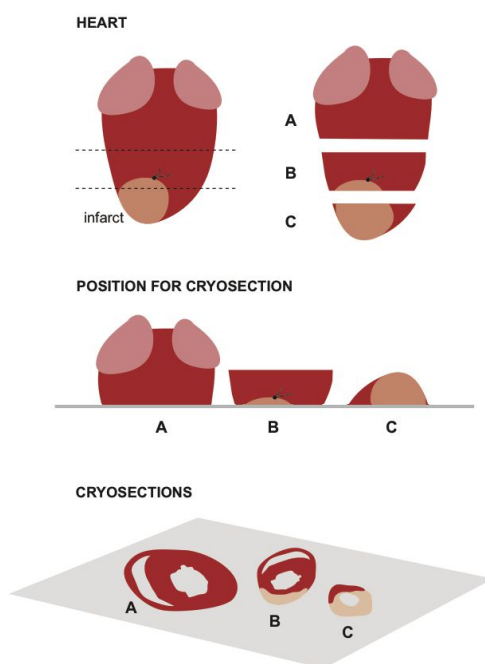


Fig. 53

Scheme of dissection of the hearts for the determination of the infarction size and histological evaluation, where A is the base, B de middle, and C the apex of the heart. The light brown colored area represents the infarcted site.

5.8.3 Cardiac function determination

Transthoracic echocardiography was performed under light sedation (1% isoflurane in O₂) (Isoflo®, ABBOTT S.A, Madrid, Spain) 2 days before surgery (baseline), 2 days after surgery (MI), and at 4 weeks after surgery (pre-sacrifice). A digital ultrasound system (Vevo 2100 Imaging System, VisualSonics) equipped with an 18-38 MHz linear-array transducer was used to take measurements. Images were obtained in B-Mode and M-Mode in the parasternal long-axis (PLAX) and short-axis (SAX) views. All data was acquired and measured by an investigator who was blinded to the treatment groups. Conventional functional parameters were measured, including left ventricle fractional shortening (FS), left ventricle ejection fraction (EF), left ventricle anterior wall, left ventricle posterior wall, left ventricle end-diastolic dimension, and left ventricle end-systolic dimension.

5.8.4 Determination of the infarction size

Mouse hearts were cross-sectioned as previously described (10µm thick sections). Sections were stained with Masson's trichrome and morphometric parameters were determined using image analysis software (Image-J, NIH) (Fig. 54). Infarct size volume, expressed as a percentage of total LV wall volume, was calculated by the sum of partial scar volumes between sections. Infarct size surface was calculated by the sum of partial endocardial infarct areas between sections and expressed as a percentage of total LV endocardial wall surface (infarct area x100 / total LV area= % infarct).

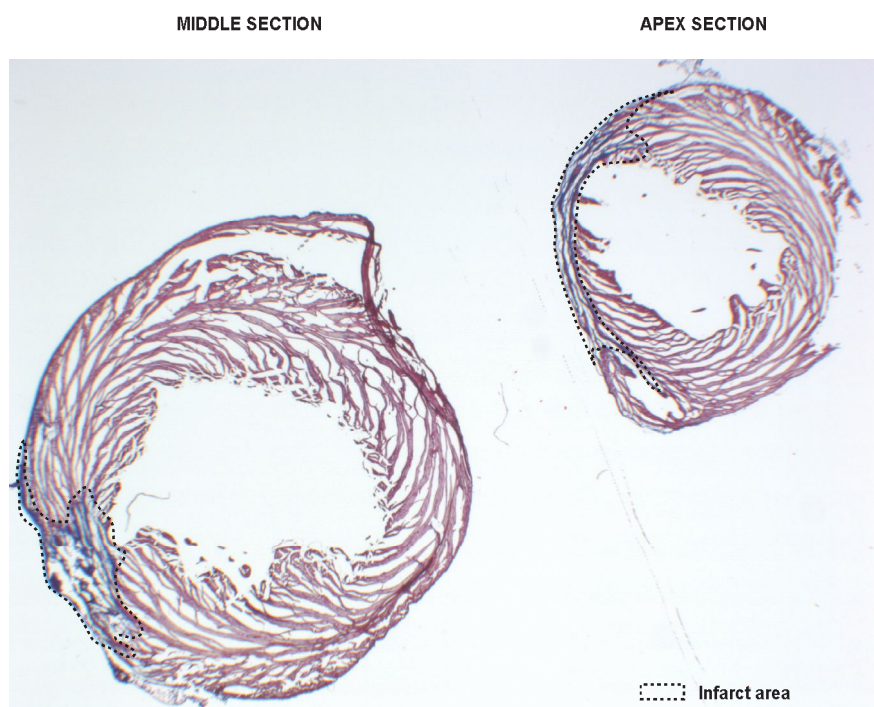


Fig. 54
Histological sections of heart's middle part and apex, stained with Masson's trichrome, showing the infarcted areas measured for each sample.

5.9 Statistical analysis

All statistical analysis were performed with GraphPad Prism 6.0 software (GraphPad Software, Inc.). Experimental data were expressed as mean $\pm$ SEM. In case of normal distribution, comparison between groups were performed using Student's T-test or one-way ANOVA followed by Bonferroni's post-hoc test. Differences were considered statistically significant when $p < 0.05$.

6. Results

6. RESULTS

6.1 Analysis of the porcine myocardium stiffness

Tensile strength assays were performed in order to obtain an elastic bulk value of the porcine myocardium, expected to be similar to that of the human heart. Young's Modulus (MY) was calculated in porcine fresh heart tissue. Porcine myocardium samples with 3 fiber orientations were analyzed (Fig. 50A). The slope of each curve was analyzed by considering only the loading section of the curve (of each stretch-relaxation cycle) (Fig. 50B). Some hysteresis appeared in relaxation part of all analyzed samples. The obtained MY value (or elasticity), which defines the relationship between the stress (Pa) and the strain (%) of a sample, was observed to vary in relation to muscle fiber orientation.

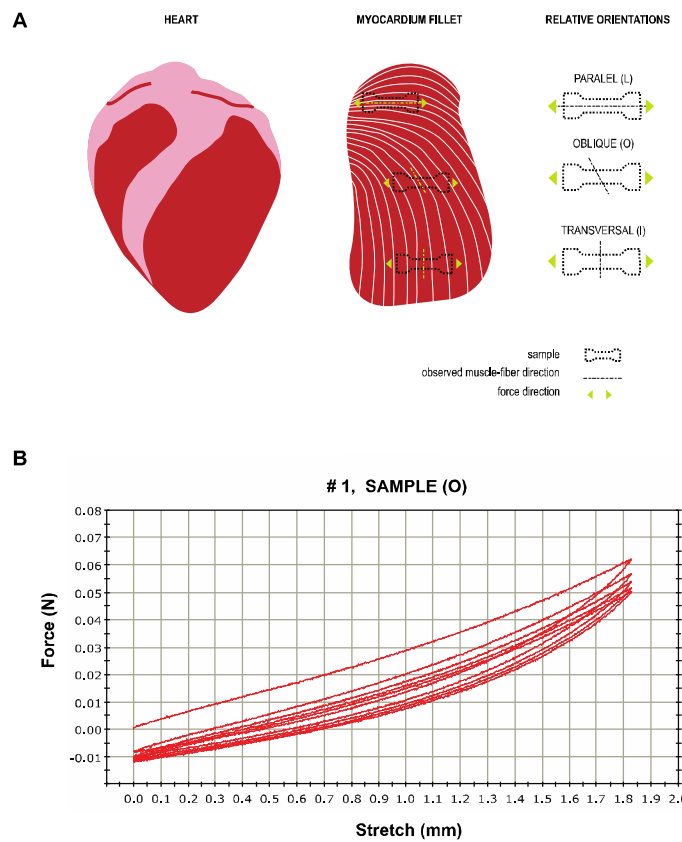


Fig. 50

(A) Representative scheme of the 3 different muscle fiber orientations of the heart samples. (B) Force (N) - stretch (mm) graph obtained from an oblique sample of heart #1, showing the 5 loading - relaxation cycles of the tensile strength assay. Each curve exhibits a marked hysteresis in the relaxation-section of the curve.

Results show great heterogeneity between hearts. Results of MY were plotted for each muscle fiber direction considering the 5 specimens, in order to see if there were significant differences between subjects (Fig. 51A-C). Statistical differences were found between the different hearts for each muscle fiber orientation (Table 8), but for the parallel orientation, in which the majority did not show any differences.

Besides, the mean of all MY values considering the muscle fiber orientations of the 5 specimens were plotted, in order to know the differences between fiber directions considering all subjects. Interestingly, statistical differences appeared only between the

transversal direction and the other two directions (Fig. 51D): $\bar{X}_{\text{transversal}} = 13,22 \text{ kPa}$, $\bar{X}_{\text{oblique}} = 19,45 \text{ kPa}$, and $\bar{X}_{\text{longitudinal}} = 32,20 \text{ kPa}$ respectively.

The total medium value (considering the 3 relative orientations for all hearts) was also calculated, $\bar{X}_{\text{TOTAL}} = 21,62 \text{ kPa}$, which was considered the general stiffness value for porcine myocardium.

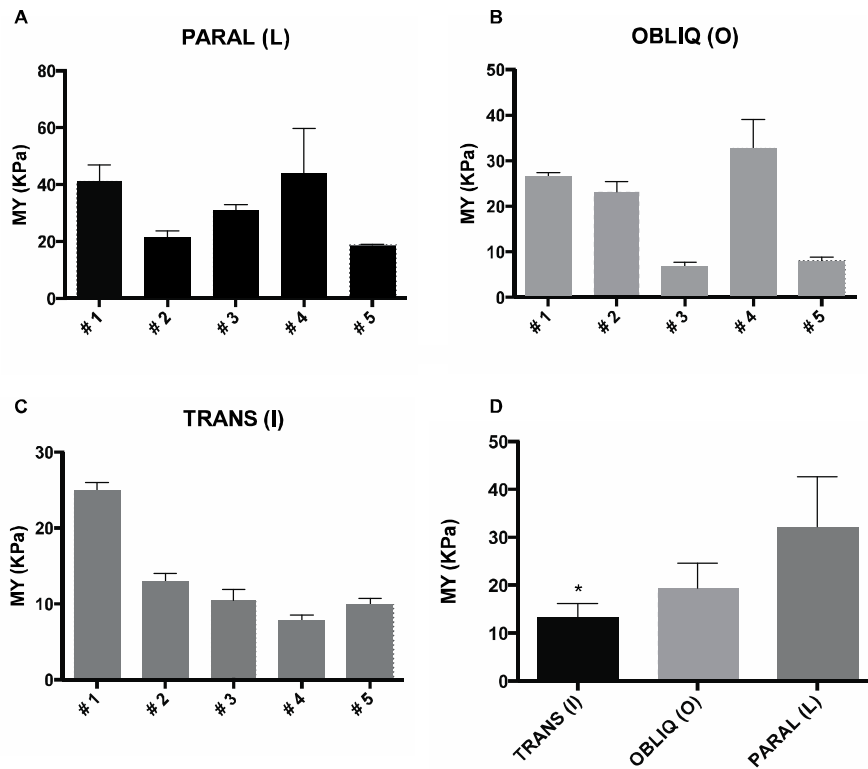


Fig. 51

Different muscle fiber orientation measures by tensile strength assays. (A) YM values for longitudinal samples (L: parallel to the muscle fiber direction). (B) YM values for oblique samples (O: 45° to the muscle fiber direction). (C) YM values for the transversal samples (I: perpendicular to the muscle fiber direction). (D) YM values of each relative orientation considering the 5 hearts. Statistical differences were only found between the transversal (I) group and the longitudinal (L) group, no statistical differences were found when the oblique (O) group was compared. (Data expressed as mean $\pm$ SEM, n= 5, *p< 0.05)

Table 8

Mean values for each heart considering muscle fiber orientations (top) and (down) table showing statistical differences found between hearts for each relative orientation (One-way ANOVA followed by Bonferroni's tests). Data expressed as mean $\pm$ SD, n= 5, *p< 0.01)

YOUNG'S MODULUS VALUES (kPa)

Heart	TRANS (I)	OBLIQ (O)	PARAL (L)	Mean
#1	24.90 $\pm$ 1.13	26.49 $\pm$ 0.89	42.94 $\pm$ 5.77	31.45 $\pm$ 9.99
#2	12.87 $\pm$ 1.10	23.17 $\pm$ 2.27	22.51 $\pm$ 2.15	19.18 $\pm$ 5.53
#3	10.52 $\pm$ 1.35	6.80 $\pm$ 0.89	30.91 $\pm$ 2.06	16.08 $\pm$ 12.98
#4	7.85 $\pm$ 0.66	32.83 $\pm$ 6.19	43.95 $\pm$ 15.80	28.21 $\pm$ 18.49
#5	9.95 $\pm$ 0.72	7.98 $\pm$ 0.87	18.71 $\pm$ 0.25	12.21 $\pm$ 5.72
Mean	13.55 $\pm$ 6.51	19.22 $\pm$ 10.55	29.35 $\pm$ 8.89	

STATISTICAL DIFFERENCES

	TRANS (I)	OBLIQ (O)	PARAL (L)
#1 VS #2	Yes	No	No
#1 VS #3	Yes	Yes	No
#1 VS #4	Yes	No	No
#1 VS #5	Yes	Yes	No
#2 VS #3	No	Yes	No
#2 VS #4	Yes	Yes	No
#2 VS #5	No	Yes	No
#3 VS #4	No	Yes	No
#3 VS #5	No	No	No
#4 VS #5	No	Yes	Yes

Elasticity mean values obtained from our study seem coherent with others published in the literature (McGarvey et al., 2016) (Pislaru et al., 2014), but there is still great variability from specimen to specimen, as previously stated, fact that suggests the great heterogeneity that real specimens can exhibit.

6.2 Generation of hydrogel scaffolds with different polymers

6.2.1 Analysis of Pluronic F127 mechanical behavior

Rheological shear stress tests were performed in order to find out hydrogels mechanical behavior. The rheological sweep tests (stress and frequency) showed an increase in hydrogel's stiffness comparing both concentrations. The medium value obtained of G' (where $E = G' + G''$) for the PF127 in PBS: was 10 kPa for the 25 % (w/v) hydrogel in stress and frequency tests (Fig. 52A & B), and ~9 kPa for the 20 % (w/v) hydrogel in both tests (Fig. 52C & D). Moreover, both hydrogels showed a reversible sol-to-gel transition at 15 °C and 19 °C, respectively. These values are close to the ones obtained for porcine myocardium and therefore PF127 represented a suitable candidate in terms of mechanical behavior, for the design of a myocardial graft

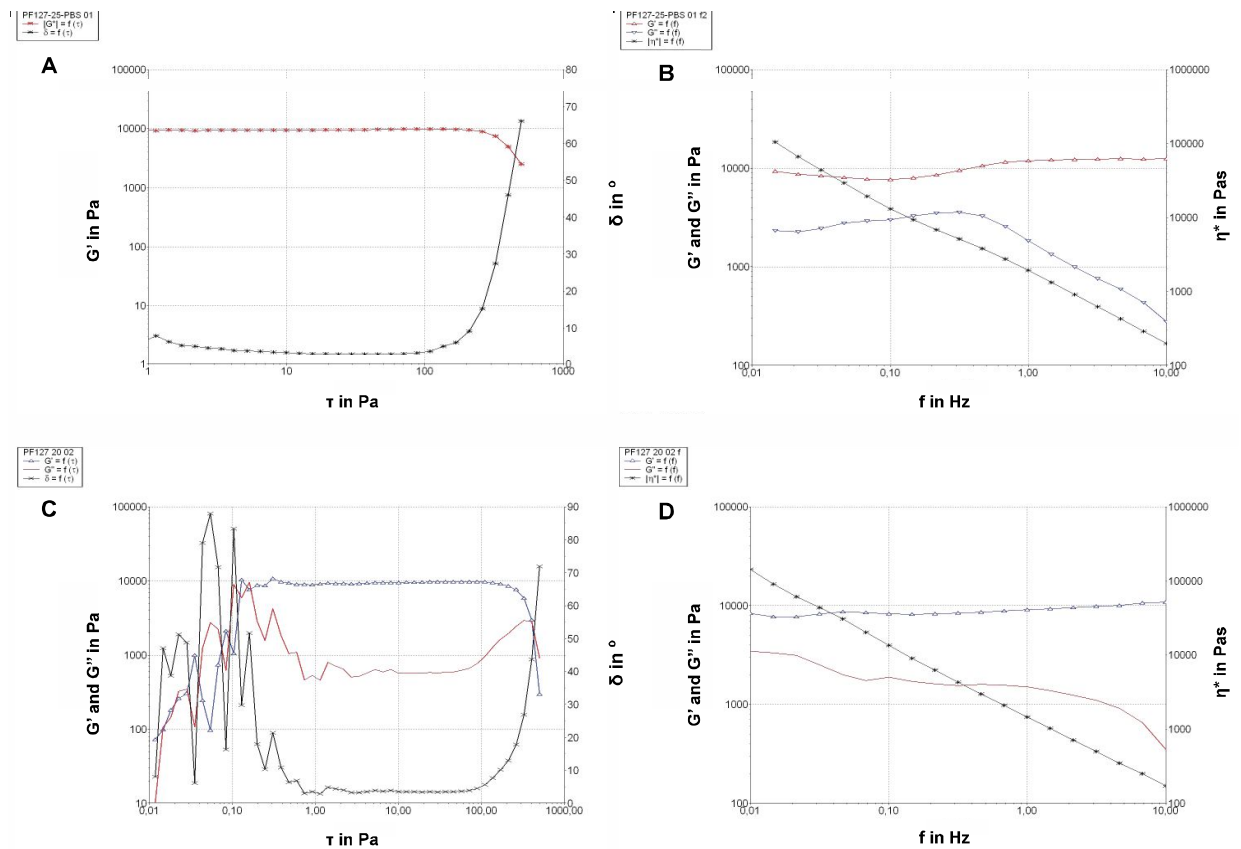


Fig. 52

Results of the rheology tests assays for PF127 hydrogels showing G' and G'' values at different concentrations. (A) Stress (τ) sweep test at 25% (w/v) of the hydrogel. (B) Frequency (Hz) sweep test at 25% (w/v) of the hydrogel. (C) Stress (τ) sweep test at 20% (w/v) of the hydrogel. (D) Frequency (Hz) sweep test at 20% (w/v) of the hydrogel.

6.2.2 Generation of Pluronic F127 hydrogel scaffold with human foreskin fibroblasts (HFF)

As the mechanical properties of the PF127 were mechanically competent to be utilized as a scaffold, we generated scaffolds with HFF. After 24 h the fabrication and incubation in the casting mold of the scaffolds with HFF, it was observed that the casting molds were almost empty (Fig. 53), as the scaffold

had melted with the medium. Consequently, cells were found to be dispersed in the culture medium for both scaffold concentrations, 20 % and 25 % (w/v). Experiment was repeated 2 more times (total n= 3) and similar results were observed. Therefore this PF127-based hydrogel was discarded to generate suitable cardiac constructs.



Fig. 53
Image of the casting mold used to fabricate the scaffold, almost without any traces of the PF127 hydrogel with HFF after 24h, incubated at 37 °C 5% CO₂.

6.2.3 Generation of a Pluronic F127- gelatin hydrogel

A new hydrogel was attempted by mixing PF127 with gelatin due to previous failure of PF127 alone, in order to generate a scaffold that did not melt in culture medium but preserved thermoreversibility. Gelatin is a water soluble protein, approved by the Food and Drug administration (FDA), which is derived from animal sources, obtained from the hydrolysis of most commonly connective-tissues skin of mammalian and fish, basically based in collagen content. It has excellent gel-forming ability, versatility (due to its alpha-amino acid composition), abundance, low cost, and great biodegradability (Martucci et al., 2011), that make gelatin an attractive protein in the biomaterials field.

An initial solution of 15% (w/v) of the PF127-gelatin new polymer in mili-Q water was prepared. The resulting solution presented a homogeneous aspect but it did not show the sol-to-gel transition at 37 °C, nor at any temperature. The experiment was repeated varying PF127-gelatin initial ratio (1:1) in MES buffer, trying 10:1, 50:1, and 100:1 but no sol-to-gel transition was observed in any of the hydrogels at any temperature between -22 °C and 60 °C (Fig. 54). After these unsuccessful attempts the strategy was abandoned, and we decided to study another hydrogels.

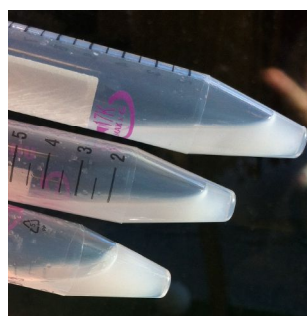


Fig. 54
Image of the 3 mixing ratios of gelatin and PF127. Top: 10:1, middle: 50:1, bottom: 100:1

6.2.4 Generation of poly-DL-lactic-co-glycolic acid (PDLLGA) with polyethylene glycol (PEG) hydrogel

The resulting polymer-film was tried to dissolve with mili-Q water and PBS, but it did not dissolve. Different temperatures were tested, from 5 to 65 °C, at different solvent volumes (1%, 10%, and 25% w/v) but no results were observed.

The experiment was repeated increasing the time of the NaOH hydrolysis, from 1h up to 24h, but still the polymer did not dissolve. In another attempt the time of EDC/NHS treatment was also prolonged, from 1h to 4h, with no differences in the results. Therefore both hydrogels were rejected for our study.

6.3 *Generation of rat engineered heart tissues (EHTs)*

6.3.1 *Morphological aspects of rat EHTs*

As previous experiments in generating our own hydrogel were unsuccessful, collagen was adopted to be the scaffold biomaterial to fabricate the constructs, following Zimmermann's protocol for EHTs.

Expression of cardiac proteins was analyzed by immunofluorescence at day 14. CMs expressed cTnT and Cx43 in either condition, controls (not subjected to mechanical stimulation) and stretched EHTs (subjected to 7 days of constant stretch). Nevertheless, Cx43 was hardly detected in the EHTs. During EHTs culture, cell arrangement was observed to change markedly in stimulated EHTs from day 1, in which CMs were found mostly randomly oriented without apparent cross striation, and day 14, in which cells were found to be aligned and lengthened in the direction of the force, with ultrastructurally discernable cross striation of the CMs. In contrast, in control EHTs cells were not found to align neither lengthen in any direction, with no ultrastructural significant striation (Fig. 55).

CMs cultured in 2D were found to proliferate during this period of time, but did not align in any particular direction. Cells were always found to be arranged in clusters, with an appreciable growth in the cluster diameters (Fig. 55). In general terms, results of the analyzed EHTs showed important statistical differences between stretched EHTs (stretchers 1 and 2) and control EHTs, considering: cell length, as cells were longer in stimulated conditions than in control conditions (Fig. 56). Regarding cell alignment, CMs were found to be aligned, parallel one to another, in the direction of the force in the stretched EHTs, and randomly oriented in the control EHTs (Fig. 57). Importantly, abundance of Z-bands in the CMs subjected to mechanical stretching was statistically enhanced in comparison with control scaffolds, which exhibited a lower number (Fig. 58).

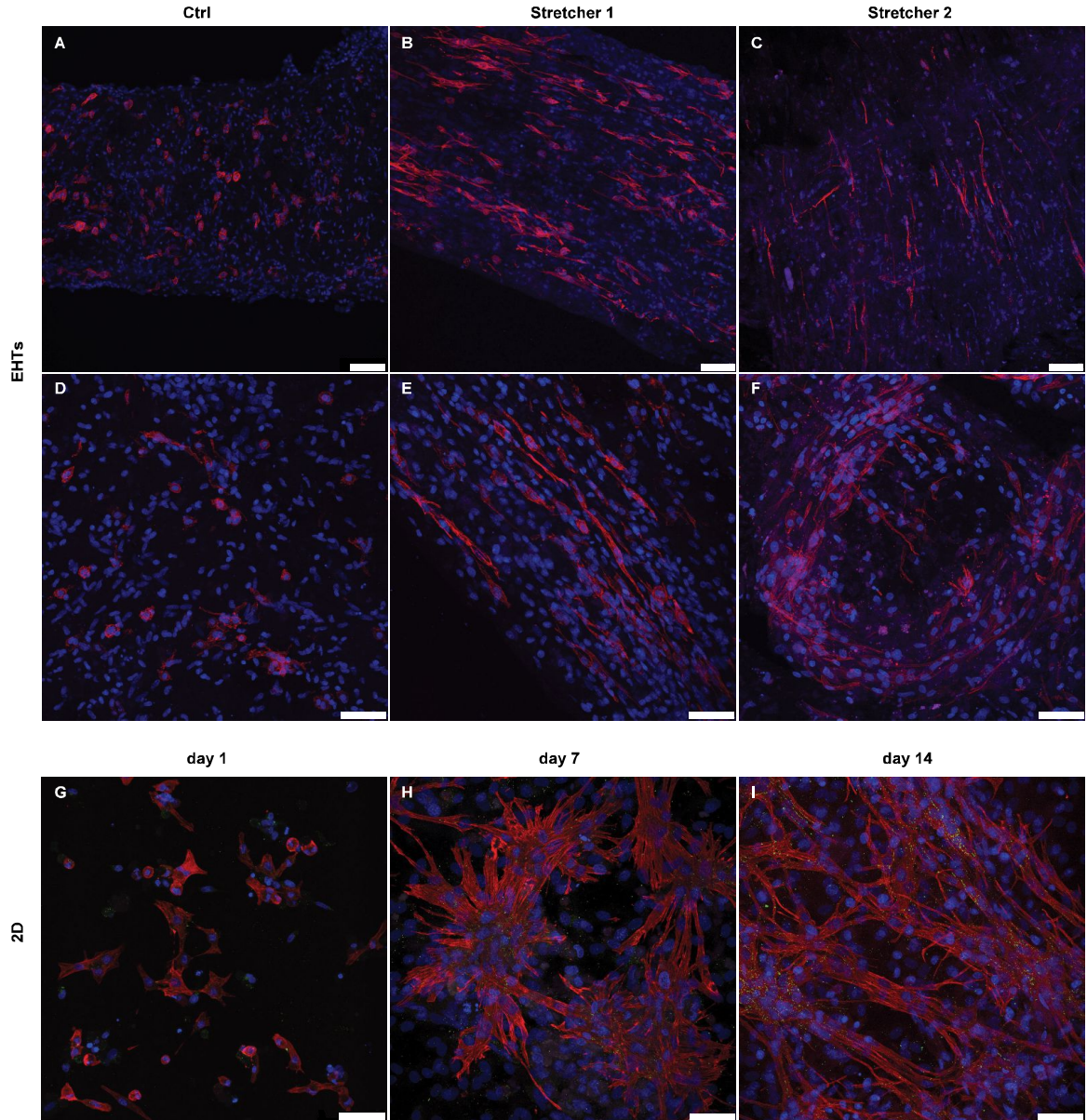


Fig. 55

Immunofluorescence detection of cardiac proteins of rat engineered heart tissues (EHTs) and conventional 2D culture control. Cells were stained for cTnT (Cy3: red) and Cx43 (Alexa-488: green), and nuclear staining was performed with DAPI (blue). Neonatal rat cardiomyocytes were detected in all scaffolds at day 14. EHT not subjected to mechanical stretch (A and D), EHT transferred to stretcher 1 (B and E), and EHT transferred to stretcher 2 (C and F). Cell alignment and lengthen were observed in stimulated EHTs with either stretcher (1 and 2), in comparison with control EHTs and 2D cultures. (G) Conventional 2D cultures of rat CMs at day 1, (H) day 7, and (I) day 14. Scale bars: A-C: 75 μ m, D-I: 50 μ m.

Cell length was found to be significantly higher in EHTs subjected to mechanical stimulation than in control conditions (stretcher 1: $\bar{X} = 62.25 \pm 22.5$ μ m, and stretcher 2: $\bar{X} = 69.62 \pm 24.7$ μ m, in contrast with the controls $\bar{X} = 26.25 \pm 11.5$ μ m, $p < 0.05$). CMs in stimulated EHTs exhibited a more elongated cytoplasm rather than EHTs in the non-stretched conditions (Fig. 56). Statistical differences were found between conditions, and also interestingly between stretchers. Nevertheless, the values in the stimulated condition (between stretchers 1 and 2) were found to be very similar.

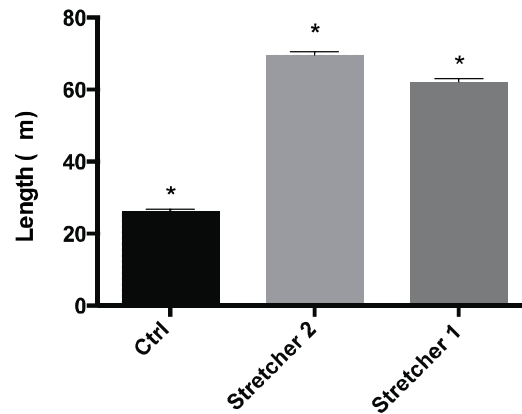


Fig. 56

Cell length of CMs in stimulated EHTs (stretcher 1 and 2) and control EHTs (Ctrl). Statistical differences were found for every group: Ctrl VS stretchers 1 and 2; and stretcher 1 VS stretcher 2. Data expressed as mean $\pm$ SEM, $n=5$, $*p<0.05$

Regarding cell alignment, angle dispersion and angle deviation were analyzed to evaluate the alignment of the CMs. In the mechanically stimulated EHTs (stretchers 1 and 2), CMs appeared aligned in the direction of the force, parallel one to another, in contrast with the randomly orientated CMs in the non-stimulated control EHTs (Fig. 57). Statistical significant differences were found between control and stimulated EHTs in angle dispersion (stretcher 1: $X=6.39 \pm 6.0$; stretcher 2: $X=6.70 \pm 6.18$; control $X=36.541 \pm 24.52$).

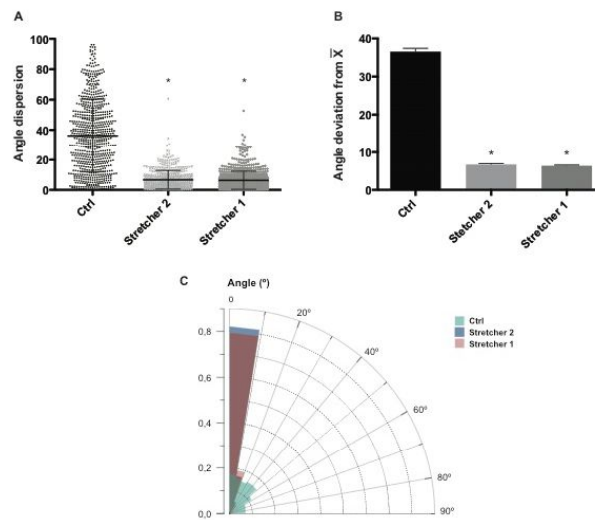


Fig. 57

CM alignment expressed as: angle dispersion (A), angle deviation (B), and polar distribution (C). (A and B) Statistical differences were found in angle dispersion (A: considering each angle measured minus the average) and angle deviation (B: considering only the mean of dispersion) between control EHTs and stimulated EHTs. (C) Polar graph representing the distribution of measured angles ($n=1$), very similar in the case of the stretched scaffolds and very disperse in the case of the controls. Data expressed as mean $\pm$ SEM, $*p<0.0001$, $n=5$.

Sarcomeric abundance and organization was analyzed in the EHTs by immunofluorescence staining of the Z-bands. CMs in EHTs subjected to mechanical stimulation exhibited more numerous and well-defined Z-bands than CMs in control EHTs not subjected to mechanical stimulation (Fig. 59). Statistical differences were found in the ALL and the SEMI groups between conditions.

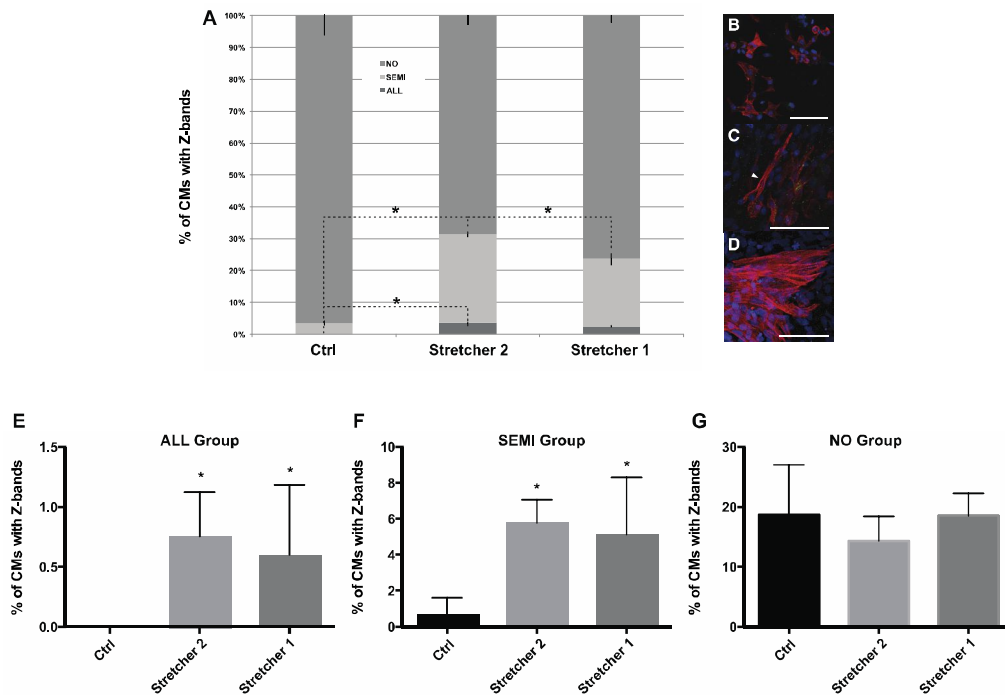


Fig. 59 Quantification of Z-bands of the CMs in the EHTs. (A) General graph showing the 3 groups for each condition: control, stretcher 2 and stretcher 1. Immunofluorescence images stained with TnT in red, Cx43 in green, and DAPI in blue, example of CMs in the (B) NO group, (C) example of CMs in the SEMI group, (D) example of CMs in the ALL group. (E) Graph with only the ALL group for the 3 conditions, (F) only the SEMI group for the 3 conditions, (G) only the NO group for the 3 conditions. Data expressed as mean $\pm$ SEM, * p < 0.05, n = 5.

6.3.2 Electrical coupling of EHTs

To analyze CM coupling with external electrical pulses, square-shaped electrical pulses (5 V/cm) were applied to the EHTs. When EHTs were stimulated at a frequency of 1 Hz, and PW of 2 ms, EHTs that were subjected to mechanical stretching showed a better synchronization of their contraction compared to non-electrically stimulated EHTs, despite no statistical differences were observed. The majority of the scaffolds showed an effective electrical coupling until the threshold of 4 Hz, over which EHTs began to beat asynchronously. Some of the scaffolds did not respond at any voltage, frequency or PW. Importantly, the contraction of the CMs was only observed in several areas of the scaffold, and rarely in the whole construct (See videos EHT rat1 and EHT rat2).

6.4 Generation of the membrane-based biomimetic stretching device (BSD)

The early idea of the development of a biomimetic stimulating device BSDe when the behavior of a pumping heart was compared to the behavior of an “automatic balloon”. Theoretically, cells on the surface of this balloon should experience a mechanical stretching very similar to the one they would experience if they were grown on the surface of a pumping heart. If cells were cultured in a scaffold on top of a pumping balloon, the whole construct would stretch and contract together with the surface of the balloon and hence, experiment a more biomimetic mechanical stimulation (Fig. 60)

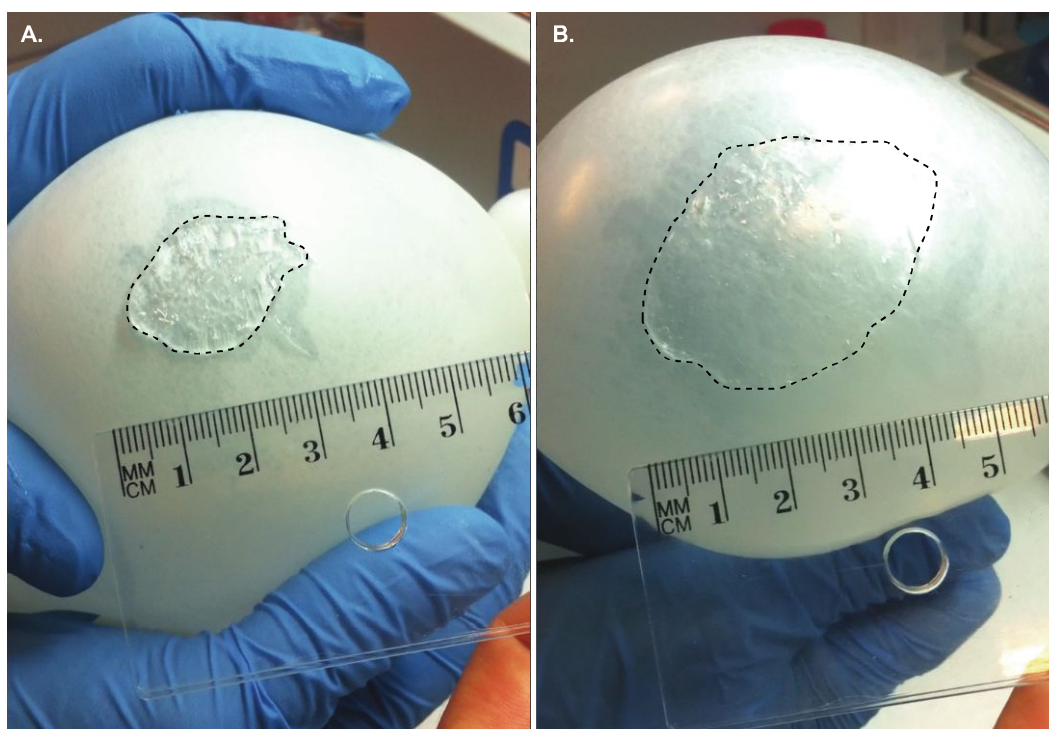


Fig. 60

Images of the first conceptual approach of the contracting automatic balloon with a PF-127 hydrogel on top. (A) Relaxed state, with a 2 cm wide scaffold. (B) Swelled state, where the scaffold measures ~5 cm wide.

In order to design the optimal BSD to our studies, different prototypes were developed until the definitive model was established (Fig. 61). First prototypes were large in diameter (~10 cm) and the scaffolds were attached to the surface of the membrane by covalent chemical binding elsewhere described (Casares et al., 2015), in order to achieve a homogeneous stretching of the scaffold whilst pumping (Fig. 61 A). In spite of this approach, our scaffolds were frequently detached when pumping began, due to their thickness (~1 mm thick) and the fact that hydrogels are not solid elastic materials, but viscoelastic materials, therefore chemical binding was only effective in the inferior surface, where as the rest of the scaffold slide from its lowest surface.

Therefore, the addition of cylindrical posts to the surface was implemented as a solution to attach the scaffold to the pumped surface, and guarantee its subsequent stretching. Different configuration and number of posts on the surface were also studied (Fig. 61 B). Finally, a number of 9 posts in a 3 x 3 matrix was chosen as the ideal configuration for our constructs (Fig. 61 C & D). In addition, the miniaturization of the device was also achieved, reducing its original diameter (~10 cm) to a 1.8 cm diameter, which permitted to place each BSD inside of a well o a 6-well plate (Fig. 61 E). This final miniaturization and implementation of the BSD allowed us to better imitate

stimulating conditions between experiments, facilitated sterilization, and replicate conditions for each experiment, as with the same resources more scaffolds could be fabricated.

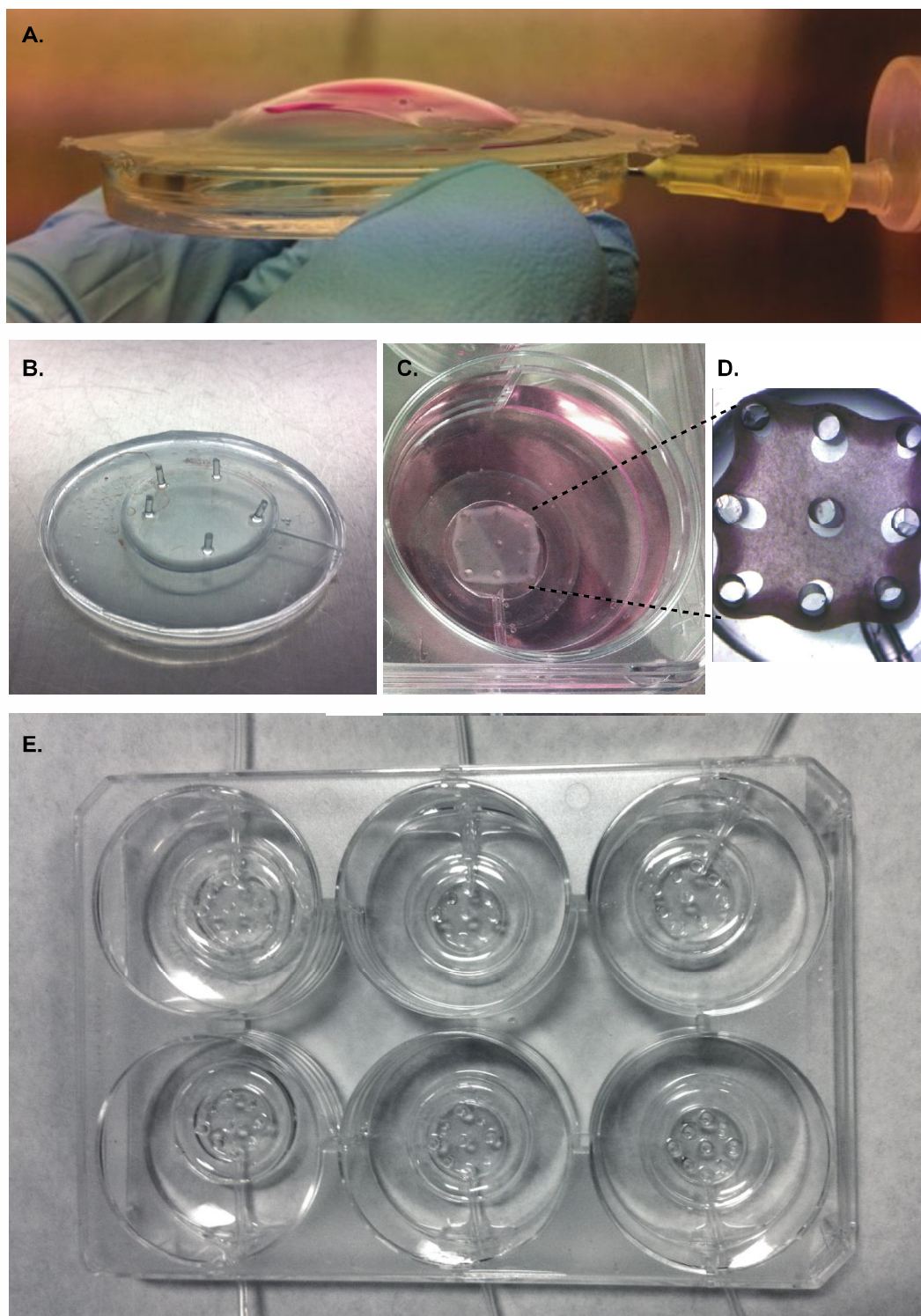


Fig. 61
 (A) Early prototype (10 cm) of the membrane-based biomimetic stretching device (BSD) with a collagen scaffold on top attached by chemical binding to the membrane. (B) Prototype (10 cm) with 5 silicone-posts attached to the surface. (C) Culture of a scaffold on the final prototype (1.8 cm) with 9 posts on its surface. (D) Detail of the construct and the posts. (E) Miniaturization and implementation of the BSD in a 6-well plate. Each BSD is connected to a tube through which the air flows.

6.4.1 Generation of membrane-based device scaffolds (BSD) with neonatal rat heart cells

In order to reproduce a more biomimetic mechanical stimulation, a BSD device was designed. Scaffolds were fabricated following the same protocol as with EHTs but applying it to the new BSD. Results of CM length, alignment and presence of Z-bands were analyzed as previously described for EHTs, following the same procedure.

6.4.1 Morphological aspects of rat BSDs

Stimulated BSD scaffolds exhibited a more compact structure at the end of the study (day 14) than control BSD scaffolds (Fig. 62).

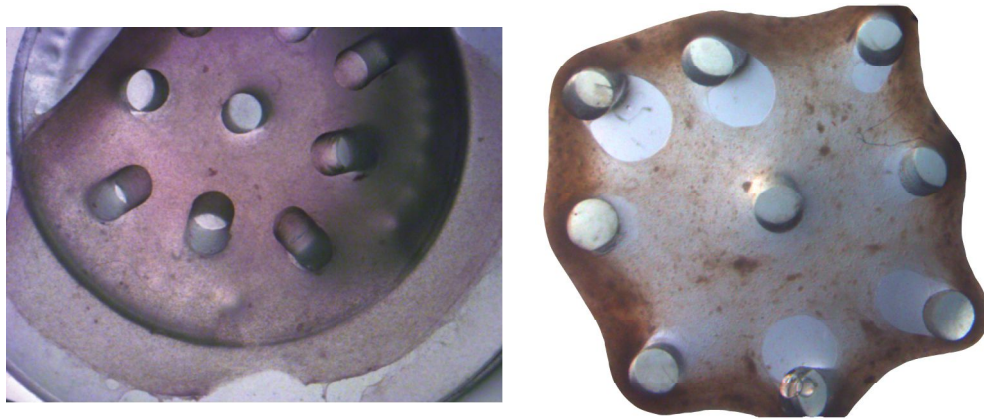


Fig. 62

BSD scaffolds on the new stimulating pneumatic device at day 14 of culture. Scaffolds stimulated with the pneumatic device were found to be more compact than control scaffolds. (A) BSD control scaffold. (B) BSD pump scaffold.

Besides, immunostaining analysis revealed a particular problem associated to in these scaffolds: cells did not follow a particular orientation in the scaffold. More precisely, cell length and alignment were observed to be zone dependent (Fig. 63).

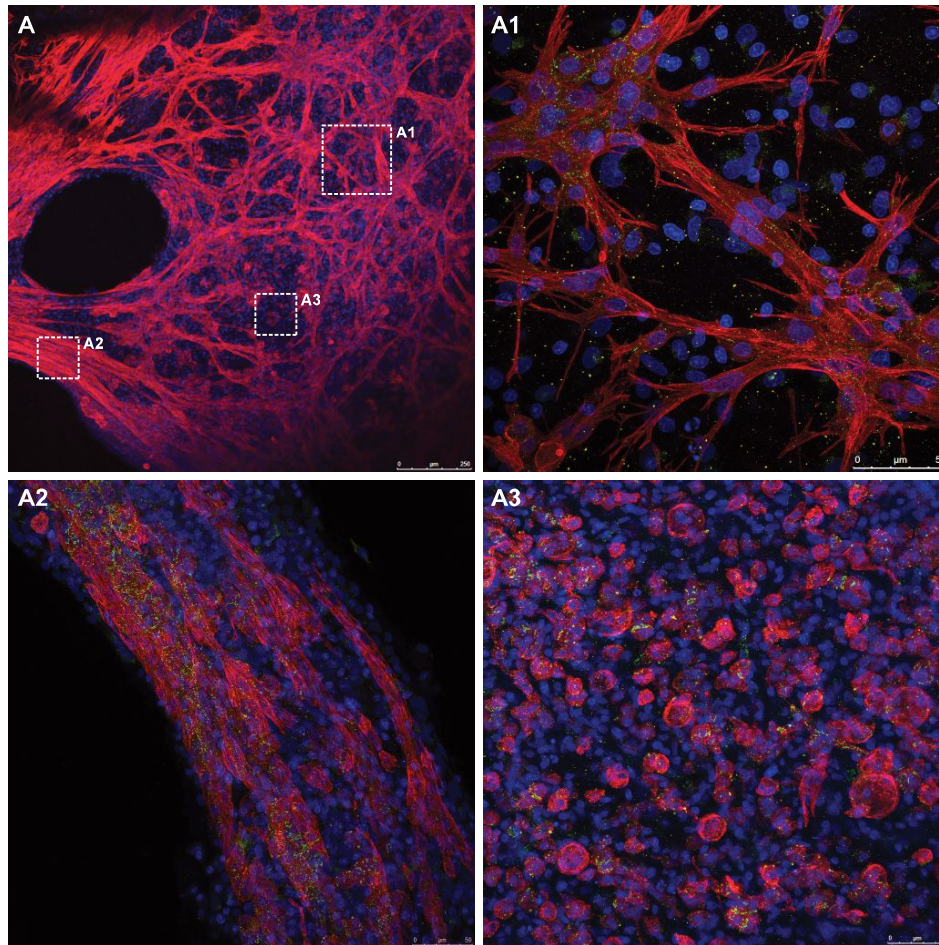


Fig. 63

Immunofluorescence detection of TnT (in red) and Cx43 (in green) cardiac proteins of BSD scaffolds, and DAPI in blue for nucleus. (A) Lateral of a scaffold, showing CMs (A1) interconnected but non-oriented in any predominant direction, (A2) highly oriented in a particular direction, and (A3) very isolated (non-interconnected nor aligned).

This was seen to be a consequence of the membrane-based stimulation system itself, as the scaffold was attached to the top of a membrane with silicon posts, when the pumping began, some zones of the scaffold were more stretched than others. Therefore cells rearranged in the scaffold accommodating their selves to the dominant direction of the force (stress-dependent). Consequently, we found that cells could be both, aligned and randomly distributed in the same scaffold, independently if they had been pumped or not. Considering previous observation, only equivalent regions of the scaffold were analyzed.

When cells were studied in a zone of the BSDs under a predominant direction of a force (uniaxial), we observed they lengthened and aligned in that same direction. In contrast, when cells were studied in a radial-force zone, where no predominant force directions existed (multiaxial or radial), they did not lengthen in any concrete direction nor follow any particular orientation. Hence, cells were found to be spread in every direction (Fig. 63 A), and lengths measured in equivalent regions of the scaffold. Despite this fact, it was observed that CMs in the BSDs that had been mechanically pumped (BSD pump) exhibited a more elongated cytoplasm than the CMs in the control BSDs (BSD ctrl), with

statistical differences between conditions (BSD pumped= $60.24 \mu\text{m} \pm 17.34$; BSD control= $39.10 \mu\text{m} \pm 17.98$) (Fig. 64).

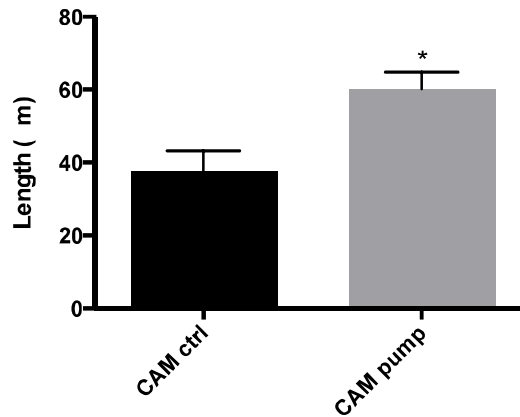


Fig. 64
Cell length of CMs of the BSD scaffolds, in non-stimulated BSD ctrl and BSD pump. Data expressed as mean $\pm$ SEM, * $p < 0.05$, $n = 3$.

As previously stated, cell alignment (together with cell length) was observed to be zone dependent within these BSD scaffolds (Fig. 63A). This fact hinders to determine a total-scaffold predominant orientation, as there cannot be such. Depending on the zone of the scaffold we studied, bundles of CMs were found to be highly aligned and parallel one to another, or completely randomly distributed (Fig. 63 B1, and C1). For the present study, we measured only CMs situated at the extremes of the BSDs (mostly oriented) as previously described. In spite of this fact, results revealed an enhanced alignment of the CMs in the stimulated BSD scaffolds in comparison with control BSD scaffolds (Fig. 65), with no statistical differences between conditions.

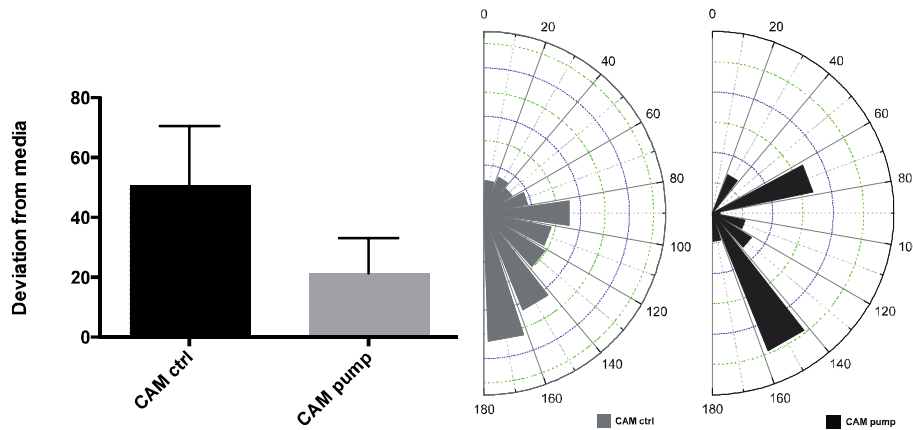


Fig. 65
Cell alignment in BSD scaffolds considering equivalent regions of the scaffold. (A) Deviation graph for cell alignment considering only extremes of the scaffolds. (B) Example of a polar graph considering angle values for one experiment ($n = 1$). Data expressed as mean $\pm$ SEM, * $p < 0.05$, $n = 3$.

Sarcomeric abundance and organization was analyzed in the BSDs by immunofluorescence staining of the Z-bands, classifying CMs (positive for TnT) into 3 groups: ALL, SEMI, NO. Results showed statistical differences in CMs in

the ALL-group, between pumped condition (BSD pump) and control condition (BSD ctrl), which exhibited an enhanced number of Z-bands (Fig. 66 and 67).

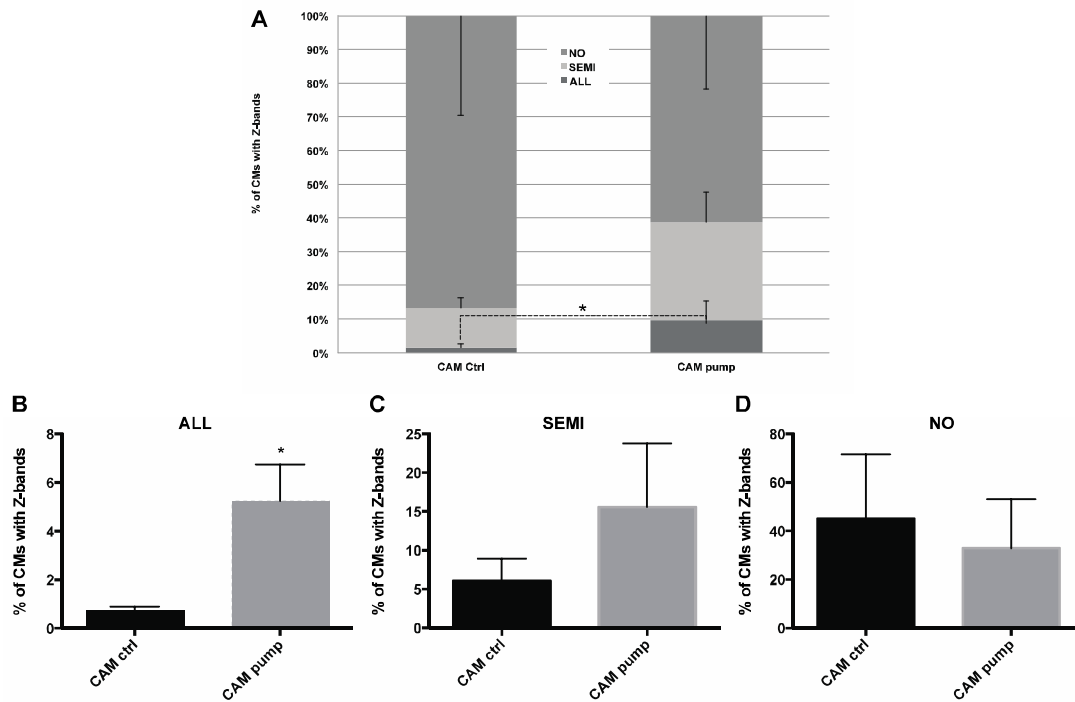


Fig. 66

Analysis of presence of Z-bands in the CMs of the BSD scaffolds. Relative percentage (%) of CMs showing Z-bands is represented (A) considering both conditions in the 3 groups. (B) Only the ALL Group, (C) only the SEMI Group, (D) only the NO Group. Data expressed as mean $\pm$ SEM, * $p < 0.05$, $n = 3$.

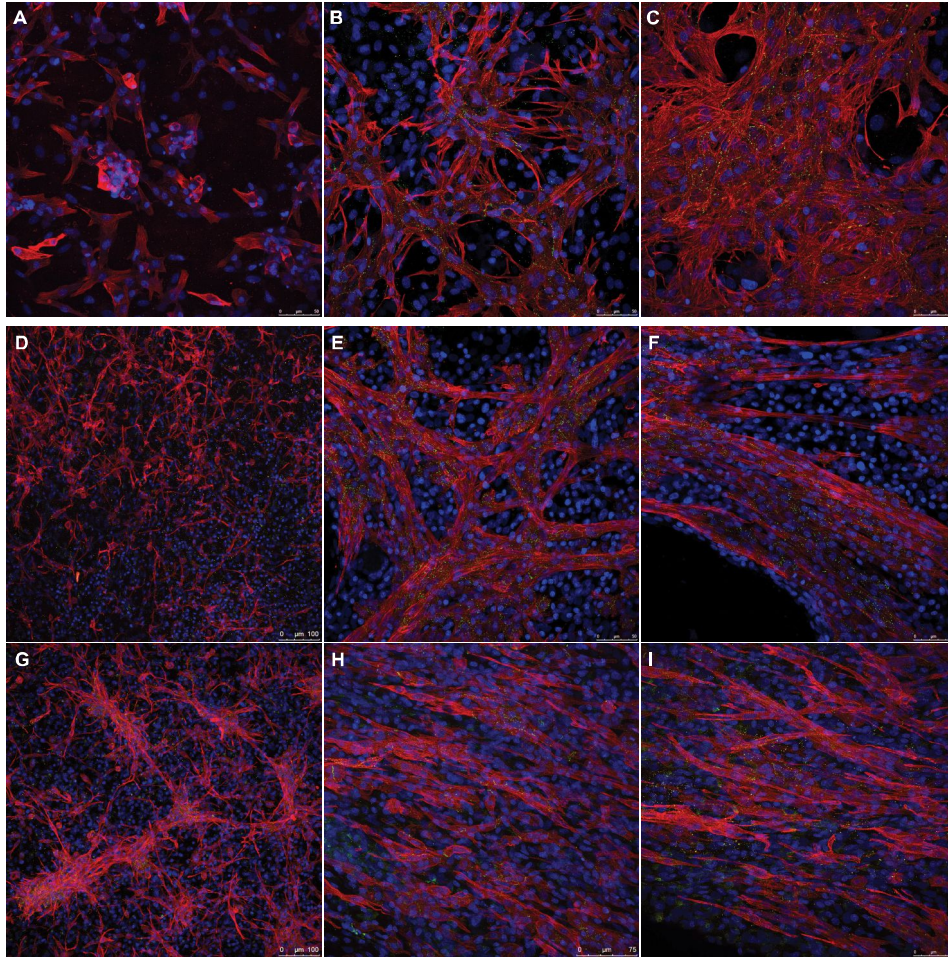


Fig. 67
Immunofluorescence images of CMs in the BSDs stained with TnT (red), Cx43 (in green), and DAPI for nucleus (in blue). CMs in stimulated BSDs exhibited statistically more striated structures in the cytoplasm than control BSDs (analyzed in equivalent regions) or 2D controls. (A-C) 2D Controls. (D-F) Control BSDs. (G-I) Pumped BSDs.

6.4.2 TEM ultrastructural analysis of BSDs compared to neonatal rat myocardium

BSD scaffolds and neonatal rat (P2 - P3) heart left ventricle were analyzed by TEM microscopy in order to study ultrastructural differences between tissues. Results showed qualitative differences in sarcomere organization, abundance and width, between scaffolds and rat ventricle. Neonatal rat ventricle was observed to be relatively thicker (Fig. 68) in comparison with the BSDs. In contrast, abundance and organization of the sarcomeres, together with presence of gap junctions were found to be similar in both groups.

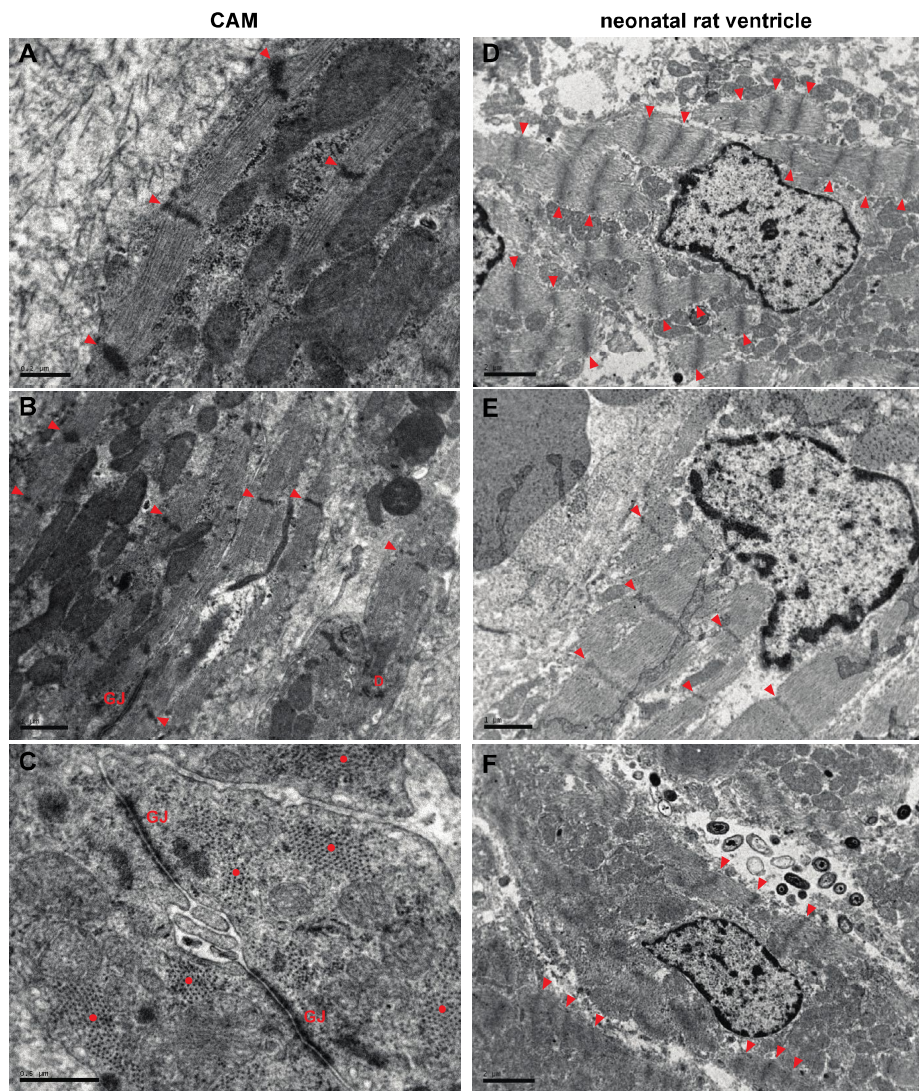


Fig. 68
TEM images of (A-C) BSD pump scaffold, and (D-F) P3 neonatal rat left ventricle. Red arrows: Z-bands, red circles: section-view of bundles of myofibrils, D: desmosomes, and GJ: gap junctions.

6.4.3 Electrical coupling of BSDs

BSD scaffolds were synchronized with electrical pulses in order to study CM electrical coupling with external pacing. A custom made chamber was utilized in

which an electric field of 5 V/cm (1 Hz, PW= 2ms) was applied. The majority of scaffolds were observed to respond to electrical pulses in every study, and remarkably, contractions of the whole construct could be observed in some of the studies for the stimulated BSD. Interesting videos were captured in which contractions of the entire construct could be (see videos BSD pump rat, and BSD ctrl rat). Despite these observations, no significant differences were observed between conditions in the majority of the experiments. A general behavior was observed to occur when BSDs were coupled to the electrical pulses; CMs showed to be sensible to distinct frequencies, ranging from 1 to 4 Hz. At low voltages (from 1.5 to 3 V/cm, CMs did not respond, excepting if PW was lengthened >

6.5 Cardiac differentiation of human keratinocyte iPSC-derived cardiomyocyte (iPSC-CM)

iPSC were obtained from retroviral reprogramming (3 factors) of human dermal keratinocytes from a healthy donor. iPSC were differentiated successfully to CMs following Gi-Wi protocol (Fig. 69). First spontaneous beating clusters appeared from day 8 to day 12 that increased over time.

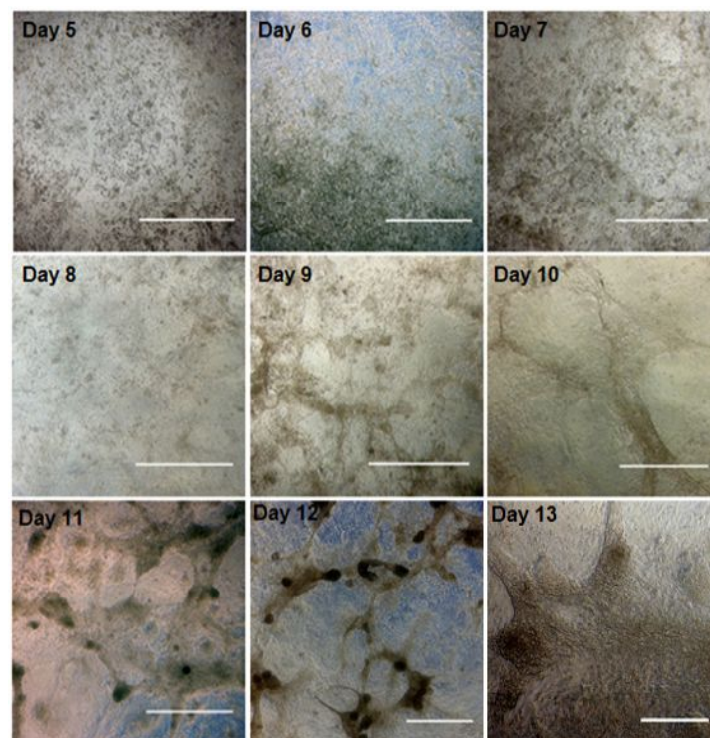


Fig. 69

Images of the KiPSC differentiation process. From day 5 cells were maintained with supplemented medium until day 19. First beating clusters appeared from day 8 to day 12. Scale bar: 0.75 mm (day 5 - 7), 1 mm (day 8 - 11), 0.5 mm (day 12 and 13).

Differentiation protocol efficacy was analyzed by flow cytometry. Live/dead analysis results showed that cell survival after differentiation protocol was over 90%, whereas flow cytometry results showed a variable efficacy from 45 % to 65 % (Fig. 70).

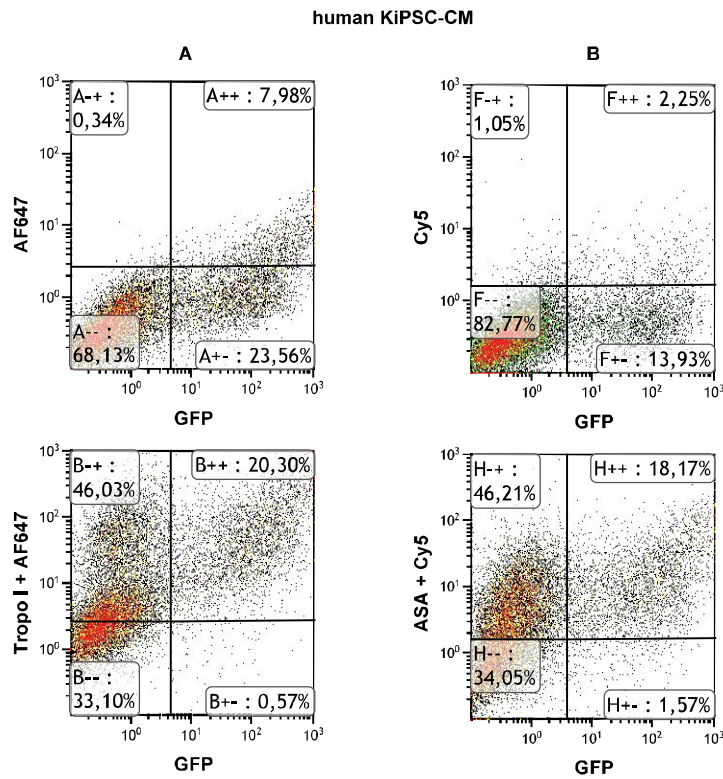


Fig. 70

Expression of cardiac proteins by flow cytometry. iPSC-CM were stained after the differentiation protocol (at day 19, before fabricating the scaffolds) with cardiac markers: Troponin I (TnI) and alpha-sarcomeric actin (ASA). (A top): control graph only with secondary antibody (AF647), (A bottom): populations positive for TnI and GFP, (B top): control graph only with secondary antibody (Cy5), (B bottom): population positive for ASA and GFP.

Cytometry studies were repeated 14 days after fabricating the scaffolds. At day 14 constructs revealed high cell viability near 65% for ring-scaffolds, resulting more increased in stretched rings (Fig. 71).

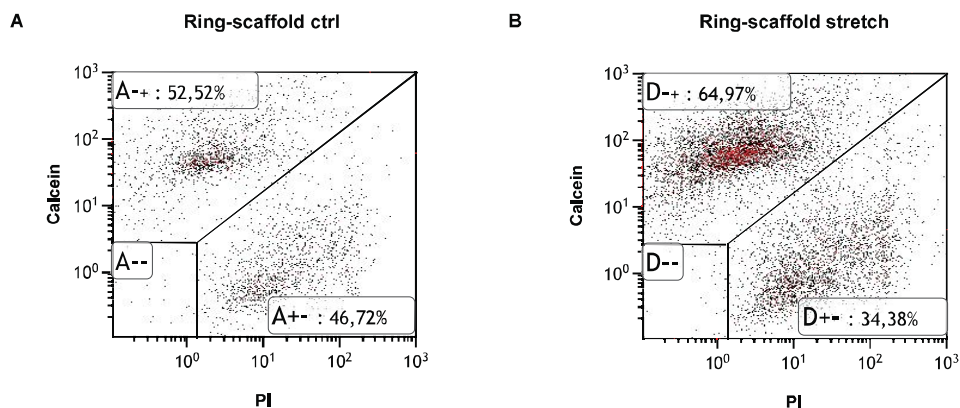


Fig. 71

Cytometry results at day 14 for cell viability in ring-scaffolds with iPSC-CM, performed with calcein and propidium iodide. (A) Control ring-scaffold with iPSC-CMs, and (B) Stretched ring-scaffold with iPSC-CMs.

Interestingly, cell viability was found to be enhanced in BSDs in comparison with ring-scaffolds, around 85 % in either condition (stimulated and controls) (Fig. 72). Besides,

a similar rate of CM population (CMs vs non-CMs) was found at the end of the experiments, ranging from 45 % to 65 %.

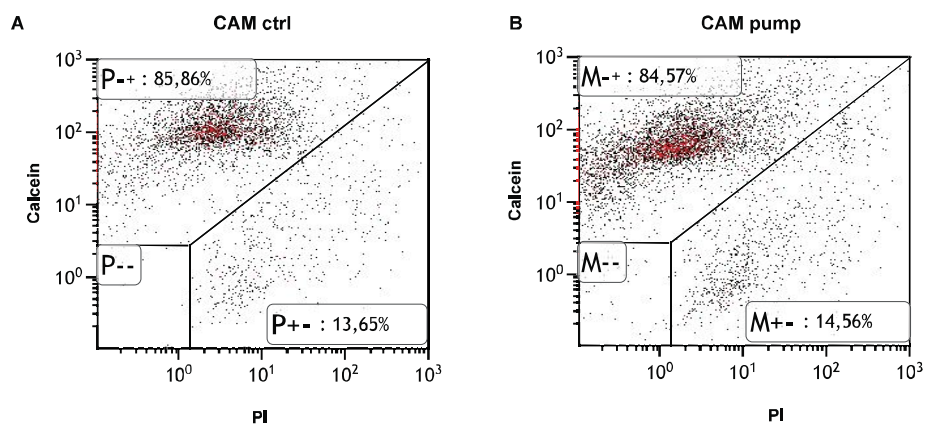


Fig. 72
Cytometry results at day 14 for cell viability in BSDs with iPSC-CM, performed with calcein and propidium iodide. (A) BSD ctrl, and (B) BSD pump.

6.6 Fabrication of ring-scaffolds with human iPSC-CM

Rings were fabricated using human iPSC-CM following the same protocol as to fabricate rat EHTs. Studies were focused on CM maturity, considering abundance and organization of Z-bands, between stretched and control conditions. Cells were selected at day 19 of the differentiation protocol, when an entire monolayer of iPSC-CMs beat synchronously on the culture wells (approximately 1 week after observing the first beating cell-clusters, which was regularly from day 12 to 14). After 7 days of culture in the molds, ring-scaffolds were transferred to the torsion springs for another 7 days of constant stretching (Fig. 73).

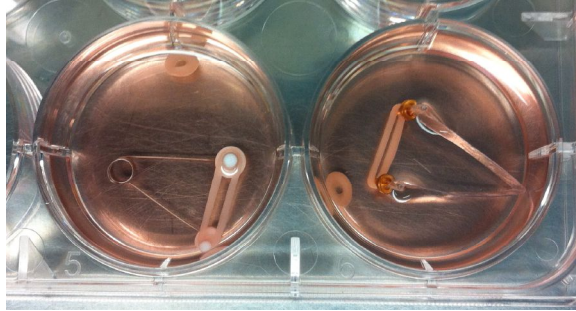


Fig. 73

Ring-scaffolds of iPSC-CM on the torsion springs in the culture wells. Left: stretcher 1 with ring-scaffold and control ring scaffold, and right: stretcher 2 with ring-scaffold and another control ring scaffold.

6.6.1 Morphological aspects of ring-shaped scaffolds with iPSC-CM

Immunofluorescence images were obtained and analyzed as previously described, where cell length and alignment could be appreciated (Fig. 74). Results, in agreement with those obtained for EHTs with rat cardiac cells, showed that human iPSC-CMs in scaffolds subjected to mechanical constant stretch exhibited more numerous and organized Z-bands than CMs in the non-stimulated control scaffolds, obtaining statistically significant differences between conditions for the ALL group (Fig. 75).

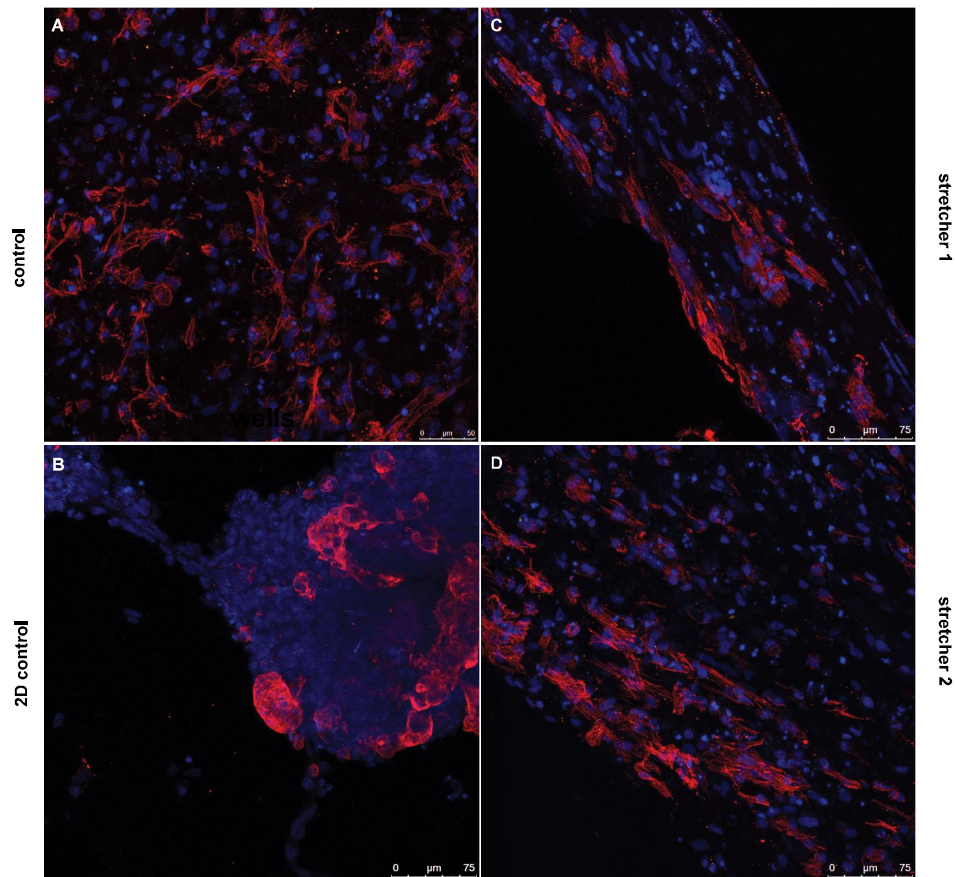


Fig. 74

Ring-shaped scaffolds with iPSC-CM analyzed by immunofluorescence microscopy, where CMs were stained with TnT in red, Cx43 in green, and DAPI in blue. (A&B) control ring-scaffold, scale bar= 50 μm, (C&D) Ring-scaffold on stretcher 1. (E&F) Ring-scaffold on stretcher 2, scale bar= 50 μm. (G) 2D control culture at day 1, when scaffolds were fabricated and, (H) at day 14, when studies ended.

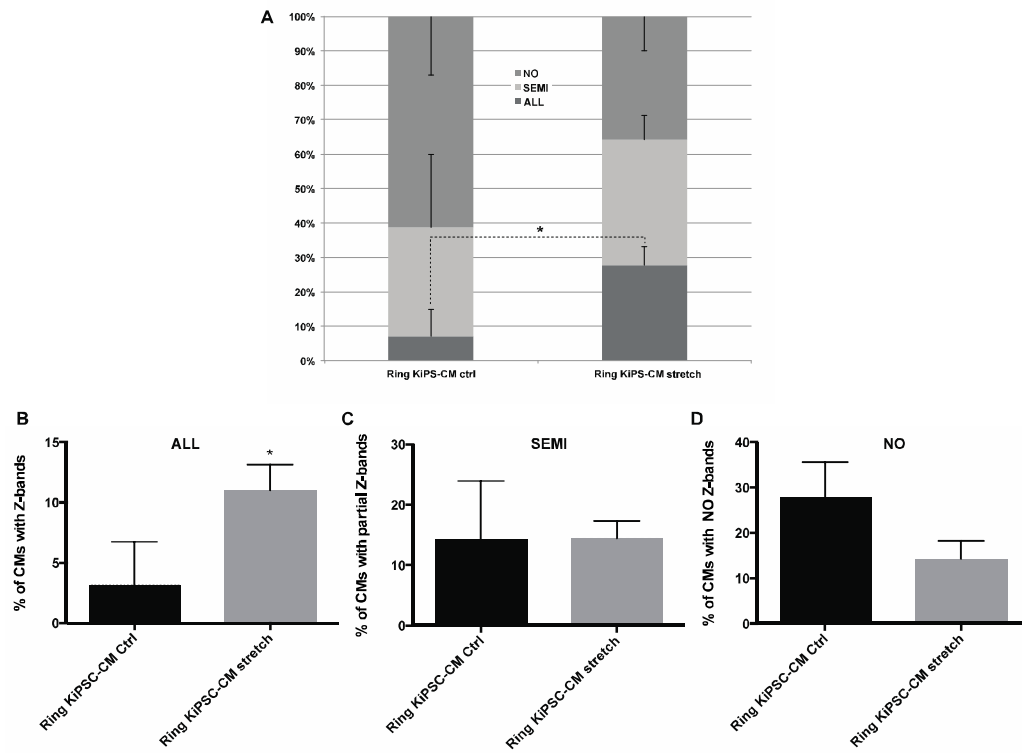


Fig. 75
Relative percentage (%) of CMs showing Z-bands is represented in these graphs between conditions, stretched and control. (A) General graph representing the 3 groups between conditions. (B) Only the ALL Group, (C) only the SEMI Group, (D) only the NO Group. Data expressed as mean $\pm$ SEM, * $p < 0.05$, $n = 4$.

6.6.2 Electrical coupling of ring-scaffolds with human iPSC-CM

Scaffolds were synchronized with electrical pulses as elsewhere described, but no effects of electrical synchronization were observed with the previous electrical parameters applied (square pulse, voltage= 10 V, F= 1 Hz, PW= 2 to 100 ms). As our initial function generator was limited to amplitude of 10 V, we switched to a biphasic electrical stimulator that allowed greater amplitudes (up to ± 50 V, biphasic) to stimulate the scaffolds. Voltage amplitude was increased gradually up to 25 V to check synchronization, keeping the rest of applied parameters the same (square wave, F= 1 Hz, PW= 2 to 100ms). At amplitudes >20 V the majority of the scaffolds were observed to contract in certain regions, with no differences in their contraction size if the voltage was raised. However, we never observed macro-contractions of the whole construct, with no qualitative differences observed between both conditions. Moreover, synchronization could not be prolonged more than few seconds due to an observed electrolysis of the culture medium (Fig. 76).

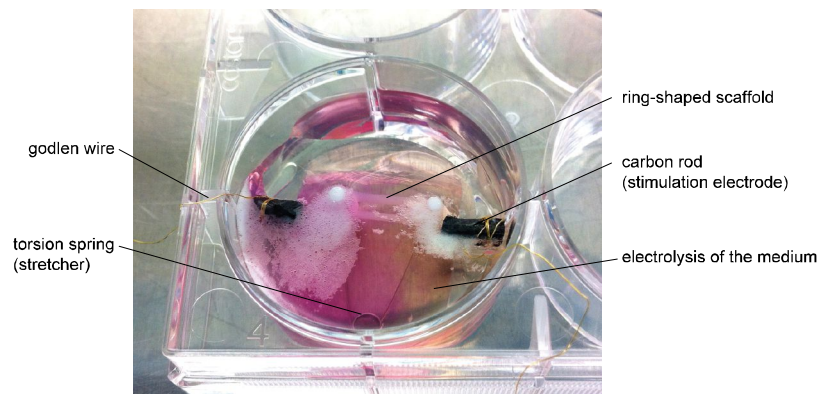


Fig. 76

Image of the electrolysis of the culture medium in the electrical chamber during stimulation at ~25 V, square pulse, F= 1 Hz, PW= 2 ms. Note the change of color of the medium due the acidification.

6.6.3 Ultrastructural analysis of ring-scaffolds with human iPSC-CM

To study the formation of mature structures in the CMs, ring-scaffolds were analyzed by TEM. In the stimulated condition (stretched scaffolds), sarcomere abundance and organization was considerably higher than in control conditions, in agreement with the immunohistochemistry results and previous results with rat EHTs, together with the presence of gap junctions (Fig. 77).

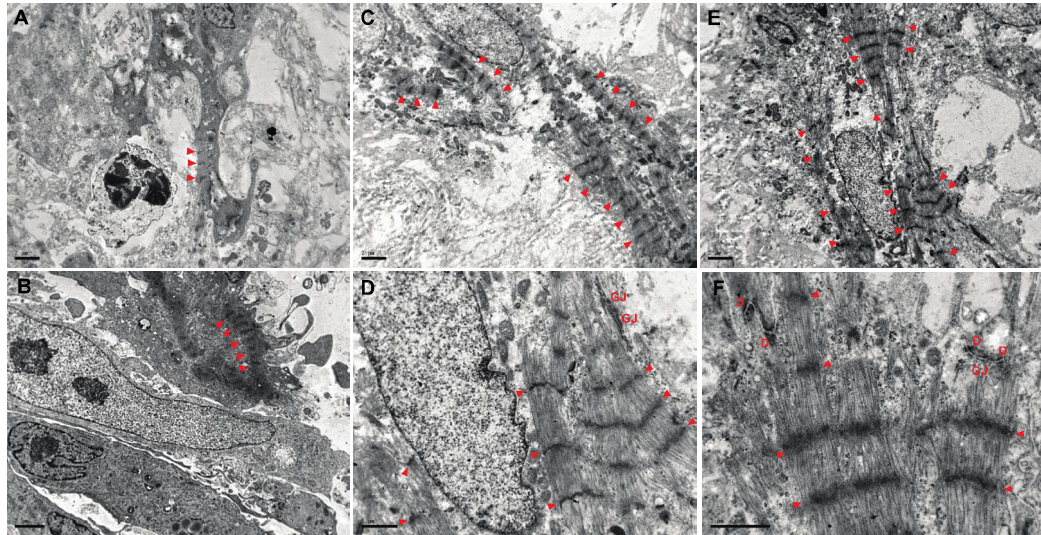


Fig. 77
TEM images of the ring-shaped iPSC-CM scaffolds comparing stretched VS controls. (A-B) Control scaffolds, (C-F) stretched scaffolds. Red arrows: Z-bands, D: desmosomes, GJ: gap junctions.

6.6.4 Expression of cardiac genes for iPSC-CM ring-scaffolds

Ring-scaffolds were analyzed by qPCR at day 14 after fabricating the scaffolds. Up-regulation of cardiac genes ACTC1, CACNA1, CONNEXIN43, cTNT, MLC2V, MYH6, MYH7, and RYR2, was studied in both conditions using human adult cardiac ventricular tissue as control. Results did not show statistical differences in gene expression when comparing both conditions, stretched and control ring-scaffolds, even though a remarkable increase in MYH7 was observed in stretched conditions. In spite of this, results exhibited a great difference when comparing gene expression of the scaffolds in either condition with human adult ventricle (Fig. 78).

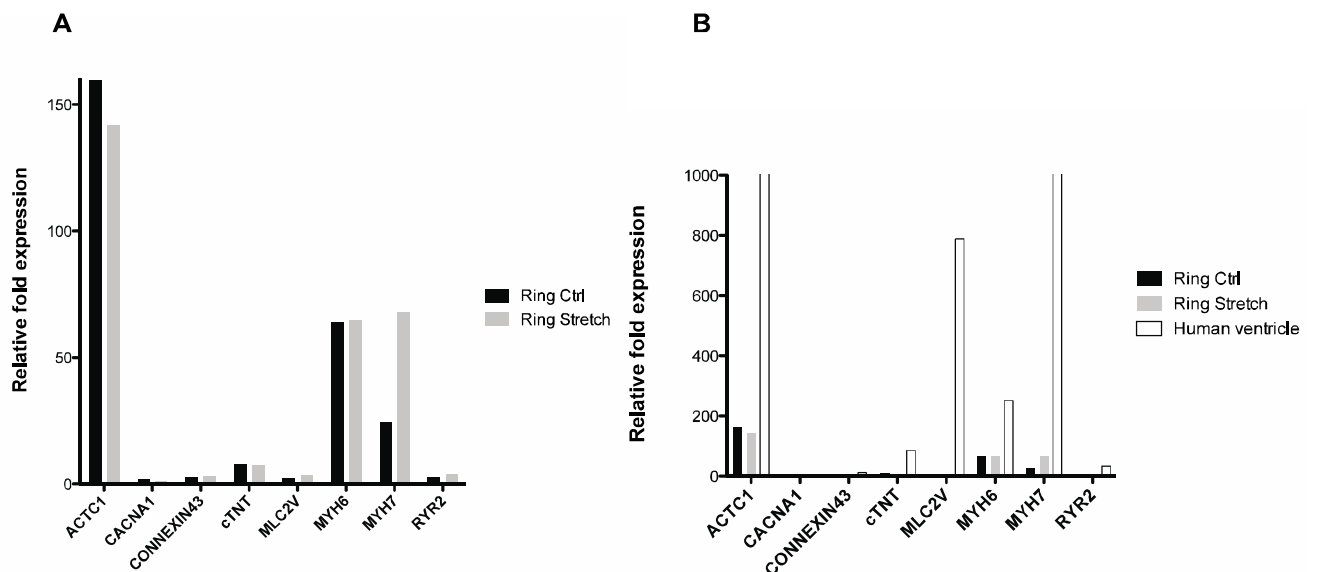


Fig. 78
qPCR results for ring-shaped scaffolds with human iPSC-CM. (A) Relative gene expression of ring-shaped scaffolds between only both conditions, and (B) both conditions in comparison with human adult left ventricle. Data expressed as mean $\pm$ SEM, * p < 0.05, n = 1.

6.7 Fabrication of BSD scaffolds with human iPSC-CM

BSD scaffolds were fabricated at 19 of differentiation protocol and seeded on top of the membrane-based device. BSDs were let 7 days of static culture and 7 more days of dynamical mechanical stimulation (pneumatic pumping), after this time scaffolds were fixed and analyzed by immunohistochemistry. Pumped BSDs exhibited a qualitative increase in CM alignment and length, together with presence of cross-striations (Z-bands) in comparison with BSD controls (Fig. 79), in accordance with previous results.

6.7.1 Morphological evaluation of BSD scaffolds

BSD scaffolds were analyzed in terms of cardiac maturity, studying sarcomere abundance and organization mainly as in previous studies (Fig. 79). CMs in pumped BSDs in comparison with control BSDs were found to exhibit a more statistically significant number of CMs with organized Z-bands (Fig. 80). Connexin was also observed to vary considerably from control BSDs to pumped BSDs despite no statistical differences were found. These results were coherent with all previous studies.

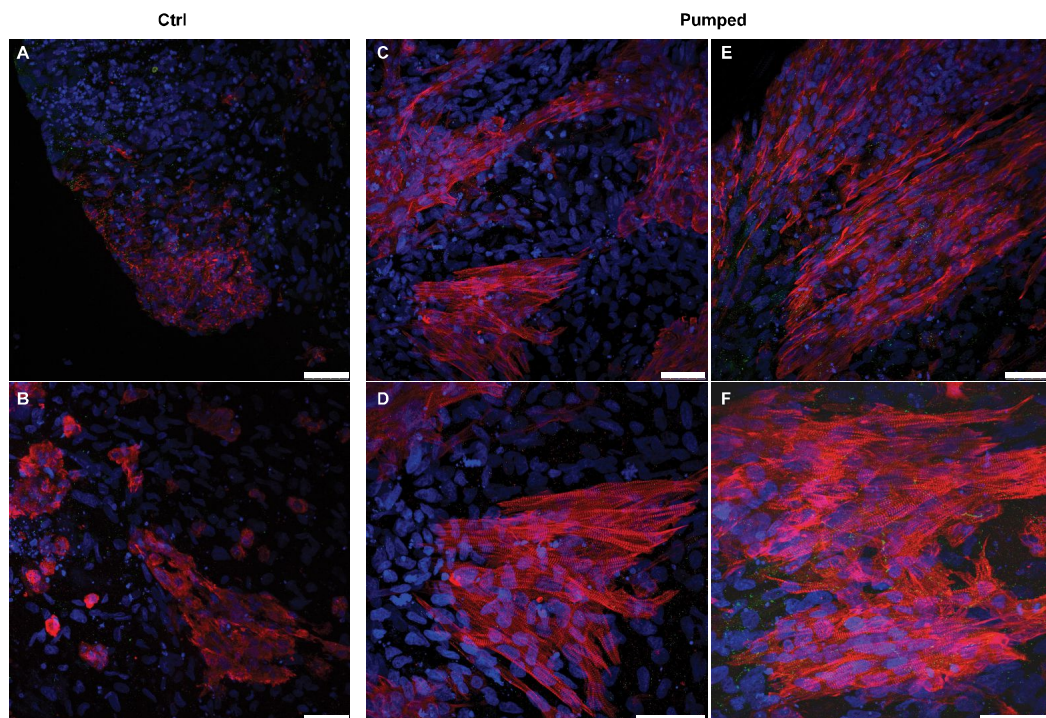


Fig. 79

Immunofluorescence detection of cardiac proteins of BSD scaffolds with iPSC-CM analyzed by immunofluorescence microscopy. CMs were stained with TnT (in red), Cx43 (in green), and DAPI for nucleus (in blue). (A&B) control BSDs, (C-F) pumped BSDs. Scale bar= 50 μ m.

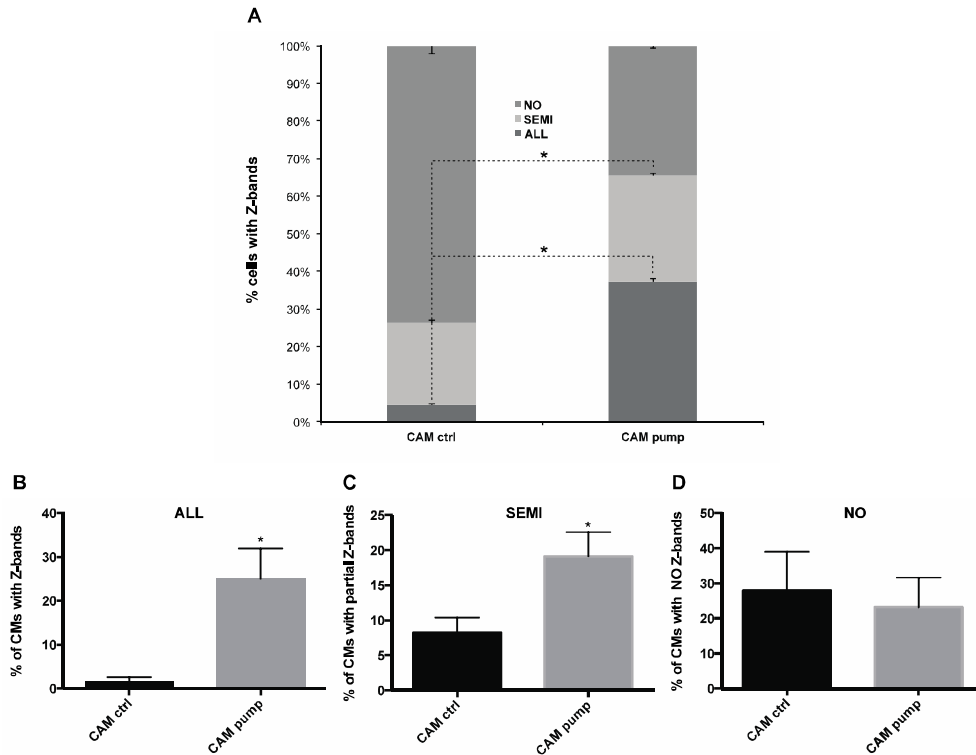


Fig. 80

Quantification of Z-bands in the iPSC-CM as a relative percentage (%) between conditions. (A) General graph showing the 3 groups at the same time between conditions, (B) Only the ALL group, (C) only the SEMI group, (D) only the NO group. Data expressed as mean $\pm$ SEM, * $p < 0.05$, $n = 5$.

6.7.2 Ultrastructural analysis of BSDs with human iPSC-CM

TEM analysis were performed in order to analyze sarcomere structure and organization of the CMs in the BSDs. Important qualitative differences were observed for sarcomere abundance and organization between conditions. In pumped BSDs, sarcomeres were more numerous and well defined in comparison with controls, besides they formed larger structures (repetition of the Z-bands) than in control conditions (Fig. 81). Moreover, pumped BSDs also presented a greater number of gap junctions along the CMs than control BSDs. Interestingly, mitochondria were also observed to be more numerous in CMs in pumped BSDs than in control BSDs. In spite of these findings, no statistical differences were obtained between conditions.

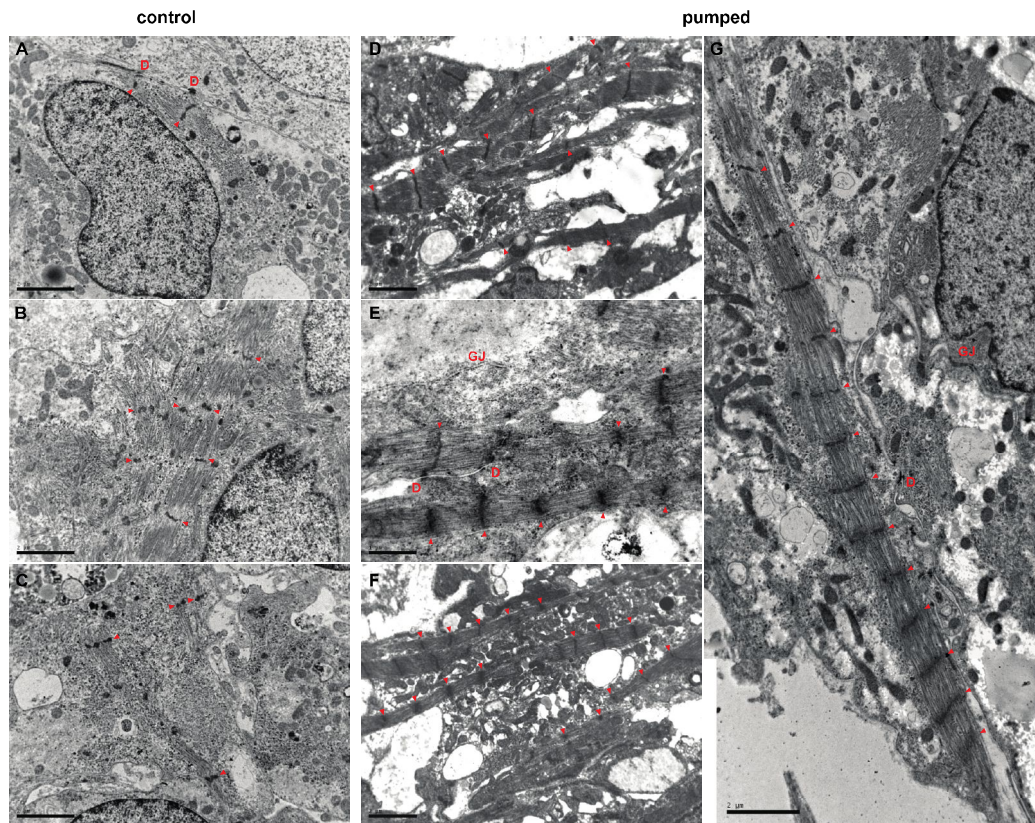


Fig. 81
TEM images of BSD scaffolds with iPSC-CM. (A-C) Control BSDs, (D-G) pumped BSDs. Red arrows: Z-bands, D: desmosomes, GJ: gap junctions.

6.7.3 Electrical coupling of BSDs with human iPSC-CM

Scaffolds were synchronized with electrical pulses as with ring-scaffolds previously described (square pulse, $V = \pm 50$ V (biphasic), $F = 1$ Hz, $PW = 2 - 50$ ms) using the same biphasic electrical stimulator. Results showed partial coupling of the scaffolds in the majority of the studies, as in previous experiments with rat cells, with some regions beating synchronously. Remarkably, some scaffolds beat entirely and synchronously with the electrical pulses, despite no statistical differences were found between conditions (see video BSD-pump-ELEC).

6.7.4 Expression of cardiac genes for BSDs with iPSC-CM

Same proteins were studied as with ring-shaped scaffolds, ACTC1, CACNAC1, CONNEXIN43, cTNT, MLC2V, MYH6, MYH7, and RYR2, using human adult left ventricle as control. Results showed similar values for most of the genes between conditions, being slightly enhanced cTNT and MYH7 in stimulated conditions in comparison with controls (Fig. 82 A). Importantly, CACNAC1, CX43, cTNT, MYH6, and RYR2 were found to be in the same order of magnitude as human's adult ventricle (Fig. 82 B).

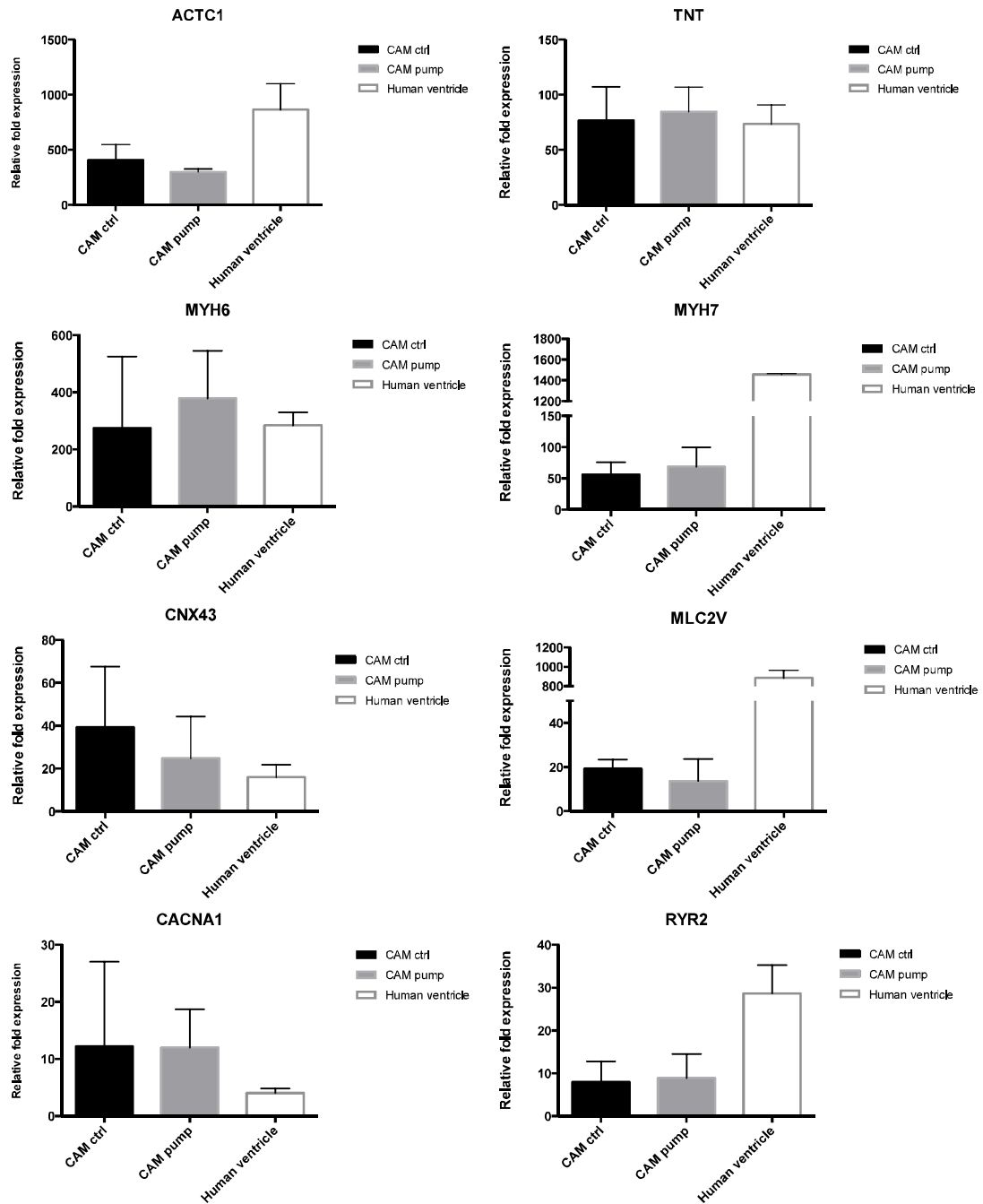


Fig. 82
qPCR results for BSD scaffolds with human iPSC-CMs showing relative gene expression comparing both conditions, pumped BSD and control BSD VS human adult left ventricle. Data expressed as mean $\pm$ SEM, * p < 0.05, n = 3.

6.7.5 Contractile behavior of BSDs with human iPSC-CM

In order to study contractile behavior of the cardiac constructs after mechanical stimulation, regions of interest (ROI) of the BSDs were analyzed in order to study relative contractions of the beating clusters. Interestingly, contraction of some ROIs of the stimulated BSDs increased significantly within time, from day 1 ($\Delta\text{ROI}_{\text{D1}} = 8,1\%$) to day 7 ($\Delta\text{ROI}_{\text{D7}} = 23,07\%$) (Fig. 83). However, no statistical differences in fractional area change were observed between stimulated BSDs and control conditions.

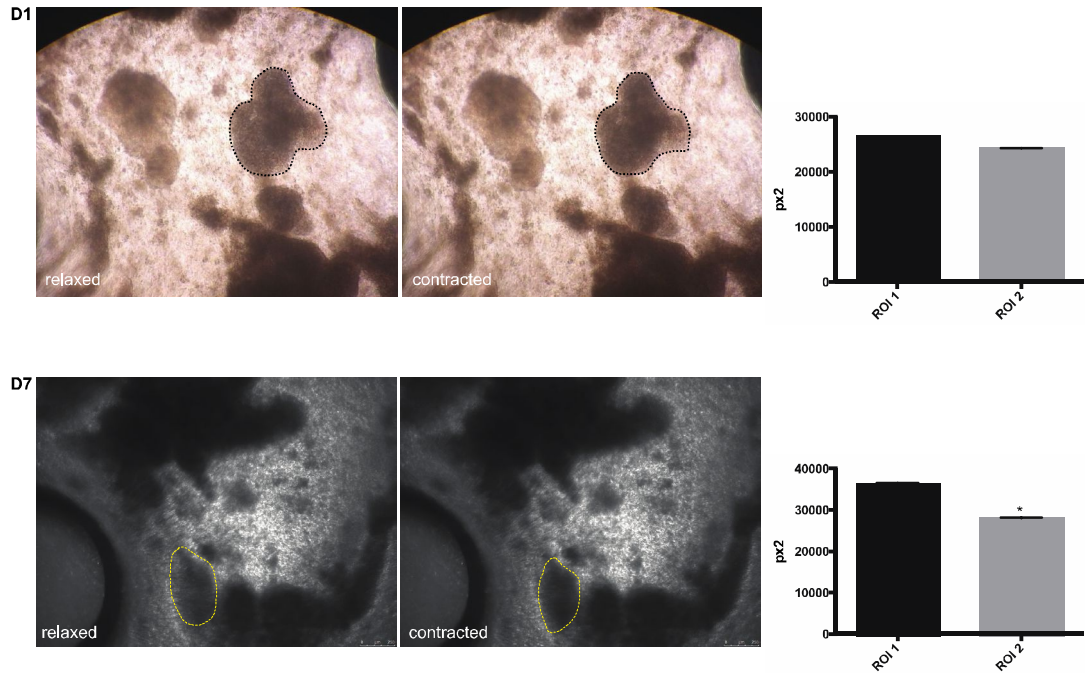


Fig. 83

Example of ROIs measured in a stimulated BSD. (D1) contraction of a ROI on day 1 ($n = 10$ contractions), and (D7) contraction of a ROI on day 7 ($n = 10$ contractions). Comparing both time points, D1 and D7, area of contraction was found to be statistically significant between relaxed and contracted state on day 7 in comparison with day 1. Data expressed as mean $\pm$ SEM, $*p < 0.05$, $n = 1$.

Moreover, contraction of a stimulated BSD was also studied by deformation field (DF) variation within time (3 s). Displacements were found to vary up to 0.7 mm in both axis (X and Y) (Fig. 84).

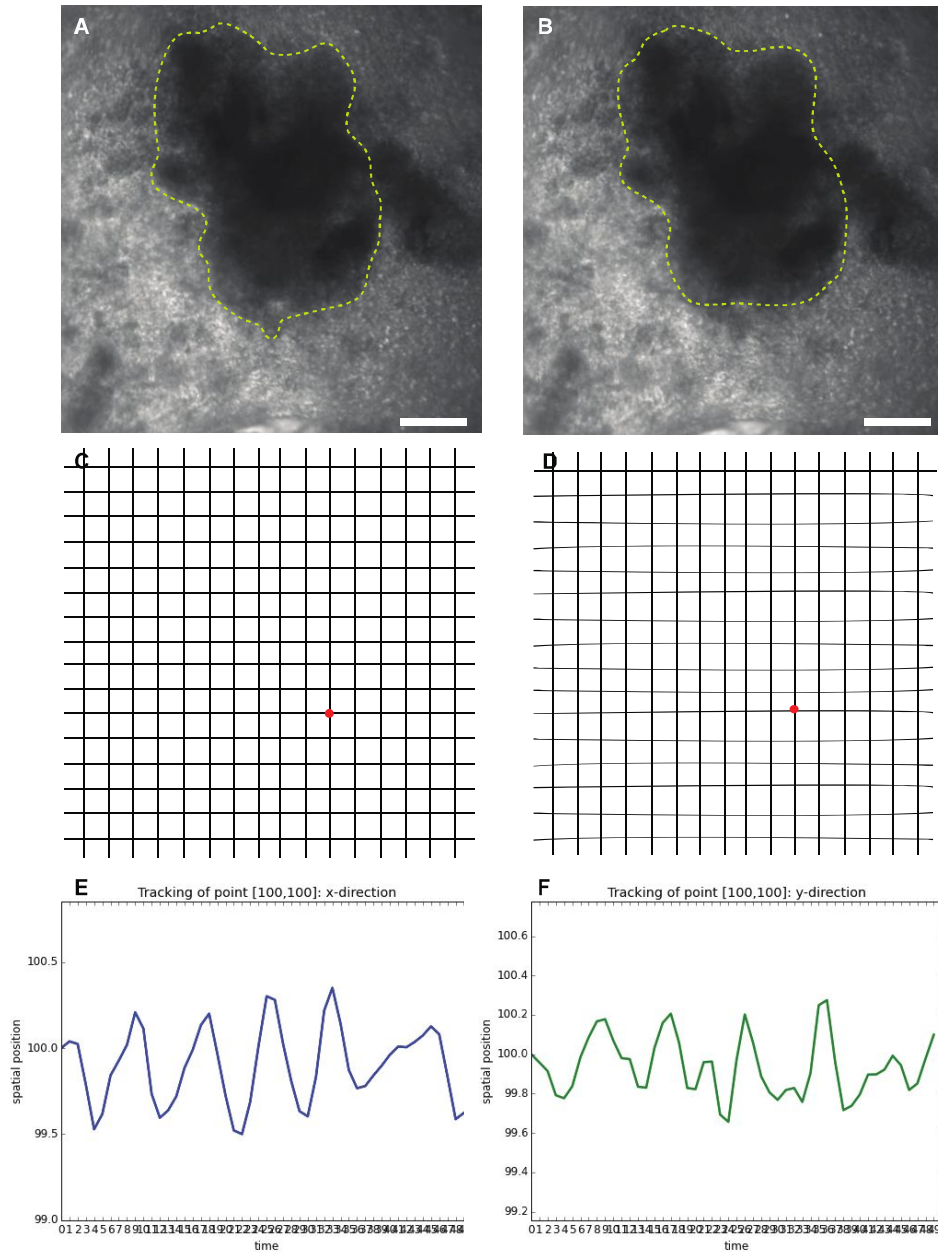


Fig. 84
TDF analysis. A and B: ROI analyzed, relaxed and contracted state respectively. Scale bar= 250 μm . C and D: Grid representing the TDF calculated displacement of the analyzed ROI at $t = 0.1$ s, relaxed and contracted state respectively. E and F: plot of the TDF displacement of a random point of the ROI ($X=100$, $Y=100$) for a certain time lapse of the video.

6.8 Regenerative potential of BSD scaffolds with human iPSC-CM in a mouse model of myocardial infarction (MI)

6.8.1 Echocardiographic evaluation of left ventricular function in a mouse model of MI

A complete characterization of LV post-infarction remodeling was performed in a mouse model of MI. Fifty-two 13 week old female immunosuppressed mice underwent permanent occlusion of the LAD coronary artery leading to MI. Four mice from the experimental group were excluded from the study: three of them died during surgery and one more 2 days after MI. Mice echocardiography was performed using a VEVO 770 before infarction (Baseline), post-infarction (PI) and 30 days after ligation. Mice were separated into 4 experimental groups: only MI mice (MI), mice with scaffolds without cells (MI+scaff), mice with BSD scaffolds with non-stimulated iPSC-CMs (KiPS ctrl), and mice with BSD scaffolds with pumped iPSC-CMs (KiPS pump).

Ultra-high resolution echocardiographies were acquired prior to sacrifice for every subject at the end-point of the experiment (30 days post-infarction). LV systolic function was evaluated by measuring the ejection fraction percentage (EF %), fractional shortening percentage (FS %), and also left ventricle anterior wall in diastole and systole (LVAWd and LVAWs), left ventricle posterior wall in diastole and systole (LVPWd and LVPWs), left ventricle interior diameter in diastole and systole (LVIDd and LVIDs), and in both short-axis and long-axis to better visualize the location of the ventricular infarction.

Results showed no statistical differences considering the 4 experimental groups, analyzing cardiac functional parameters (Table 9), excepting in the case of the LVAW, in which significant differences were found ($p=0.041$) between studied groups. Statistical differences were also found when comparing temporal evolution of each experimental group, in which MI and MI+scaffold groups were found to worsen their EF and FS functions (significant worsening), in contrast with the KiPS control and KiPS pump group, which maintained both functions (Table 9 Temporal Evolution) (Fig. 85).

Table 9

Cardiac functional values obtained from the echocardiographic analysis at baseline (2 days prior to surgery), post MI (2 days after surgery) and 30 days post implantation (prior to sacrifice). LVAWd: left ventricle anterior wall diastole, LVAWs: left ventricle anterior wall systole, LVPWd: left ventricle posterior wall diastole, LVPWs: left ventricle posterior wall systole, LVIDd: left ventricle interior diameter diastole, LVIDs: left ventricle interior diameter systole, FS: left ventricle fractional shortening, EF: left ventricle ejection fraction, $n=48$. Results represent the mean $\pm$ SD, $p<0.05$).

EF	66.07 $\pm$ 9.04	49.37 $\pm$ 6.09	33.61 $\pm$ 9.20	60.93 $\pm$ 6.18	51.77 $\pm$ 8.36	42.53 $\pm$ 10.26
	KiPS ctrl			KiPS pump		
	Baseline	Post MI	30 days	Baseline	Post MI	30 days
LVAWd	0.69 $\pm$ 0.08	0.63 $\pm$ 0.08	0.68 $\pm$ 0.08	0.75 $\pm$ 0.10	0.68 $\pm$ 0.14	0.65 $\pm$ 0.13
LVAWs	1.09 $\pm$ 0.14	0.93 $\pm$ 0.20	1.07 $\pm$ 0.16	1.14 $\pm$ 0.14	1.36 $\pm$ 0.23	1.13 $\pm$ 0.20
LVPWd	0.80 $\pm$ 0.19	0.89 $\pm$ 0.20	0.94 $\pm$ 0.23	0.84 $\pm$ 0.15	0.86 $\pm$ 0.20	0.82 $\pm$ 0.24
LVPWs	1.24 $\pm$ 0.40	1.20 $\pm$ 0.18	1.08 $\pm$ 0.30	1.13 $\pm$ 0.14	1.28 $\pm$ 0.24	1.06 $\pm$ 0.40
LVIDd	3.27 $\pm$ 0.30	3.32 $\pm$ 0.40	3.66 $\pm$ 0.37	3.34 $\pm$ 0.27	3.26 $\pm$ 0.35	3.88 $\pm$ 0.49
LVIDs	2.26 $\pm$ 0.40	2.19 $\pm$ 0.29	2.69 $\pm$ 0.48	2.36 $\pm$ 0.24	2.46 $\pm$ 0.32	2.62 $\pm$ 0.52
FS	34.47 $\pm$ 0.20	28.92 $\pm$ 8.80	24.59 $\pm$ 4.97	38.51 $\pm$ 4.83	28.52 $\pm$ 6.02	19.68 $\pm$ 5.36

EF	67.76±9.45	51.75±12.56	48.84±8.29	64.78±3.27	45.99±5.85	37.58±7.66
----	------------	-------------	------------	------------	------------	------------

	TEMPORAL EVOLUTION	
	EF %	FS %
	<i>Post-MI VS 30 days</i>	<i>Post-MI VS 30 days</i>
MI	p=0.008	p=0.007
MI+scaff	p=0.034	p=0.044
KiPS ctr	p=0.633	p=0.712
KiPS pump	p=0.064	p=0.090

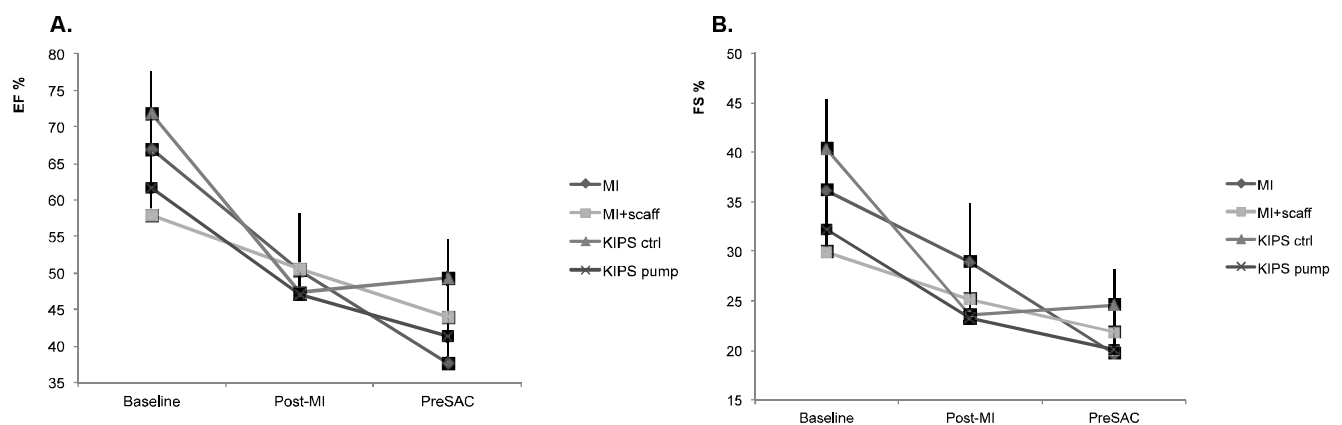


Fig. 85

Functional values (A: EF %, and B: FS %) of the 4 experimental groups measured at baseline, 2 after MI (Post-MI) and 30 days after implantation. Statistically significant differences were found evaluating temporal recovery iwhere KiPS ctr and KiPS pump groups maintained their functions in comparison with controls. Data expressed as mean $\pm$ SD, * $p < 0.05$, $n = 48$.

6.8.2 Measurement of left ventricle remodeling

Functional parameters analyzed 30 days post-implantation by echocardiography showed no statistical differences between the 4 experimental groups when comparing EF % and FS %. Besides, several sections of 10 μ m thick of each heart were studied to measure infarct size as previously described. The analyzed infarct areas showed no statistical differences between groups (Fig. 86 and 87).

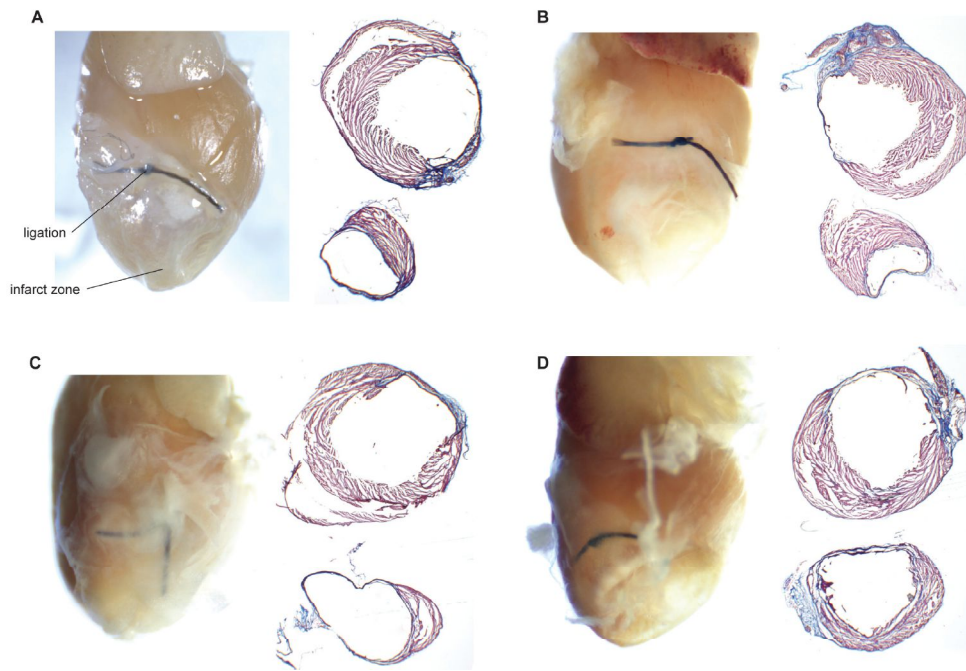


Fig. 86
Frontal anterior view example hearts of the 4 experimental groups, collected at day 30 after implantation, and representative sections of the middle and the apex of the heart. Notice ligation and infarct zone in every specimen with no remarkable differences between them. A: MI heart, B: MI+matrix heart, C: MI+KiPS ctrl heart, D: MI+KiPS pump heart.

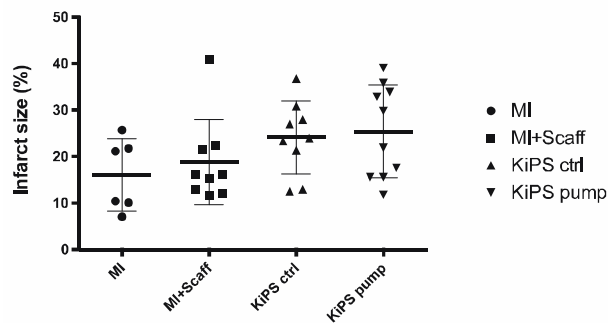


Fig. 87
Infarct area 30 days after implantation expressed as a relative percentage (%) of the entire LV area. No statistical differences were found between groups. Data expressed as mean $\pm$ SD, * p < 0.05, n = 48.

6.8.3 Tissue engraftment

Engraftment of the iPSC-CM in the host tissue was analyzed by histochemistry with Masson's trichrome and immunohistochemistry against GFP. Scaffolds (alone or with iPSC-CM) were found to be adhered to host myocardium, and all of them contributed to avoid LV wall thinning in comparison with MI group despite no statistical differences were found. In the case of BSD scaffolds with iPSC-CMs, stimulated or not, it was observed a partial engraftment of the cells into the native myocardium, as trichrome studies revealed (Fig. 88) and also corroborated by GFP (Fig. 89).

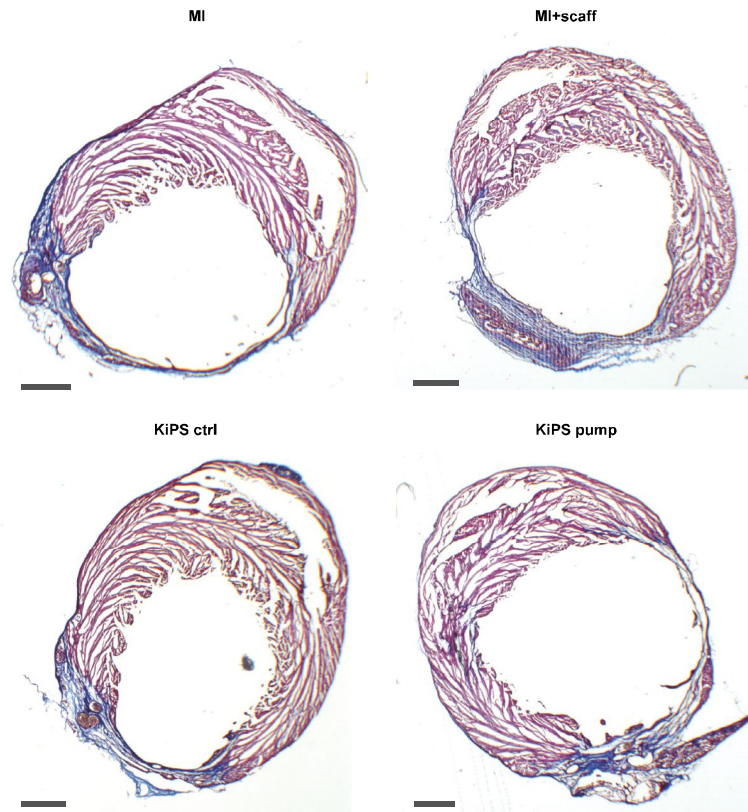


Fig. 88

Masson's trichrome staining of different heart sections from the 4 different experimental groups. In MI group a considerable thinning of the LV wall can be appreciated, whereas in the MI+scaffold group the scaffold alone contributed to avoid LV wall thinning. KiPS ctrl and KiPS pump images show partial cell engraftment in host myocardium (red tissue is continuous from side to side of the wall, being more evident in the KiPS pump group). Scale bar= 1mm.

Moreover, 30 days post-transplantation iPSC-CM-GFP⁺ were also stained for AAS and CX43 and detected in BSD scaffolds (both control and pumped) in every individual. Many iPSC-CM-GFP⁺ exhibited organized Z-bands confirming their cardiac phenotype (Fig. 89). Interestingly, in some individuals, iPSC-CM-GFP⁺ were found within the native tissue, out of the BSD scaffold, considering this fact as a sign of cell migration to host myocardium.

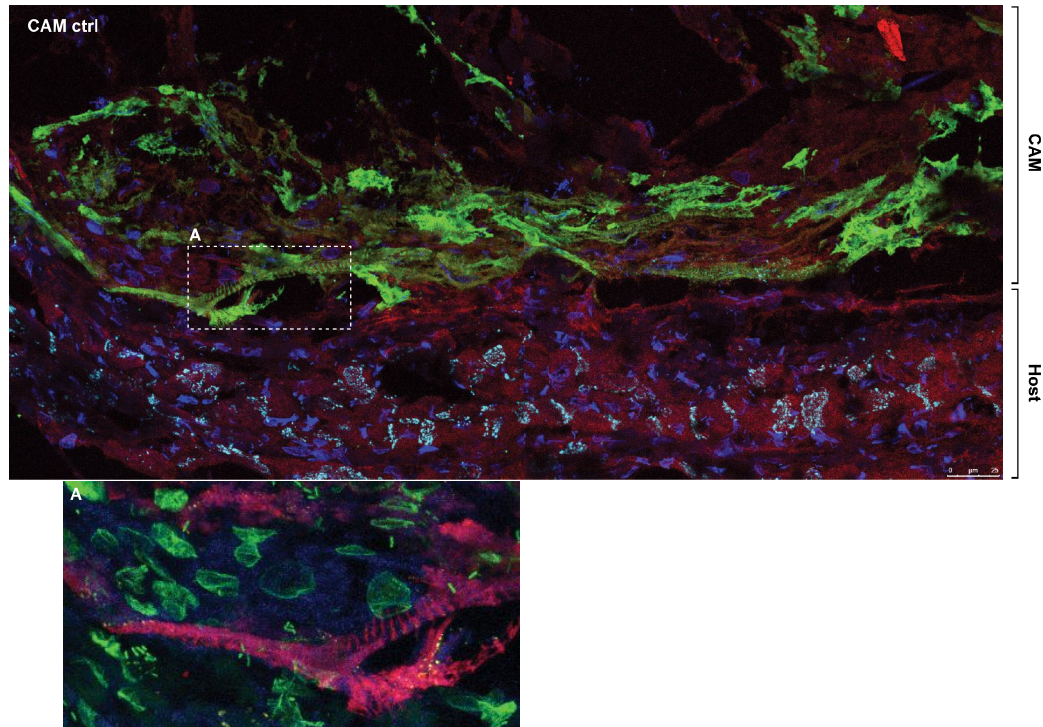


Fig. 89

Presence GFP⁺ transplanted iPSC-CMs was confirmed by immunoconfocal microscopy for both groups, BSD ctrl and BSD pump (n= 24). iPSC-CM-GFP⁺ in green, CMs were stained with alpha-actinin sarcomeric (AAS) in red, Cx43 in turquoise, and nucleus in blue with DAPI. Implanted iPSC-CM-GFP⁺ could be co-localized with AAS confirming their cardiac phenotype.

In addition, BSD scaffolds were also analyzed parallel to the in-vivo in order to analyze morphology and distribution of iPSC-CM-GFP⁺ in the scaffold, as for previous experiments. GFP was observed to be expressed in numerous iPSC-CM but not in all of them, as some cells were found positive for TNT and negative to GFP (Fig. 90). Interestingly, captured videos showed a complete synchronization of the cells in all of BSDs, exhibiting a huge contraction appreciable to the eye before were implanted in the mice (see videos KiPS-10x-Bf-PUMP, KiPS-10x-Bf-CTRL, and KiPS-10x-GFP-PUMP)

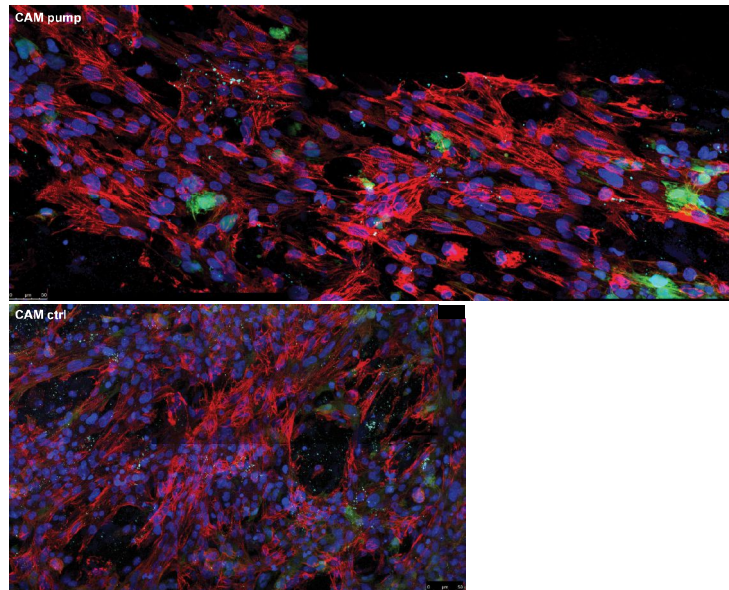


Fig. 90

Immunofluorescence images BSD scaffolds control and pumped with iPSC-CM-GFP⁺ analyzed by immunoconfocal microscopy. CMs were stained with TnT (in red), Cx43 (in turquoise), GFP (in green), and DAPI for nucleus (in blue). iPSC-CMs were found to be more aligned in pumped BSDs than in controls, in agreement with previous results.

In summary, our results indicate the feasibility of the transplantation of prefabricated BSD scaffolds with iPSC-CMs, mechanically stimulated or not, in a mouse model of acute MI, with a high cell survival after 30 days of engraftment. Implanted BSDs suggest beneficial effects by inducing a positive tissue remodeling and an increase in the function recovery, despite no statistical differences were found between stimulated and control BSDs. The promising features of these stimulated scaffolds require further research on stimulating iPSC-CMs and new strategies that potentiate their engraftment and survival in the heart tissue, contributing in a greater manner to the cardiac tissue regeneration.

7. Discussion

7. DISCUSSION

Porcine myocardium assays

It is well known how porcine heart anatomy and functional values resemble human (Milani-Nejad & Janssen, 2014) (Crick et al., 1998), not only at an anatomical level but at a histological level (Wainwright et al., 2010). Regarding this similarity, a pig's heart can be then considered as a useful platform to study functional and histological parameters, akin to human. Results of our tensile strength assays with porcine myocardium showed a high anisotropy of the tissue, where mechanical behavior strictly depended on the relation between the applied force direction and the muscle's fiber orientation, as other studies have also reported (Giles et al., 2007), and with other animals (Gupta et al., 1994) (Mirsky & Parmley, 1973). This fact is attributed to myocardium's muscle cell-fibers distribution and organization, in which there is no predominant direction throughout the thickness of myocardial tissue, but a continuous rotating distribution of the muscle bundles, that swivels from one muscle layer to another (Fig. 91) (Marcé-Nogué et al., 2013) (Zhang et al., 2015) (Dervaux & Ben Amar, 2008).

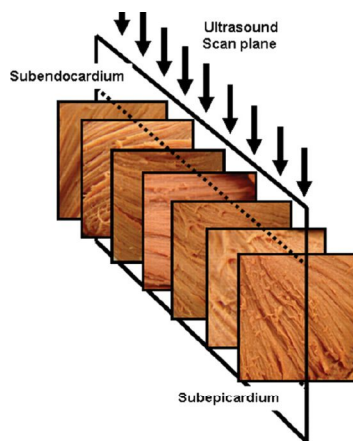


Fig. 91

Image of the aligned structure of CMs in the myocardium obtained by echography technique. The alignment is uniform in each layer with a gradual transition between layers from the endocardium to the epicardium. (Mor-Avi et al., 2011)

In our initial hypothesis, as myocardium's mechanical behavior is highly anisotropic (Chaturvedi et al., 2010) (Costa et al., 2001), we assumed that the direction of the force applied in relation with the muscle-fiber orientation could influence myocardium's mechanical behavior. Our results showed similar values when comparing average directions with statistical differences only in the transversal one. This is due to the fact that the thickness of our myocardium samples included several muscle layers, and therefore no single predominant direction could be found. When average of the test directions of the porcine hearts (transversal, oblique and parallel) were compared one to another, interestingly, the values were found to be similar. In contrast, when the hearts were compared between them considering only one tested direction, the differences between were statistically significant for many specimens, fact that suggests a great variability between individuals.

Pluronic and hydrogels assays

Considering stiffness results of the porcine myocardium, the design of a hydrogel that met the mechanical properties of the heart was the next goal. It has been previously demonstrated how synthetic and natural hydrogels can contribute to the healing of an infarcted myocardium. Hydrogels, besides of keeping the cells in the site of interest and give mechanical support to the damaged area, can also provide a biomimetic 3D

milieu for the cells, and a temporal shelter from hostile environment. Among others, ideal biomaterials should be biodegradable, biocompatible, biomechanically and biochemically compatible with the tissue and may include bioactive features such as induce angiogenesis or being capable of delivering bioactive molecules (Arnal-Pastor et al., 2013). Particularly, thermoreversible hydrogels were found to be highly interesting, as elsewhere explained, for the development our myocardial graft. Initially, thermoreversibility was a non-avoidable constriction for our study, together with adequate mechanical elasticity. From the selected candidates, PF127 seemed to be the most suitable, due to its elasticity results obtained in the rheological assays. Nevertheless, PF127 dissolved in every study when culturing it with medium, with the consequent drifting of the HFFs in the medium, hence the candidate was discarded. Our assays with PF127 crosslinked with gelatin did not show sol-to-gel transition at any temperature, despite other studies seem to prove the opposite (Kim et al., 2010) and consequently, we abandoned the approach. Our results for PDLLGA with PEG showed that the resulting polymer film was not capable of being dissolved in an aqueous solution, therefore, it resulted impossible to form a hydrogel, despite some evidence can be found in the literature (Hermanson, 2008) (Radhakrishnan et al., 2014). After these unsuccessful attempts, we decided to fabricate our scaffolds following Zimmermann's protocol, based in collagen rings. Recently, at the end of this research, we have found the collaborating group of Dr. C. Ciccione from the Tissue Engineering Lab at Rome's Biomedical Campus, which are currently working on a new polymer of PF127 crosslinked with collagen that may meet our 2 main requirements: mechanical elasticity and thermoreversibility. Unfortunately it cannot be included in this study because its design has not been finished at this point.

EHTs and BSDs with rat neonatal cardiac cells

As previously seen, the use of scaffolds composed of natural biodegradable matrices represents an attractive strategy to circumvent the maturation of CMs by applying mechanical or electrical stimulation. Numerous studies have proven with rat cardiac cell scaffolds (Zimmermann et al., 2002) (Fink et al., 2000) (Vunjak Novakovic et al., 2014) how mechanical stimulation provides CMs of a more matured-like phenotype in comparison with non-stimulated controls. In the current study, EHTs and CAMs scaffolds were fabricated and stimulated for 7 days as elsewhere described, and we demonstrated how CMs in scaffolds subjected to mechanical stretch, constant and auxotonic in the case of the springs, or dynamic and pulsed in the case of the CAMs, exhibited more abundant and organized Z-bands in comparison with controls, together with a more elongated cytoplasm, and an enhanced degree of alignment between them within the scaffolds. Importantly, in our study we have developed a novel method to evaluate CMs sarcomere abundance by diving cTnT-positive CM population into 3 different groups: ALL, SEMI, and NO, depending on the percentage of CMs showing Z-bands. In stimulated scaffolds, the percentage of CMs exhibiting Z-bands in the ALL, and also the SEMI-group was statistically higher in comparison with control scaffolds in all of our studies. This apparent subjective criterion for CM's classification regarding sarcomere abundance was reinforced by the evaluation of 3 different colleagues of the laboratory, blinded to prior results, who analyzed numerous scaffolds. Their results were in accordance to ours considering percentage of cells exhibiting Z-bands and their classification into the 3 groups (Fig. 92). Hence, we could successfully validate this technique to apply it to further studies. Moreover, it is known how the degree of sarcomere intracellular organization can be directly related with the CM stage of maturity, as recent studies suggest (Pasqualini et al., 2015) (Bedada, Wheelwright, & Metzger, 2015) (D. Zhang et al., 2013), together with techniques related to measure sarcomere length (Vunjak Novakovic et al., 2014) (Iribe & Kohl, 2008). Besides, new computational techniques are being developed to automatize similar approaches to measure sarcomere abundance and organization in the cell.

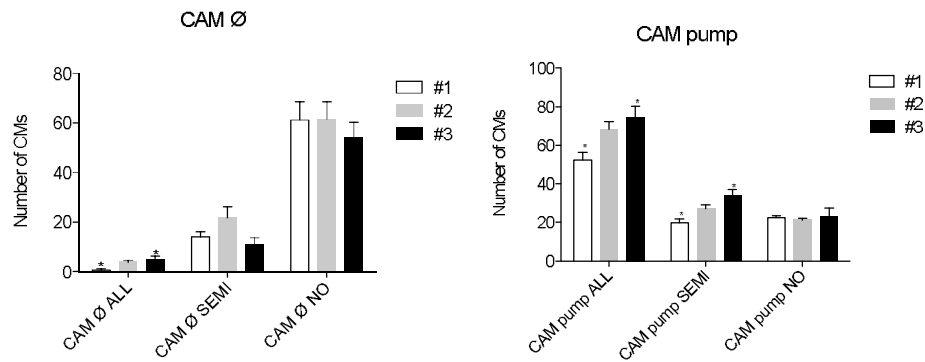
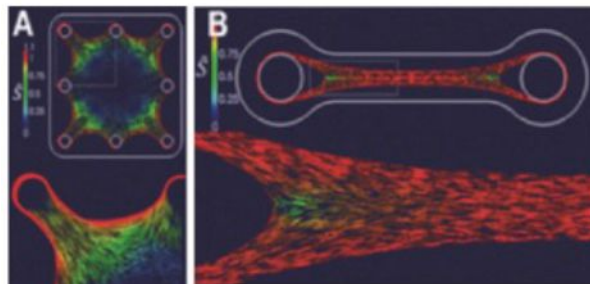


Fig. 92

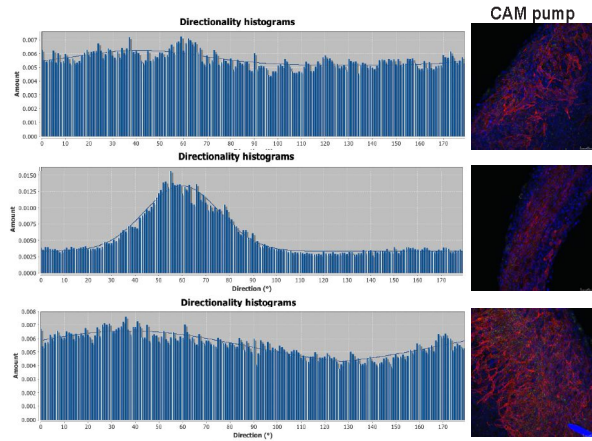
Presence of Z-bands in ALL, SEMI and NO groups, evaluated by 3 different colleagues of the laboratory: #1, #2, and #3. (A) control CAM, (B) stimulated CAM.

In addition, our study proved that CMs exhibited a longer cytoplasm and were distributed in an aligned pattern in mechanically stimulated scaffolds than in controls, in which CMs were found to be significantly shorter and randomly distributed instead. These EHT results were in accordance with the ones published by Zimmermann's group, which demonstrated the effect of mechanical constant strain to rat cardiac cells scaffolds by analyzing electro-mechanical functional parameters of the scaffolds, and also with histology, immunohistochemistry and TEM studies. Importantly, in our study a new technique was developed to quantify presence of Z-bands that may help other researchers to measure CM maturation.

Once we demonstrated the effects of mechanical stretching to EHTs with the torsion springs, the same hypothesis was evaluated with our novel biomimetic pumping system (CAM), which offers an innovative dynamic pulsed stimulation platform, similar to the one cells could find in native myocardium. Results proved that our novel stimulation system enhanced the presence of CMs showing abundant and organized Z-bands in the mechanically pumped CAM scaffolds in comparison with controls. Interestingly, the device lead CMs to experience more tension in certain zones of the scaffold, and consequently, cells were found to be more aligned in stressed zones, and randomly distributed in stress-free zones. This stress mediated alignment, which has been recently studied in a similar setup (Sirabella et al., 2015) (D. Zhang et al., 2013), was overcome in our study by analyzing equivalent regions in the scaffolds in order to uniquely compare stress-mediated CM populations (Fig. 93). Moreover,



C.



D.

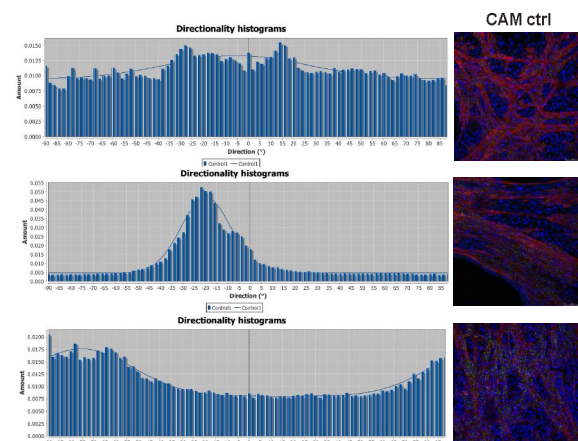


Fig. 93

Microtissues mathematical modeling similar in geometry to our (A) CAM scaffold and (B) EHT scaffold exhibiting stress-mediated alignment of the cells. Extracted from (Sirabella et al., 2015). Angle histograms of different regions of the scaffolds (CAM scaffolds) measured in (C) pumped CAMs, and (D) CAM controls. Both conditions exhibited highly orientated and random oriented CMs.

Moreover, cell lengthening and alignment were found to be a characteristic of a stretched tissue rather than a sign of maturity. Cells could be stretched and therefore lengthen and parallel in the direction of the exerted force, with no signs of cardiac maturation (Tulloch et al., 2011).

According to literature (Kostin et al., 2004), Cx43 is found to be clustered at both extremes of an adult CM when analyzed by immunohistochemistry. Hence, the more mature-like phenotype a CM had, the more clustered and polarized Cx43 should appear (Y. Zhang et al., 2009). In our studies, no significant differences were observed between stretched and control conditions in EHTs or CAMs. In our studies, Cx43 clusters were rarely found at both ends of CMs, but distributed among the cell, probably due to an insufficient level of gap-junction organization, and thus CM maturation as some studies agree (Zhuang, Yamada, Saffitz, & Kléber, 2000) (Kostin, Hein, Bauer, & Schaper, 1999).

Regarding TEM results, remarkable differences were observed between stretched and control conditions, mainly found in the enhanced presence, size and organization of sarcomeres in stimulated scaffolds, as other studies (Mihic et al., 2014) (Zimmermann et al., 2002), together with the presence of gap-junctions and the increased number of mitochondria.

Electrical coupling of EHTs and BSDs with rat cells

Many studies have reported the ability of rat cardiac cells when cultured in scaffolds to couple with electrical pulses (Thavandiran, Nunes, Xiao, & Radisic, 2013) (Miklas et al., 2014) (Zimmermann et al., 2000). In our study, we hypothesized that despite we could not observe macroscopic contractions of our entire constructs, fact attributed to a probable lack of an interconnected network between CMs, they might couple with an external electrical pulse if they had reached certain level of maturity (Radisic et al., 2004). Our results showed that the majority of constructs successfully coupled partially with electrical pulses, in spite of considerable variability in synchronization between experiments. By varying voltage and pulse width (PW) most of the scaffolds could be

synchronized in several regions but still, some did not. This fact may suggest a relative low percentage of CMs in the inside the scaffold, and therefore no contraction can be appreciated, or a critical survival of the CM population. Interestingly, when scaffolds exhibited large contractions before applying electrical pulses, tension and PW thresholds could be decreased down to a minimum of 4 V and 2 ms. Fact in agreement with other studies (Marsano et al., 2008), in which they demonstrate the more adult-like phenotype a CM had, the lower voltage threshold it needed to be excited. In contrast, if scaffolds did not show evident coupling prior to electrical synchronization, threshold voltage and PW had to be increased (up to 10 V and 100 ms) in order to observe electrical coupling. Importantly, when pulse width (>50 ms) and potential (>10 V) were increased over a certain threshold, electrolysis of the culture medium was observed, reaching the point that if voltage was over 25 V, culture medium suddenly changed color (accelerated electrolysis), and therefore, experiment stopped as cell viability could be seriously compromised. Further studies would be needed to understand the great variability of the electrical response of our 3D scaffolds when applying external pulses, as some authors suggest (Godier-Furnémont et al., 2015).

Differentiation efficacy & morphological evaluation of iPSC-CM scaffolds (rings and BSDs)

We demonstrated how mechanical stimulation contributed to enhance maturity of rat CMs, in constant stimulation conditions (torsion springs), and in dynamical stimulation conditions (CAMs). Our next achievement was then to explore the usefulness of these stimulation techniques applied to human iPS cells and analyze their contribution to CM maturation. As previously explained, iPS cells due to their embryonic-like properties, are able to differentiate in-vitro towards almost any cell type, including CMs. Cardiac differentiation protocols present great changeability considering cell source as some studies point (Streckfuss-Bomeke et al., 2013). Moreover, it is known that most differentiation protocols result in CMs that lack a mature ventricular phenotype (Xi et al., 2010) (Iglesias-García et al., 2015), fact which is also affected by the selected iPS cell line. In our study, flow cytometry results revealed a CM-deriving efficacy near 40 %, relatively low in comparison with other published studies (Lian et al., 2012), probably due to the nature of our iPS cell line generated from keratinocytes, as it has been suggested in other studies (Blanpain et al., 2012). Despite cardiac differentiation protocol was optimized for our study and efficacy did still vary from one study to another, scaffolds with iPSC-CM were always fabricated with an homogeneous cell population.

Regarding iPSC-CM maturity, our results demonstrated that Z-bands were also found to be statistically more numerous and organized in CAM or ring scaffolds subjected to mechanical stimulation (constant or dynamic) than in control scaffolds, in concordance with previous results obtained for rat cardiac cells. Immunofluorescence studies also exhibited lower levels of Cx43 expression in comparison with neonatal rat cardiac cells, with neither relevant difference between conditions. This fact is attributed to a general lack of maturity of human iPSC-CMs in spite of mechanical stimulation, exhibiting a more fetal-like phenotype, probably due to the insufficient duration of the experiments , as some studies suggest (Bedada et al., 2015). TEM results also showed great differences between conditions, being more abundant and bigger in size sarcomeres in the mechanically stimulated scaffolds than in the controls.

Electrical coupling of ring-shaped scaffolds and BSDs with iPSC-CM

Akin to rat cardiac cell scaffolds, human iPSC-CM showed great variability in cell coupling with electrical pulses (Halbach et al., 2013). In our study, human CMs were found to be considerably more difficult to couple than rat cells. Voltage and PW needed

to be increased in order to obtain a contractive response in certain regions of the scaffold. However, an entire synchronized macroscopic contraction was never observed when applying electrical pulses. As the differentiation protocol efficacy was highly variable, it is probable that the low number of actual CMs impeded a continuous cell-to-cell contact, and therefore a macroscopic contraction of the whole scaffold, besides a possible insufficient degree of maturity in certain cases. In contrast, some studies have demonstrated electrical coupling of cell populations for several days (Nunes et al., 2013) (Sirabella et al., 2015), but in our study it was not evident if mechanical preconditioning affected the electrical coupling of the scaffolds. Moreover, electrolysis of the culture medium when synchronizing the constructs at such levels of voltage and PW obliged us to stimulate only for a few minutes, as we did not want to compromise cell viability. Further studies on cell coupling would be highly valuable to understand 3D electrical behavior, as it radically differs from 2D cultures as some studies mention (Breckwoldt et al., 2015) (Vunjak Novakovic et al., 2014).

qPCR results

To investigate the effect of mechanical stimulation in iPS cardiac cells, the expression of cardiac proteins was analyzed between conditions. We observed an up-regulation of cTnT in the stimulated scaffolds in comparison with control scaffolds, despite no statistical differences were found between conditions. Interestingly, this expression of cTnT was in the same order of magnitude as human adult ventricle (Kamakura et al., 2013). MYH7 was also found to be more expressed in stimulated scaffolds, but still far from adult ventricle values (Lundy et al., 2013) (Tulloch et al., 2011). ACTC1, CACNAC1 (Sirabella et al., 2015), CX43 (Lux et al., 2015), MYH6, and RYR2 (Godier-Furnémont et al., 2015) levels were found to be slightly under the ones of human adult ventricle. In our study, no variations of the isoforms of MYH6 and MYH7 were observed between conditions, although MYH6/MYH7 ratio is known to change from fetal to adult stages (Reiser et al., 2001), probably due to the fact that our human iPS cells exhibit a more fetal-like phenotype (Feric & Radisic, 2015). Overall, our human iPS cells gene expression is still far from adult human ventricle. These results suggest that iPSC-CM maturation in our study is more structural (related with the arrangement of certain proteins in the cell, such as TnT) than expressional (related with the quantity of expressed transcripts). In addition, the duration of the experiments should be prolonged, as the degree of maturation CMs could reach within our experimental time-lapse (7 days of pumping) was probably insufficient in order to find statistical differences between conditions in gene expression, as some studies suggest (Passier et al., 2008). Moreover, fetal and infant human left ventricle controls would result highly useful in forthcoming studies to better characterize the stage of maturation our human iPS cells are.

Calcium imaging results for ring-scaffolds and BSDs with iPSC-CM

Interestingly, when the cardiac functionality of the scaffolds was analyzed by calcium imaging, important qualitative differences in the dynamics of calcium transients were observed between conditions (see videos CALCIUM CAM pump and CAM Ctrl), despite further analysis would be needed to confirm these observations. Considering future studies, it would be a highly promising technique to measure and evaluate cardiac tissue maturity from a functional point of view, as it has been elsewhere suggested (Herron et al., 2012).

Fractional area change analysis

Analyzed ROIs showed variations in fractional area shortening (FAS) within time of the experiment, as other studies suggest (Tulloch et al., 2011). Scaffolds progressively increased FAS over days, until they reached a stationary state, in which no increments of the contracted areas could be observed. Statistical differences were found between FAS at day 1 and FAS at day 7 for ROI of a CAM pump scaffold. Despite such effect was only found once, further experiments would be needed in our study to corroborate such evidence. Previous studies proved the effect of electrical stimulation (Radisic et al., 2009) (Tandon et al., 2011), and mechanical stimulation (Tulloch et al., 2011) (Zimmermann et al., 2002) in human stem cells derived-CM constructs, but did not evaluate response within time. Hence, further studies should consider analyzing variations over time into the human cardiac constructs, comparing stimulated conditions and controls to study iPSC-CM maturation in these sort of constructs.

Regenerative potential of the BSDs in a mouse model of MI

As we demonstrated the contribution of mechanical stimulation to enhance human CM maturity, we then studied the therapeutic benefit of the iPSC-CM scaffolds in a mouse model of MI. Few studies have demonstrated the beneficial effects derived from stem cell therapy for the treatment of myocardial infarction (MI) (Passier et al., 2008) (Behfar et al., 2014), and particularly with human iPSC-CM in collagen scaffolds (Tulloch et al., 2011). Cells injected alone possess a critical survival in injured hearts due not only to the injection methods, but also to the ischemic environment and the lack of cell-to-cell and cell-to matrix contact with the following loss of survival signals (Terrovitis, Smith, & Marb??n, 2010). Therefore, cells in scaffold offer a greater framework to develop human cardiac tissues, in spite of problems of coupling to prearrangement of cells in the inside and non-controlled 3D niche (Breckwoldt et al., 2015).

In our study, human cardiac tissues with pre-stimulated (and controls) iPSC-CMs in collagen scaffolds were implanted onto the myocardium of the left ventricle to study the different contributions of pre-mechanically stimulated scaffolds in comparison with controls to the cardiac recovery after a MI in a mouse model. Mice were analyzed by high-resolution ultrasound imaging equipment, with which structural measurements were acquired in both, paraesternal short-axis and long-axis views, in order to better visualize the infarct area. Functional results did not show general statistical differences between groups, but for the LVAW, a physical effect probably due to the presence of the scaffolds in the wall of the ventricle, as other studies report (Lakshmanan, Krishnan, & Sethuraman, 2012) (Serpooshan et al., 2014). Interestingly, a preservation of the cardiac function was observed for the mice with CAMs with iPSC-CMs (KiPS ctrl and KiPS pump groups) in comparison with the control groups (MI and MI+scaffold) when analyzing temporal evolution of the EF % and the FS %, fact that suggests a temporal contribution of the cells in the scaffolds to the performance of the heart, in comparison with the acellular groups. In spite of this observation, infarct sizes were found to be similar between groups 30 days after post-implantation. The overall lack of functional contribution of the scaffolds to regenerate injured myocardium in our study could be due to experimental limitations. First, the duration of the in-vivo experiment could be insufficient in order to appreciate some functional significant changes, as other studies have suggested (Iglesias-García et al., 2015). This fact should be carefully considered as scaffold composition and cell types vary considerably from one study to another; in spite of some studies have demonstrated beneficial effects of implanting iPSC-CM-scaffolds (Bearzi et al., 2014), very few have attempted a mechanical pre-stimulation of the cells prior to implantation, as ours attempted. Second, iPSC-CM were transplanted after several days of differentiation and preconditioning in the scaffolds, a fact that might have impaired optimal engraftment of

the constructs into the damaged myocardium, as some studies suggest (Laflamme et al., 2007). Highly differentiated and matured cardiomyocytes are more sensible to hypoxic environments together with a reduced cellular plasticity and reduced capacity to establish new connections to native cells (Laflamme & Murry, 2011), therefore it might hindered the success of our scaffolds in the experiment. Importantly, the approach chosen to attach the scaffold on the myocardium may have influenced the engraftment of the construct in the myocardium (Gerbin & Murry, 2015). If the scaffolds move or did not contact entirely the LV wall, could not obviously couple effectively to host tissue, and hence, could not contribute to the regeneration of the damaged tissue.

Regarding cell migration, we demonstrated that a few number of cells were observed to have migrated from the scaffold to host myocardium. It is likely that our scaffolds, despite they are a useful system to stimulate and increase maturation in CMs, may not constitute an optimal platform to allow cell engraftment in an infarcted myocardium. After several days of culture and stimulation in the scaffolds, many CMs have become hypertrophied and established strong interconnections between them, being this fact possible impede to their migration to host tissue. In our scaffolds, iPSC-CMs were arranged in the scaffold prior to engraftment, and even maturity, could suppose a disadvantage in cell migration, as iPSC-CMs were functional (see videos KiPS-10x-Bf-PUMP and CTRL) and fully coupled between them as immunoconfocal studies demonstrate, and this prior configuration of our cardiac tissues, may not easily allow to establish new attachments to host ECM and new cell-to-cell contact (Zhu, Hauch, Xu, & Laflamme, 2009). In contrast, some studies have demonstrated the establishment of cellular interconnections between transplanted stem cells and native cells, when cells were injected in a cell suspension, despite the critical survival of the population (Lakshmanan et al., 2012) (Reinecke, Zhang, Bartosek, & Murry, 1999). Successfully in our study, numerous iPSC cells survived after 30 days in the scaffold, exhibiting Z-bands, overcoming the hurdle of the limited oxygen supply in the ischemic area, despite a massive death is known to occur within the first week after engraftment, as other studies suggest (Kutschka et al., 2006) (Kawamura et al., 2012). Further experiments with iPSC-CMs in 3D scaffolds at different stages of maturation would be highly useful to deepen in the integration of such constructs in a damaged myocardium. In addition, more studies could be proposed in which cardiac constructs could be prepared also with early-stage differentiated iPSC-CMs and compare the degree of engraftment between the early-stage iPSC-CMs and matured iPSC-CMs, combining mechanical with electrical stimulation (Stoppel, Kaplan, & Black, 2015).

In general terms, more extensive studies would also be necessary to assess functional integration and electrophysiological coupling of cardiac constructs in-vivo, when scaffolds had been previously mechanically stimulated, and also study co-cultures with different cell types, such as endothelial cells, as some studies suggest (Radisic & Christman, 2013). Comparative in-vivo studies between electrical and mechanical contribution to the engraftment of iPSC-CMs in 3D scaffolds, would be highly profitable for the scientific community. Angiogenesis, together with optimal electromechanical coupling of the implanted construct with host myocardium, still represent a major hurdle for a durable and effective myocardial graft. The improvement of the survival of foreign CMs in the damaged myocardium, besides inducing their integration and ulterior maturation are necessary features that would need more extensive research. Importantly, some other questions in the field of cardiac tissue engineering would need general consensus (Hirt et al., 2014), such as more quantitative methods to evaluate CM maturation with adequate control references, definition of normal values and deviations; and even enhanced robustness in the differentiation protocols, so frequently found to be cell-line dependent as elsewhere stated (Kaichi et al., 2010). Many of the advantages of 3D engineered tissues compared to 2D cultures remain theoretical, as no comparable references are available (Breckwoldt et al., 2015).

Uniform criteria in an international framework to compare maturation in 3D from different research groups would result highly profitable. The 3D heart muscle engineering has undergone exciting progress for the past decade, and is rapidly moving to new horizons. The understanding of cardiac regeneration remains a challenge, as the hope of restoring cardiac functional tissue that the heart itself does not regenerate, represents a major approach in biomedical engineering.

In summary, we have demonstrated that human functional cardiac tissue can be successfully fabricated with human iPSC-CMs, obtaining improved degrees of CM maturation by providing mechanical cyclical stimulation in a novel biomimetic system, and the feasibility of preserving cardiac functions after a MI for 30 days in a mouse model. This research, provides an interesting framework in order to evaluate human iPS derived-cardiomyocyte improved maturation induced by mechanical stimuli, which could be highly useful to broaden the study of cardiac maturation process itself, or to study pharmacological response, together with the study of the fundamentals of a functional human cardiac patch in an in-vivo approach (Fig. 94).

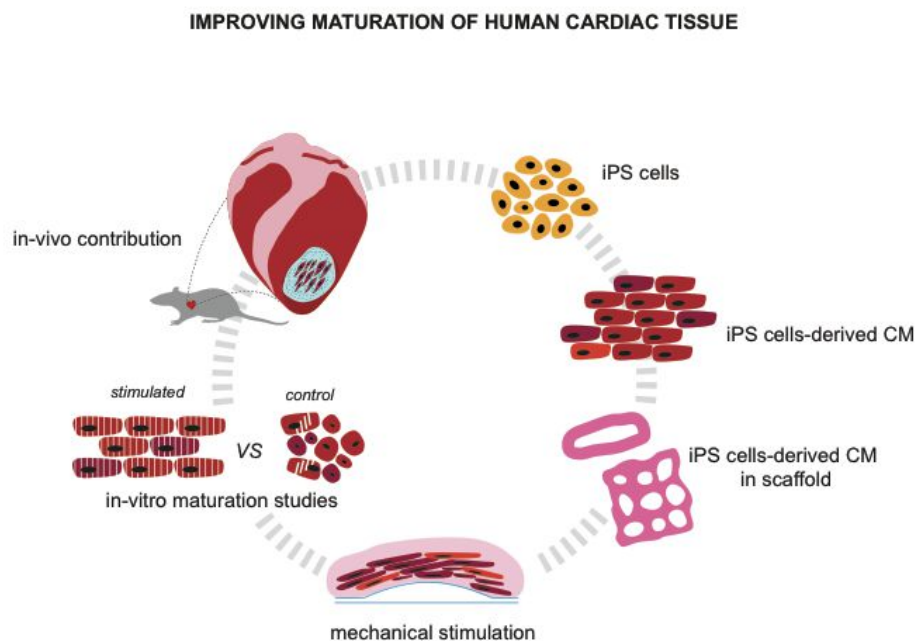


Fig. 94

iPS cells-derived cardiomyocyte in collagen scaffolds subjected to mechanical stretch, show an enhancement of maturity in comparison with control conditions. Moreover, engrafted in a damaged heart tissue exert a positive effect including the preservation of cardiac remodeling and tissue viability.

8. Conclusions

8. CONCLUSIONS

- 1.** Porcine myocardium tensile strength assays presented an increased stiffness value when loaded parallel to the longitudinal direction of the muscle direction in comparison with the oblique or transversal directions, with an average value of ~22 kPa.
- 2.** We have designed, implemented and fabricated a novel biomimetic system for culturing cardiomyocytes embedded in collagen-based hydrogels, which provides controlled cyclic mechanical stimulation consisting on a 3-dimensional biomimetic radial pumped stretching, to stimulate rat and human iPS cells.
- 3.** Our novel device induces CMs maturation, as stimulated cells were found to exhibit a more matured phenotype under dynamic pulsed pumping in comparison with controls.
- 4.** A new method to objectively evaluate CM maturation was developed to measure and classify cells exhibiting cross-striation.
- 5.** The implantation of human iPSC-CM scaffolds in a model of mice MI did not evidenced an improvement of the cardiac function, despite a temporal recovery of subjects with treatment was observed.
- 6.** Altogether, these findings show the potential of our novel biomimetic stretching device to increase maturation in cardiac scaffolds and represent a suitable platform to fabricate and study human myocardial grafts.

9. Bibliography

- Abraham, C. M. (2014). A brief historical perspective on dental implants, their surface coatings and treatments. *The Open Dentistry Journal*, 8, 50–5. <http://doi.org/10.2174/1874210601408010050>
- Ahuja, P., Sdek, P., & Maclellan, R. W. (2007). Cardiac Myocyte Cell Cycle Control in Development, Disease and Regeneration. *Physiol. Rev.*, 87(2), 521–544. <http://doi.org/10.1152/physrev.00032.2006.Cardiac>
- Almeida, H., Amaral, M. H., Lobão, P., & Lobo, J. M. S. (2012). Pluronic® F-127 and Pluronic Lecithin Organogel (PLO): main features and their applications in topical and transdermal administration of drugs. *Journal of Pharmacy & Pharmaceutical Sciences: A Publication of the Canadian Society for Pharmaceutical Sciences, Société Canadienne Des Sciences Pharmaceutiques*, 15(4), 592–605.
- Arnal-Pastor, M., Chachques, J. C., Monleón Pradas, M., & Valles-Lluch, A. (2013). Biomaterials for Cardiac Tissue Engineering. *Regenerative Medicine and Tissue Engineering*, 275–323. <http://doi.org/10.5772/56076>
- Badylak, S. F., Taylor, D., & Uygun, K. (2011). Whole-Organ Tissue Engineering: Decellularization and Recellularization of Three-Dimensional Matrix Scaffolds. *Annual Review of Biomedical Engineering*, 13(1), 27–53. <http://doi.org/10.1146/annurev-bioeng-071910-124743>
- Bao, L., He, L., Chen, J., Wu, Z., Liao, J., Rao, L., ... Xiao, L. (2011). Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors. *Cell Research*, 21(4), 600–8. <http://doi.org/10.1038/cr.2011.6>
- Barash, Y., Dvir, T., Tandeitnik, P., Ruvinov, E., Guterman, H., & Cohen, S. (2010). Electric field stimulation integrated into perfusion bioreactor for cardiac tissue engineering. *Tissue Engineering. Part C, Methods*, 16(6), 1417–1426. <http://doi.org/10.1089/ten.tec.2010.0068>
- Bartman, T., & Hove, J. (2005). Mechanics and function in heart morphogenesis. *Developmental Dynamics*, 233(2), 373–381. <http://doi.org/10.1002/dvdy.20367>
- Baumgartner, S., Halbach, M., Krausgrill, B., Maass, M., Srinivasan, S. P., Sahito, R. G. A., ... Hescheler, J. (2015). Electrophysiological and morphological maturation of murine fetal cardiomyocytes during electrical stimulation in vitro. *Journal of Cardiovascular Pharmacology and Therapeutics*, 20(1), 104–112. <http://doi.org/10.1177/1074248414536273>

- Bearzi, C., Gargioli, C., Baci, D., Fortunato, O., Shapira-Schweitzer, K., Kossover, O., ... Rizzi, R. (2014). PIGF-MMP9-engineered iPS cells supported on a PEG-fibrinogen hydrogel scaffold possess an enhanced capacity to repair damaged myocardium. *Cell Death & Disease*, 5(2), e1053. <http://doi.org/10.1038/cddis.2014.12>
- Becker, M. J. (1999). Ancient dental pathology. The international Journal of Oral & Maxillofacial Implants.
- Bedada, F. B., Chan, S. S.-K., Metzger, S. K., Zhang, L., Zhang, J., Garry, D. J., ... Metzger, J. M. (2014). Acquisition of a Quantitative, Stoichiometrically Conserved Ratiometric Marker of Maturation Status in Stem Cell-Derived Cardiac Myocytes. *Stem Cell Reports*, 3(4), 594–605. <http://doi.org/10.1016/j.stemcr.2014.07.012>
- Bedada, F. B., Wheelwright, M., & Metzger, J. M. (2015). Maturation status of sarcomere structure and function in human iPSC-derived cardiac myocytes. *Biochimica et Biophysica Acta*. <http://doi.org/10.1016/j.bbamcr.2015.11.005>
- Behfar, A., Crespo-Diaz, R., Terzic, A., & Gersh, B. J. (2014). Cell therapy for cardiac repair[mdash]lessons from clinical trials. *Nat Rev Cardiol*, 11(4), 232–246. Retrieved from <http://dx.doi.org/10.1038/nrcardio.2014.9>
- Bercea, M., Darie, R. N., Nita, L. E., & Morariu, S. (2011). Temperature responsive gels based on Pluronic F127 and poly(vinyl alcohol). *Industrial and Engineering Chemistry Research*, 50(7), 4199–4206. <http://doi.org/10.1021/ie1024408>
- Bersell, K., Arab, S., Haring, B., & Kühn, B. (2009). Neuregulin1/ErbB4 Signaling Induces Cardiomyocyte Proliferation and Repair of Heart Injury. *Cell*, 138(2), 257–270. <http://doi.org/10.1016/j.cell.2009.04.060>
- Blanpain, C., Daley, G. Q., Hochedlinger, K., Passegué, E., Rossant, J., & Yamanaka, S. (2012). Stem cells assessed. *Nature Reviews Molecular Cell Biology*, 13(7), 471–476. <http://doi.org/10.1038/nrm3371>
- Blazeski, A., & Tung, L. (2012). Electrophysiological and contractile function of cardiomyocytes derived from human embryonic stem cells, 18(9), 1199–1216. <http://doi.org/10.1016/j.micinf.2011.07.011>.Innate
- Blazeski, A., Zhu, R., Hunter, D. W., Weinberg, S. H., Boheler, K. R., Zambidis, E. T., & Tung, L. (2012). Electrophysiological and contractile function of cardiomyocytes derived from human embryonic stem cells. *Progress in Biophysics and Molecular*

- Biology*, 110(2-3), 178–95. <http://doi.org/10.1016/j.pbiomolbio.2012.07.012>
- Borbely, A. (2005). Cardiomyocyte Stiffness in Diastolic Heart Failure. *Circulation*, 111(6), 774–781. <http://doi.org/10.1161/01.CIR.0000155257.33485.6D>
- Breckwoldt, K., Weinberger, F., & Eschenhagen, T. (2015). Heart regeneration. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. <http://doi.org/10.1016/j.bbamcr.2015.11.010>
- Brochhausen, C., Lehmann, M., Halstenberg, S., Meurer, A., Klaus, G., & Kirkpatrick, C. J. (2009). Signalling molecules and growth factors for tissue engineering of cartilage – what can we learn from the growth plate?#. *Journal of Tissue Engineering and Regenerative Medicine*, 2(July), 408–417. <http://doi.org/10.1002/term>
- Buckingham, M., Meilhac, S., & Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nature Reviews Genetics*, 6(11), 826–837. <http://doi.org/10.1038/nrg1710>
- Burridge, P. W., Keller, G., Gold, J. D., & Wu, J. C. (2012). Production of de novo cardiomyocytes: human PSC differentiation and direct reprogramming, 10(1), 16–28. <http://doi.org/10.1016/j.stem.2011.12.013.Production>
- Burridge, P. W., Thompson, S., Millrod, M. a, Weinberg, S., Yuan, X., Peters, A., ... Zambidis, E. T. (2011). A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PloS One*, 6(4), e18293. <http://doi.org/10.1371/journal.pone.0018293>
- Calvo, B., Sierra, M., Grasa, J., Muñoz, M. J., & Peña, E. (2014). Determination of passive viscoelastic response of the abdominal muscle and related constitutive modeling: Stress-relaxation behavior. *Journal of the Mechanical Behavior of Biomedical Materials*, 36, 47–58. <http://doi.org/10.1016/j.jmbbm.2014.04.006>
- Camelliti, P., Borg, T., & Kohl, P. (2005). Structural and functional characterisation of cardiac fibroblasts. *Cardiovascular Research*, 65(1), 40–51. <http://doi.org/10.1016/j.cardiores.2004.08.020>
- Carpenter, L., Carr, C., Yang, C. T., Stuckey, D. J., Clarke, K., & Watt, S. M. (2012). Efficient differentiation of human induced pluripotent stem cells generates cardiac cells that provide protection following myocardial infarction in the rat. *Stem Cells and Development*, 21(6), 977–86.

<http://doi.org/10.1089/scd.2011.0075>

Carrier, R. L., Papadaki, M., Rupnick, M., Schoen, F. J., Bursac, N., Langer, R., Freed, L. E., and Vunjak-Novakovic, G. (1999). *Biotechnology and Bioengineering.*, 64, 580.

Casares, L., Vincent, R., Zalvidea, D., Campillo, N., Navajas, D., Arroyo, M., & Trepac, X. (2015). Hydraulic fracture during epithelial stretching. *Nature Materials*, 14(3), 343–351. <http://doi.org/10.1038/nmat4206>

Chaturvedi, R. R., Herron, T., Simmons, R., Shore, D., Kumar, P., Sethia, B., ... Kentish, J. C. (2010). Passive stiffness of myocardium from congenital heart disease and implications for diastole. *Circulation*, 121(8), 979–988. <http://doi.org/10.1161/CIRCULATIONAHA.109.850677>

Chaudhuri, J., & Al-Rubeai, M. (2005). *Bioreactors for tissue engineering*. Springer. <http://doi.org/10.1017/CBO9781107415324.004>

Chen, Q.-Z., Harding, S. E., Ali, N. N., Lyon, A. R., & Boccaccini, A. R. (2008). Biomaterials in cardiac tissue engineering: Ten years of research survey. *Materials Science and Engineering: R: Reports*, 59, 1–37. <http://doi.org/10.1016/j.mser.2007.08.001>

Chiu, L. L. Y., Iyer, R. K., King, J.-P., & Radisic, M. (2011). Biphasic electrical field stimulation aids in tissue engineering of multicell-type cardiac organoids. *Tissue Engineering. Part A*, 17(11-12), 1465–77. <http://doi.org/10.1089/ten.tea.2007.0244>

Cimetta, E., Godier-Furnémont, A., & Vunjak-Novakovic, G. (2013). Bioengineering heart tissue for in vitro testing. *Current Opinion in Biotechnology*, 24(5), 926–932. <http://doi.org/10.1016/j.copbio.2013.07.002>

Collado-Vázquez, S., & Carrillo, J. M. (2014). Cranial trepanation in The Egyptian. *Neurología (Barcelona, Spain)*, 29(7), 433–40. <http://doi.org/10.1016/j.nrl.2011.05.012>

Costa, K. D., Holmes, J. W., & McCulloch, a. D. (2001). Modelling cardiac mechanical properties in three dimensions. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, 359(1783), 1233–1250. <http://doi.org/10.1098/rsta.2001.0828>

Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole

- organ decellularization processes. *Biomaterials*, 32(12), 3233–43.
<http://doi.org/10.1016/j.biomaterials.2011.01.057>
- Crick, S. J., Sheppard, M. N., Ho, S. Y., Gebstein, L., & Anderson, R. H. (1998). Anatomy of the pig heart: comparisons with normal human cardiac structure. *Journal of Anatomy*, 193 (Pt 1, 105–19. <http://doi.org/10.1046/j.1469-7580.1998.19310105.x>
- Davis, P. J., Cladis, F. P., Monotoyama, E. K. (2010). "Smith's Anesthesia for Infants and Children", *Elsevier*, 8th Ed.
- Dervaux, J., & Ben Amar, M. (2008). Morphogenesis of Growing Soft Tissues. *Physical Review Letters*, 101(6), 068101. <http://doi.org/10.1103/PhysRevLett.101.068101>
- Desmoulière, a, Redard, M., Darby, I., & Gabbiani, G. (1995). Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *The American Journal of Pathology*, 146(1), 56–66.
- Domanski, M. J., Cunnion, R. E., & Roberts, W. C. (2016). Analysis of fractional area change at various levels in the normal left ventricle. *American Journal of Cardiology*, 70(15), 1367–1368. [http://doi.org/10.1016/0002-9149\(92\)90777-V](http://doi.org/10.1016/0002-9149(92)90777-V)
- Eminli, S., Utikal, J., Arnold, K., Jaenisch, R., & Hochedlinger, K. (2008). Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. *Stem Cells*, 26(10), 2467–2474. <http://doi.org/10.1634/stemcells.2008-0317>
- Engler, A., Carag-Krieger, C., Johnson, C., Raab, M., Tang, H.-Y., Speicher, D., ... Discher, D. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *Journal of Cell Science*, 121(Pt 22), 3794–802. <http://doi.org/10.1242/jcs.029678>
- Epstein, J. (2010). Cardiac Development and Implications for Heart Disease. *New England Journal of Medicine*, 363(17), 1638–1647. <http://doi.org/10.1056/NEJMra1003941>
- Erik Willems, Sean Spiering, Herman Davidovics¹, Marion Lanier, Zebin Xia, M., & Dawson, John Cashman, M. M. (2011). Small molecule inhibitors of the Wnt pathway potently promote cardiomyocytes from human embryonic stem cell derived mesoderm, 18(9), 1199–1216.

- <http://doi.org/10.1016/j.micinf.2011.07.011>. *Innate*
- Ertl, G., & Frantz, S. (2005). Healing after myocardial infarction. *Cardiovascular Research*, 66(1), 22–32. <http://doi.org/10.1016/j.cardiores.2005.01.011>
- Eschenhagen, T., Didié, M., Münzel, F., Schubert, P., Schneiderbanger, K., & Zimmermann, W.-H. (2002). 3D engineered heart tissue for replacement therapy. *Basic Research in Cardiology*, 97 Suppl 1, I146–I152. <http://doi.org/10.1007/s003950200043>
- Eschenhagen, T., Fink, C., Remmers, U., Scholz, H., Wattchow, J., Weil, J., ... Elson, E. L. (1997). Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 11(8), 683–694.
- Eschenhagen, T., & Zimmermann, W. H. (2005). Engineering myocardial tissue. *Circulation Research*, 97(12), 1220–1231. <http://doi.org/10.1161/01.RES.0000196562.73231.7d>
- Esteban, M. A., Xu, J., Yang, J., Peng, M., Qin, D., Li, W., ... Pei, D. (2009). Generation of Induced Pluripotent Stem Cell Lines from Tibetan Miniature Pig. *Journal of Biological Chemistry*, 284(26), 17634–17640. <http://doi.org/10.1074/jbc.M109.008938>
- Fakirov, S., Sarac, Z., Anbar, T., Boz, B., Bahar, I., Evstatiev, M., ... Kloczkowski, A. (1997). Mechanical properties and transition temperatures of crosslinked-oriented gelatin. *Colloid and Polymer Science*, 275(4), 307–314. <http://doi.org/10.1007/s003960050087>
- Feld, Y., Melamed-Frank, M., Kehat, I., Tal, D., Marom, S., & Gepstein, L. (2002). Electrophysiological modulation of cardiomyocytic tissue by transfected fibroblasts expressing potassium channels: a novel strategy to manipulate excitability. *Circulation*, 105(4), 522–529. <http://doi.org/10.1161/hc0402.102661>
- Feric, N. T., & Radisic, M. (2015). Maturing human pluripotent stem cell-derived cardiomyocytes in human engineered cardiac tissues. *Advanced Drug Delivery Reviews*. <http://doi.org/10.1016/j.addr.2015.04.019>
- Fink, C., Ergün, S., Kralisch, D., Remmers, U., Weil, J., & Eschenhagen, T. (2000). Chronic stretch of engineered heart tissue induces hypertrophy and functional

- improvement. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, 14(5), 669–679.
- Gerbin, K. A., & Murry, C. E. (2015). The winding road to regenerating the human heart. *Cardiovascular Pathology*, 24(3), 133–140.
<http://doi.org/10.1016/j.carpath.2015.02.004>
- Gessert, S., & Kühl, M. (2010). The multiple phases and faces of Wnt signaling during cardiac differentiation and development. *Circulation Research*, 107(2), 186–199.
<http://doi.org/10.1161/CIRCRESAHA.110.221531>
- Giles, J. M., Black, A. E., & Bischoff, J. E. (2007). Anomalous rate dependence of the preconditioned response of soft tissue during load controlled deformation. *Journal of Biomechanics*, 40(4), 777–785.
<http://doi.org/10.1016/j.jbiomech.2006.03.017>
- Godier-Furnémont, A. F. G., Tiburcy, M., Wagner, E., Dewenter, M., Lämmle, S., El-Armouche, A., ... Zimmermann, W.-H. (2015). Physiologic force-frequency response in engineered heart muscle by electromechanical stimulation. *Biomaterials*, 60, 82–91. <http://doi.org/10.1016/j.biomaterials.2015.03.055>
- Goldstein, A. L., Hannappel, E., Sosne, G., & Kleinman, H. K. (2012). Thymosin β_4 : a multi-functional regenerative peptide. Basic properties and clinical applications. *Expert Opinion on Biological Therapy*, 12(1), 37–51.
<http://doi.org/10.1517/14712598.2012.634793>
- Gupta, K. B., Ratcliffe, M. B., Fallert, M. A., Edmunds, L. H., & Bogen, D. K. (1994). Changes in passive mechanical stiffness of myocardial tissue with aneurysm formation. *Circulation*, 89(5), 2315–2326. <http://doi.org/10.1161/01.CIR.89.5.2315>
- Halbach, M., Peinkofer, G., Baumgartner, S., Maass, M., Wiedey, M., Neef, K., ... Müller-Ehmsen, J. (2013). Electrophysiological integration and action potential properties of transplanted cardiomyocytes derived from induced pluripotent stem cells. *Cardiovascular Research*, 100, 432–440. <http://doi.org/10.1093/cvr/cvt213>
- Hermanson, G. T. (2008). *Bioconjugate techniques. Bioconjugate chemistry* (Vol. 1).
<http://doi.org/10.1017/CBO9781107415324.004>
- Herron, T. J., Lee, P., & Jalife, J. (2012). Optical Imaging of Voltage and Calcium in Cardiac Cells & Tissues. *Circulation Research*, 110(4), 609–623.
<http://doi.org/10.1161/CIRCRESAHA.111.247494>

- Hirt, M. N., Hansen, A., & Eschenhagen, T. (2014). Cardiac Tissue Engineering: State of the Art. *Circulation Research*, 114(2), 354–367.
<http://doi.org/10.1161/CIRCRESAHA.114.300522>
- Hirt, M. N., Hansen, A., & Eschenhagen, T. (2014). Cardiac tissue engineering: state of the art. *Circulation Research*, 114(2), 354–367.
<http://doi.org/10.1161/CIRCRESAHA.114.300522>
- Huang, M., Tian, H., Wu, J., Matsubayashi, K., Weisel, R. D., & Li, R. (2006). Myometrial cells induce angiogenesis and salvage damaged myocardium. *American Journal of Physiology. Heart and Circulatory Physiology*, 291(5), H2057–66. <http://doi.org/10.1152/ajpheart.00494.2006>
- Husse, B., & Wussling, M. (1996). Developmental changes of calcium transients and contractility during the cultivation of rat neonatal cardiomyocytes. *Molecular and Cellular Biochemistry*, 163–164, 13–21.
- Iglesias-García, O., Baumgartner, S., Macrí-Pellizzeri, L., Rodriguez-Madoz, J. R., Abizanda, G., Guruceaga, E., ... Prósper, F. (2015). Neuregulin-1 β induces mature ventricular cardiac differentiation from induced pluripotent stem cells contributing to cardiac tissue repair. *Stem Cells and Development*, 24(4), 484–96.
<http://doi.org/10.1089/scd.2014.0211>
- Iribe, G., & Kohl, P. (2008). Axial stretch enhances sarcoplasmic reticulum Ca²⁺ leak and cellular Ca²⁺ reuptake in guinea pig ventricular myocytes: Experiments and models. *Progress in Biophysics and Molecular Biology*, 97(2-3), 298–311.
<http://doi.org/10.1016/j.pbiomolbio.2008.02.012>
- Jeon, H., Kim, E., & Grigoropoulos, C. (2011). Measurement of contractile forces generated by individual fibroblasts on self-standing fiber scaffolds. *Biomedical Microdevices*, 13(1), 107–115. <http://doi.org/10.1007/s10544-010-9475-5>
- Jugdutt, B. I. (2003). Ventricular Remodeling After Infarction and the Extracellular Collagen Matrix: When Is Enough Enough? *Circulation*, 108(11), 1395–1403.
<http://doi.org/10.1161/01.CIR.0000085658.98621.49>
- Jugdutt, B. I., Menon, V., Kumar, D., & Idikio, H. (2002). Vascular remodeling during healing after myocardial infarction in the dog model. *Journal of the American College of Cardiology*, 39(9), 1538–1545. [http://doi.org/10.1016/S0735-1097\(02\)01805-3](http://doi.org/10.1016/S0735-1097(02)01805-3)

- Kaichi, S., Hasegawa, K., Takaya, T., Yokoo, N., Mima, T., Kawamura, T., ... Heike, T. (2010). Cell line-dependent differentiation of induced pluripotent stem cells into cardiomyocytes in mice. *Cardiovascular Research*, 88(2), 314–323. <http://doi.org/10.1093/cvr/cvq189>
- Kamakura, T., Makiyama, T., Sasaki, K., Yoshida, Y., Wuriyanghai, Y., Chen, J., ... Kimura, T. (2013). Ultrastructural Maturation of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes in a Long-Term Culture. *Circulation Journal*, 77(5), 1307–1314. <http://doi.org/10.1253/circj.CJ-12-0987>
- Katz, a M. (2001). A growth of ideas: role of calcium as activator of cardiac contraction. *Cardiovascular Research*, 52(1), 8–13. [http://doi.org/10.1016/S0008-6363\(01\)00405-9](http://doi.org/10.1016/S0008-6363(01)00405-9)
- Kawamura, M., Miyagawa, S., Miki, K., Saito, A., Fukushima, S., Higuchi, T., ... Sawa, Y. (2012). Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation*, 126(11 SUPPL.1). <http://doi.org/10.1161/CIRCULATIONAHA.111.084343>
- Kim, D. H., Heo, S.-J., Shin, J.-W., Mun, C. W., Park, K. M., Park, K. D., & Jee, K. S. (2010). Preparation of thermosensitive gelatin-pluronic copolymer for cartilage tissue engineering. *Macromolecular Research*, 18(4), 387–391. <http://doi.org/10.1007/s13233-010-0405-4>
- Kim, K., Doi, a, Wen, B., Ng, K., Zhao, R., & Cahan, P. (2010). Epigenetic memory in induced pluripotent stem cells K. *Nature*, 467(7313), 285–290. <http://doi.org/10.1038/nature09342>.Epigenetic
- Konstantopoulou, A., Tsikrikas, S., Asvestas, D., Korantzopoulos, P., & Letsas, K. P. (2013). *World Journal of Cardiology*, 5(6), 175–185. <http://doi.org/10.4330/wjc.v5.i6.175>
- Kostin, S. (2004). Connexin 43 expression and distribution in compensated and decompensated cardiac hypertrophy in patients with aortic stenosis. *Cardiovascular Research*, 62(2), 426–436. <http://doi.org/10.1016/j.cardiores.2003.12.010>
- Kostin, S., Hein, S., Bauer, E. P., & Schaper, J. (1999). Spatiotemporal development and distribution of intercellular junctions in adult rat cardiomyocytes in culture. *Circulation Research*, 85(1d), 154–167. <http://doi.org/10.1161/01.RES.85.2.154>

- Kraehenbuehl, T. P., Langer, R., & Ferreira, L. S. (2011). Three-dimensional biomaterials for the study of human pluripotent stem cells. *Nature Methods*, 8(9), 731–736. <http://doi.org/10.1038/nmeth.1671>
- Kutschka, I., Chen, I. Y., Kofidis, T., Arai, T., Von Degenfeld, G., Sheikh, A. Y., ... Robbins, R. C. (2006). Collagen matrices enhance survival of transplanted cardiomyoblasts and contribute to functional improvement of ischemic rat hearts. *Circulation*, 114(SUPPL. 1). <http://doi.org/10.1161/CIRCULATIONAHA.105.001297>
- Kwon, C., Arnold, J., Hsiao, E. C., Taketo, M. M., Conklin, B. R., & Srivastava, D. (2007). Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proceedings of the National Academy of Sciences of the United States of America*, 104(26), 10894–9. <http://doi.org/10.1073/pnas.0704044104>
- Laflamme, M. a, Chen, K. Y., Naumova, A. V., Muskheli, V., Fugate, J. a, Dupras, S. K., ... Murry, C. E. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnology*, 25(9), 1015–1024. <http://doi.org/10.1038/nbt1327>
- Laflamme, M. a, & Murry, C. E. (2011). Heart regeneration. *Nature*, 473(7347), 326–335. <http://doi.org/10.1038/nature10147>
- Lafortune, P., & Aris, R. (2012). Coupled electromechanical model of the heart: parallel finite element formulation. *International Journal for Numerical Methods in Biomedical Engineering*, 28(1), 72–86. <http://doi.org/10.1002/cnm>
- Lakshmanan, R., Krishnan, U. M., & Sethuraman, S. (2012). Living cardiac patch: The elixir for cardiac regeneration. *Expert Opinion on Biological Therapy*, 1–18. <http://doi.org/10.1517/14712598.2012.721770>
- Lam, M. L., Bartoli, M., & Claycomb, W. C. (2002). The 21-day postnatal rat ventricular cardiac muscle cell in culture as an experimental model to study adult cardiomyocyte gene expression. *Molecular and Cellular Biochemistry*, 229(1-2), 51–62.
- Landa, N., Miller, L., Feinberg, M. S., Holbova, R., Shachar, M., Freeman, I., ... Leor, J. (2008). Effect of injectable alginate implant on cardiac remodeling and function after recent and old infarcts in rat. *Circulation*, 117(11), 1388–1396. <http://doi.org/10.1161/CIRCULATIONAHA.107.727420>

- Lang, R. M., Bierig, M., Devereux, R. B., Flachskampf, F. A., Foster, E., Pellikka, P. A., ... Stewart, W. J. (2005). Recommendations for Chamber Quantification: A Report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, Developed in Conjunction with the European Association of Echocardiography. *Journal of the American Society of Echocardiography*, 18(12), 1440–1463. <http://doi.org/http://dx.doi.org/10.1016/j.echo.2005.10.005>
- Langer, R., & Vacanti, J. P. (1993). *Tissue Engineering* (Vol. 260).
- Lanza, R., Langer, R., and Vacanti, J.P. (2002). "Principles of Tissue Engineering." 2nd Ed.
- Lee, J., Bae, Y. H., Sohn, Y. S., & Jeong, B. (2006). Thermogelling Aqueous Solutions of Alternating Multiblock Copolymers of Poly(L-lactic acid) and Poly(ethylene glycol). *Biomacromolecules*, 7(6), 1729–1734. <http://doi.org/10.1021/bm0600062>
- Lee, L. S., Ghanta, R. K., Mokashi, S. a., Coelho-Filho, O., Kwong, R. Y., Bolman, R. M., & Chen, F. Y. (2010). Ventricular restraint therapy for heart failure: The right ventricle is different from the left ventricle. *Journal of Thoracic and Cardiovascular Surgery*, 139(4), 1012–1018. <http://doi.org/10.1016/j.jtcvs.2009.09.064>
- Li, R. K., Mickle, D., Weisel, R. D., Carson, S., Omar, S., Tumati, L. C., ... Williams, W. G. (1996). Human pediatric and adult ventricular cardiomyocytes in culture: assessment of phenotypic changes with passaging. *Cardiovascular Research*, 32(2), 362–73. [http://doi.org/10.1016/0008-6363\(96\)00079-X](http://doi.org/10.1016/0008-6363(96)00079-X)
- Li, Z., & Guan, J. (2011a). Hydrogels for cardiac tissue engineering. *Polymers*, 3(2), 740–761. <http://doi.org/10.3390/polym3020740>
- Li, Z., & Guan, J. (2011b). Hydrogels for cardiac tissue engineering. *Polymers*, 3(2), 740–761. <http://doi.org/10.3390/polym3020740>
- Li, Z., Guo, X., Palmer, A. F., Das, H., & Guan, J. (2012). High-efficiency matrix modulus-induced cardiac differentiation of human mesenchymal stem cells inside a thermosensitive hydrogel. *Acta Biomaterialia*, 8(10), 3586–3595. <http://doi.org/10.1016/j.actbio.2012.06.024>
- Li, Z., Wang, F., Roy, S., Sen, C. K., & Guan, J. (2009). Injectable, highly flexible, and thermosensitive hydrogels capable of delivering superoxide dismutase. *Biomacromolecules*, 10(12), 3306–16. <http://doi.org/10.1021/bm900900e>

- Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L. B., Azarin, S. M., ... Palecek, S. P. (2012). PNAS Plus: Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the National Academy of Sciences*, 109(27), E1848–E1857. <http://doi.org/10.1073/pnas.1200250109>
- Liang, G., & Zhang, Y. (2013). Embryonic stem cell and induced pluripotent stem cell: an epigenetic perspective. *Cell Research*, 23(1), 49–69. <http://doi.org/10.1038/cr.2012.175>
- Liao, J., Cui, C., Chen, S., Ren, J., Chen, J., Gao, Y., ... Xiao, L. (2009). Generation of Induced Pluripotent Stem Cell Lines from Adult Rat Cells. *Cell Stem Cell*, 4(1), 11–15. <http://doi.org/10.1016/j.stem.2008.11.013>
- Lippens, E., Swennen, I., Gironès, J., Declercq, H., Vertenten, G., Vlamincx, L., ... Cornelissen, R. (2013). Cell survival and proliferation after encapsulation in a chemically modified Pluronic(R) F127 hydrogel. *Journal of Biomaterials Applications*, 27(7), 828–39. <http://doi.org/10.1177/0885328211427774>
- Liu, H., Zhu, F., Yong, J., Zhang, P., Hou, P., Li, H., ... Deng, H. (2008). Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell*, 3(6), 587–590. <http://doi.org/10.1016/j.stem.2008.10.014>
- Llucià-Valldeperas, A., Sanchez, B., Soler-Botija, C., Gálvez-Montón, C., Prat-Vidal, C., Roura, S., ... Bayes-Genis, A. (2013). Electrical stimulation of cardiac adipose tissue-derived progenitor cells modulates cell phenotype and genetic machinery. *Journal of Tissue Engineering and Regenerative Medicine*, 2(February 2013), 408–417. <http://doi.org/10.1002/term>
- Lundy, S. D., Zhu, W.-Z., Regnier, M., & Laflamme, M. A. (2013). Structural and Functional Maturation of Cardiomyocytes Derived from Human Pluripotent Stem Cells. *Stem Cells and Development*, 22(14), 1991–2002. <http://doi.org/10.1089/scd.2012.0490>
- Lux, M., Andrée, B., Horvath, T., Nosko, A., Manikowski, D., Hilfiker-Kleiner, D., ... Hilfiker, A. (2015). In vitro maturation of large-scale cardiac patches based on a perfusable starter matrix by cyclic mechanical stimulation. *Acta Biomaterialia*. <http://doi.org/10.1016/j.actbio.2015.11.006>
- Marcé-Nogué, J., Fortuny, G., Ballester-Rodés, M., Carreras, F., & Roure, F. (2013). Computational modeling of electromechanical propagation in the helical

- ventricular anatomy of the heart. *Computers in Biology and Medicine*, 43(11), 1698–1703. <http://doi.org/10.1016/j.combiomed.2013.07.016>
- Marsano, A., Maidhof, R., Tandon, N., Gao, J., Wang, Y., & Vunjak-Novakovic, G. (2008). Engineering of functional contractile cardiac tissues cultured in a perfusion system. *Conference Proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference, 2008*, 3590–3. <http://doi.org/10.1109/IEMBS.2008.4649982>
- Martucci, J. F., Accareddu, a. E. M., & Ruseckaite, R. a. (2011). Preparation and characterization of plasticized gelatin films cross-linked with low concentrations of Glutaraldehyde. *Journal of Materials Science*, 47(7), 3282–3292. <http://doi.org/10.1007/s10853-011-6167-3>
- Matsui, Y., Morimoto, J., & Uede, T. (2010). Role of matricellular proteins in cardiac tissue remodeling after myocardial infarction. *World Journal of Biological Chemistry*, 1(5), 69–80. <http://doi.org/10.4331/wjbc.v1.i5.69>
- Matsuo, T., Masumoto, H., Tajima, S., Ikuno, T., Katayama, S., Minakata, K., ... Yamashita, J. K. (2015). Efficient long-term survival of cell grafts after myocardial infarction with thick viable cardiac tissue entirely from pluripotent stem cells. *Scientific Reports*, 5(October), 16842. <http://doi.org/10.1038/srep16842>
- Mauritz, C., Schwanke, K., Reppel, M., Neef, S., Katsirntaki, K., Maier, L. S., ... Martin, U. (2008). Generation of Functional Murine Cardiac Myocytes From Induced Pluripotent Stem Cells. <http://doi.org/10.1161/CIRCULATIONAHA.108.778795>
- McGann, C. L., Levenson, E. a., & Kiick, K. L. (2013). Resilin-based hybrid hydrogels for cardiovascular tissue engineering. *Macromolecular Chemistry and Physics*, 214(2), 203–213. <http://doi.org/10.1002/macp.201200412>
- McGarvey, J. R., Mojsejenko, D., Dorsey, S. M., Nikou, A., Burdick, J. A., Gorman III, J. H., ... Wenk, J. F. (2016). Temporal Changes in Infarct Material Properties: An In-Vivo Assessment Using Magnetic Resonance Imaging and Finite Element Simulations. *The Annals of Thoracic Surgery*, 100(2), 582–589. <http://doi.org/10.1016/j.athoracsur.2015.03.015>
- Meyer, U., Meyer, T., Handschel, J., & Wiesemann, H. P. (2009). *Fundamentals of tissue engineering and regenerative medicine*. Springer (Vol. 1). <http://doi.org/10.1017/CBO9781107415324.004>

- Mihic, A., Li, J., Miyagi, Y., Gagliardi, M., Li, S.-H., Zu, J., ... Li, R.-K. (2014). The effect of cyclic stretch on maturation and 3D tissue formation of human embryonic stem cell-derived cardiomyocytes. *Biomaterials*, 35(9), 2798–2808. <http://doi.org/10.1016/j.biomaterials.2013.12.052>
- Miklas, J. W., Nunes, S. S., Sofla, A., Reis, L. A., Pahnke, A., Xiao, Y., ... Radisic, M. (2014). Bioreactor for modulation of cardiac microtissue phenotype by combined static stretch and electrical stimulation. *Biofabrication*, 6(2), 024113. <http://doi.org/10.1088/1758-5082/6/2/024113>
- Milani-Nejad, N., & Janssen, P. M. L. (2014). Small and large animal models in cardiac contraction research: advantages and disadvantages. *Pharmacology & Therapeutics*, 141(3), 235–49. <http://doi.org/10.1016/j.pharmthera.2013.10.007>
- Mirsky, I., & Parmley, W. W. (1973). Assessment of passive elastic stiffness for isolated heart muscle and the intact heart. *Circulation Research*, 33(2), 233–243. <http://doi.org/10.1161/01.RES.33.2.233>
- Mor-Avi, V., Lang, R. M., Badano, L. P., Belohlavek, M., Cardim, N. M., Derumeaux, G., ... Zamorano, J. L. (2011). Current and evolving echocardiographic techniques for the quantitative evaluation of cardiac mechanics: ASE/EAE consensus statement on methodology and indications endorsed by the Japanese society of echocardiography. *European Journal of Echocardiography*, 12(3), 167–205. <http://doi.org/10.1093/ejechocard/jer021>
- Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., ... Turner, M. B. (2016). *AHA Statistical Update Heart Disease and Stroke Statistics – 2016 Update A Report From the American Heart Association WRITING GROUP MEMBERS*. <http://doi.org/10.1161/CIR.0000000000000350>
- Nagueh, S. F. (2004). Altered Titin Expression, Myocardial Stiffness, and Left Ventricular Function in Patients With Dilated Cardiomyopathy. *Circulation*, 110(2), 155–162. <http://doi.org/10.1161/01.CIR.0000135591.37759.AF>
- Nakano, K., Sugawara, M., Ishihara, K., Kanazawa, S., Corin, W. J., Denslow, S., ... Carabello, B. a. (1990). Myocardial stiffness derived from end-systolic wall stress and logarithm of reciprocal of wall thickness. Contractility index independent of ventricular size. *Circulation*, 82(4), 1352–61. <http://doi.org/10.1161/01.CIR.82.4.1352>
- Navajas, D., Roca-cusachs, P., Sunyer, R., & Farre, R. (2008). Mapping Cell-Matrix

- Stresses during Stretch Reveals Inelastic Reorganization of the Cytoskeleton, 95(July), 464–471. <http://doi.org/10.1529/biophysj.107.124180>
- Nelson, T. J., Martinez-Fernandez, A., Yamada, S., Perez-Terzic, C., Ikeda, Y., & Terzic, A. (2009). Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. *Circulation*, 120(5), 408–16. <http://doi.org/10.1161/CIRCULATIONAHA.109.865154>
- Nenadic, I., Urban, M. W., & Greenleaf, J. F. (2009). Ex vivo measurements of mechanical properties of myocardium using lamb and Rayleigh wave dispersion velocities. *Proceedings - IEEE Ultrasonics Symposium*, 2785–2788. <http://doi.org/10.1109/ULTSYM.2009.5441411>
- Nicola, S., Bollini, S., Dubé, K., Vieira, J. M., Zhou, B., Davidson, S., ... Riley, P. R. (2011). De novo cardiomyocytes from within the activated adult heart after injury. *Nature*, 18(9), 1199–1216. <http://doi.org/10.1016/j.micinf.2011.07.011>.Innate
- Noailly, J., Van Oosterwyck, H., Wilson, W., Quinn, T. M., & Ito, K. (2008). A poroviscoelastic description of fibrin gels. *Journal of Biomechanics*, 41(15), 3265–3269. <http://doi.org/10.1016/j.jbiomech.2008.09.002>
- Nunes, S., Miklas, J., Liu, J., & Aschar-Sobbi, R. (2013). Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Nature ...*, 10(8), 1–6. <http://doi.org/10.1038/NMETH.2524>
- Nunes, S. S., Miklas, J. W., Liu, J., Aschar-Sobbi, R., Xiao, Y., Zhang, B., ... Radisic, M. (2013). Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Nature Methods*, 10(8), 781–787. <http://doi.org/10.1038/nmeth.2524>
- Otsuka, F., Kramer, M. C. a., Woudstra, P., Yahagi, K., Ladich, E., Finn, A. V., ... Virmani, R. (2015). Natural progression of atherosclerosis from pathologic intimal thickening to late fibroatheroma in human coronary arteries: A pathology study. *Atherosclerosis*, 241(2), 1–11. <http://doi.org/10.1016/j.atherosclerosis.2015.05.011>
- Paradis, Alexandra N., Maresha S. Gay, and and Lubo Zhang. 2014. “Binucleation of Cardiomyocytes: The Transition from a Proliferative to a Terminally Differentiated State Alexandra.” 19(2): 161–69.
- Park, K. M., Joung, Y. K., Park, K. D., Lee, S. Y., & Lee, M. C. (2008). RGD-Conjugated chitosan-pluronic hydrogels as a cell supported scaffold for articular cartilage

- regeneration. *Macromolecular Research*, 16(6), 517–523.
<http://doi.org/10.1007/BF03218553>
- Pasqualini, F. S., Sheehy, S. P., Agarwal, A., Aratyn-Schaus, Y., & Parker, K. K. (2015). Structural phenotyping of stem cell-derived cardiomyocytes. *Stem Cell Reports*, 4(3), 340–7. <http://doi.org/10.1016/j.stemcr.2015.01.020>
- Passier, R., van Laake, L. W., & Mummery, C. L. (2008). Stem-cell-based therapy and lessons from the heart. *Nature*, 453(7193), 322–329.
<http://doi.org/10.1038/nature07040>
- Pasumarthi, K. B. S. (2002). Cardiomyocyte Cell Cycle Regulation. *Circulation Research*, 90(10), 1044–1054.
<http://doi.org/10.1161/01.RES.0000020201.44772.67>
- Pellegrini, S., Ungaro, F., Mercalli, A., Melzi, R., Sebastiani, G., Dotta, F., ... Sordi, V. (2015). Human induced pluripotent stem cells differentiate into insulin-producing cells able to engraft in vivo. *Acta Diabetologica*, 1025–1035.
<http://doi.org/10.1007/s00592-015-0726-z>
- Perea-Gil, I., Prat-Vidal, C., & Bayes-Genis, A. (2015). In vivo experience with natural scaffolds for myocardial infarction: the times they are a-changin'. *Stem Cell Research & Therapy*, 6(1), 248. <http://doi.org/10.1186/s13287-015-0237-4>
- Pinnell, J., Turner, S., & Howell, S. (2007). Cardiac muscle physiology. *Continuing Education in Anaesthesia, Critical Care & Pain*, 7(3), 85–88.
<http://doi.org/10.1093/bjaceaccp/mkm013>
- Piryaee, A., Soleimani, M., Heidari, M. H., Saheli, M., Rohani, R., & Almasieh, M. (2015). Ultrastructural maturation of human bone marrow mesenchymal stem cells-derived cardiomyocytes under alternative induction of 5-azacytidine. *Cell Biology International*, 39(5), 519–530. <http://doi.org/10.1002/cbin.10421>
- Pislaru, C., Urban, M. W., Pislaru, S. V., Kinnick, R. R., & Greenleaf, J. F. (2014). Viscoelastic properties of normal and infarcted myocardium measured by a multifrequency shear wave method: Comparison with pressure-segment length method. *Ultrasound in Medicine and Biology*, 40(8), 1785–1795.
<http://doi.org/10.1016/j.ultrasmedbio.2014.03.004>
- Planell, J. A. (2013). Elastine Like Recombinant Polymers for Wound Healing Applications 1 . PROJECT ABSTRACT (limited to 500 words) 2 . CLINICAL

NEED ADDRESSED BY THE PROJECT (limited to 500 words), 2013, 1–13.

- Radhakrishnan, J., Krishnan, U. M., & Sethuraman, S. (2014). Hydrogel based injectable scaffolds for cardiac tissue regeneration. *Biotechnology Advances*. <http://doi.org/10.1016/j.biotechadv.2013.12.010>
- Radisic, M., & Christman, K. L. (2013). Materials science and tissue engineering: repairing the heart. *Mayo Clinic Proceedings*, 88(8), 884–98. <http://doi.org/10.1016/j.mayocp.2013.05.003>
- Radisic, M., & D. Black III, L. (2014). *Cardiac Tissue Engineering. Methods in Molecular Biology* (Human Pres, Vol. 1181). <http://doi.org/10.1007/978-1-4939-1047-2>
- Radisic, M., Fast, V. G., Sharifov, O. F., Iyer, R. K., Park, H., & Vunjak-Novakovic, G. (2009). Optical Mapping of Impulse Propagation. *Tissue Engineering. Part A*, 15(4), 851–860.
- Radisic, M., Malda, J., Epping, E., Geng, W., Langer, R., & Vunjak-Novakovic, G. (2006). Oxygen gradients correlate with cell density and cell viability in engineered cardiac tissue. *Biotechnology and Bioengineering*, 93(2), 332–343. <http://doi.org/10.1002/bit.20722>
- Radisic, M., Marsano, A., Maidhof, R., Wang, Y., & Vunjak-Novakovic, G. (2008). Cardiac tissue engineering using perfusion bioreactor systems. *Nature Protocols*, 3(4), 719–738. <http://doi.org/10.1038/nprot.2008.40>
- Radisic, M., Park, H., Shing, H., Consi, T., Schoen, F. J., Langer, R., ... Vunjak-Novakovic, G. (2004). Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proceedings of the National Academy of Sciences of the United States of America*, 101(52), 18129–18134. <http://doi.org/10.1073/pnas.0407817101>
- Rane, A. A., & Christman, K. L. (2011). Biomaterials for the Treatment of Myocardial Infarction: A 5-Year Update: A 5-Year Update. *Journal of the American College of Cardiology*, 58(25), 2615–2629. <http://doi.org/10.1016/j.jacc.2011.11.001>
- Reddy, K., Khaliq, A., & Henning, R. J. (2015). World Journal of Cardiology, 7(5), 243–276. <http://doi.org/10.4330/wjc.v7.i5.243>
- Reinecke, H., Zhang, M., Bartosek, T., & Murry, C. E. (1999). A Study in Normal and Injured Rat Hearts, (1).

- Reis, L. A., Chiu, L. L. Y., Feric, N. T., Fu, L., & Radisic, M. (2014). Biomaterials in myocardial tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*, 2, 408–417. <http://doi.org/10.1002/term>
- Reiser, P. J., Portman, M. a, Ning, X. H., & Schomisch Moravec, C. (2001). Human cardiac myosin heavy chain isoforms in fetal and failing adult atria and ventricles. *American Journal of Physiology. Heart and Circulatory Physiology*, 280(4), H1814–H1820.
- Ren, Y., Lee, M. Y., Schliffke, S., Paavola, J., Amos, P. J., Ge, X., ... Ehrlich, B. E. (2011). Small Molecule Wnt Inhibitors Enhance the Efficiency of BMP-4-Directed Cardiac Differentiation of Human Pluripotent Stem Cells. *Molecular Cell*, 51(3), 280–287. <http://doi.org/10.1016/j.jymcc.2011.04.012.Small>
- Salameh, A., Haunschild, J., Bräuchle, P., Peim, O., Seidel, T., Reitmann, M., ... Dähnert, I. (2014). On the role of the gap junction protein Cx43 (GJA1) in human cardiac malformations with Fallot-pathology. a study on paediatric cardiac specimen. *PloS One*, 9(4), e95344. <http://doi.org/10.1371/journal.pone.0095344>
- Samchenko, Y., Ulberg, Z., & Korotych, O. (2011). Multipurpose smart hydrogel systems. *Advances in Colloid and Interface Science*, 168(1-2), 247–262. <http://doi.org/10.1016/j.cis.2011.06.005>
- Sarró, E., Lecina, M., Fontova, A., Solà, C., Gòdia, F., Cairó, J. J., & Bragós, R. (2012). Electrical impedance spectroscopy measurements using a four-electrode configuration improve on-line monitoring of cell concentration in adherent animal cell cultures. *Biosensors and Bioelectronics*, 31(1), 257–263. <http://doi.org/10.1016/j.bios.2011.10.028>
- Schlüter, K. D., & Piper, H. M. (1999). Regulation of growth in the adult cardiomyocytes. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 13 Suppl, S17–22. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10352141>
- Serpooshan, V., Zhao, M., Metzler, S., Weo, K., Shah, P. B., Wang, A., ... Ruiz-Lozano, P. (2014). Use of bio-mimetic 3D technology in therapeutics for heart disease. *Bioengineered*, 5(3). <http://doi.org/10.4161/bioe.27751>
- Shachaf, Y., Gonen-Wadmany, M., & Seliktar, D. (2010). The biocompatibility of PluronicF127 fibrinogen-based hydrogels. *Biomaterials*, 31(10), 2836–47. <http://doi.org/10.1016/j.biomaterials.2009.12.050>

- Sharma, S., Jackson, P. G., & Makan, J. (2004). Cardiac troponins, 1025–1026.
<http://doi.org/10.1136/jcp.2003.015420>
- Shimizu, T., Yamato, M., Isoi, Y., Akutsu, T., Setomaru, T., Abe, K., ... Okano, T. (2002). Fabrication of Pulsatile Cardiac Tissue Grafts Using a Novel 3-Dimensional Cell Sheet Manipulation Technique and Temperature-Responsive Cell Culture Surfaces. *Circulation Research*, 90(3), 40e–48.
<http://doi.org/10.1161/hh0302.105722>
- Singelyn, J. M., & Christman, K. L. (2010). Injectable materials for the treatment of myocardial infarction and heart failure: The promise of decellularized matrices. *Journal of Cardiovascular Translational Research*, 3(5), 478–486.
<http://doi.org/10.1007/s12265-010-9202-x>
- Sinha Roy, R., Soni, S., Harfouche, R., Vasudevan, P. R., Holmes, O., de Jonge, H., ... Sengupta, S. (2010). Coupling growth-factor engineering with nanotechnology for therapeutic angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 13608–13613.
<http://doi.org/10.1073/pnas.1006007107>
- Sirabella, D., Cimetta, E., & Vunjak-Novakovic, G. (2015). “The state of the heart”: Recent advances in engineering human cardiac tissue from pluripotent stem cells. *Experimental Biology and Medicine*, 240(8), 1008–1018.
<http://doi.org/10.1177/1535370215589910>
- Smyth, J. W., Vogan, J. M., Buch, P. J., Zhang, S.-S., Fong, T. S., Hong, T.-T., & Shaw, R. M. (2012). Actin Cytoskeleton Rest Stops Regulate Anterograde Traffic of Connexin 43 Vesicles to the Plasma Membrane. *Circulation Research*, 110(7), 978–989. <http://doi.org/10.1161/CIRCRESAHA.111.257964>
- Soler-Botija, C., Bagó, J. R., Llucià-Valldeperas, A., Vallés-Lluch, A., Castells-Sala, C., Martínez-Ramos, C., ... Bayes-Genis, A. (2014). Engineered 3D bioimplants using elastomeric scaffold, self-assembling peptide hydrogel, and adipose tissue-derived progenitor cells for cardiac regeneration. *American Journal of Translational Research*, 6(3), 291–301.
- Song, B., Gu, Y., Pu, J., Reid, B., Zhao, Z., & Zhao, M. (2007). Application of direct current electric fields to cells and tissues in vitro and modulation of wound electric field in vivo. *Nat. Protocols*, 2(6), 1479–1489. Retrieved from <http://dx.doi.org/10.1038/nprot.2007.205>

- Stoppel, W. L., Kaplan, D. L., & Black, L. D. (2015). Electrical and mechanical stimulation of cardiac cells and tissue constructs. *Advanced Drug Delivery Reviews*, 96, 135–155. <http://doi.org/10.1016/j.addr.2015.07.009>
- Streckfuss-Bomeke, K., Wolf, F., Azizian, A., Stauske, M., Tiburcy, M., Wagner, S., ... Guan, K. (2013). Comparative study of human-induced pluripotent stem cells derived from bone marrow cells, hair keratinocytes, and skin fibroblasts. *European Heart Journal*, 34(33), 2618–2629. <http://doi.org/10.1093/eurheartj/ehs203>
- Sun, J.-Y., Zhao, X., Illeperuma, W. R. K., Chaudhuri, O., Oh, K. H., Mooney, D. J., ... Suo, Z. (2012). Highly stretchable and tough hydrogels. *Nature*, 489(7414), 133–136. <http://doi.org/10.1038/nature11409>
- Sun, Y., Kiani, M. F., Postlethwaite, A. E., & Weber, K. T. (2002). Infarct scar as living tissue. *Basic Research in Cardiology*, 97(5), 343–347. <http://doi.org/10.1007/s00395-002-0365-8>
- Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, 126(4), 663–676. <http://doi.org/10.1016/j.cell.2006.07.024>
- Takashi Furusawa, Mark Rochman, Leila Taher, Emiliós K. Dimitriadis, K., Nagashima, S. A., & Michael Bustin. (2015). Chromatin De-Compaction By The Nucleosomal Binding Protein HMGN5 Impairs Nuclear Sturdiness. *Nature Communications*.
- Takeuchi, J. K., & Bruneau, B. G. (2009). Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature*, 459(7247), 708–711. <http://doi.org/10.1038/nature08039>
- Tandon, N., Cannizzaro, C., Chao, P.-H. G., Maidhof, R., Marsano, A., Au, H. T. H., ... Vunjak-Novakovic, G. (2009). Electrical stimulation systems for cardiac tissue engineering. *Nature Protocols*, 4(2), 155–173. <http://doi.org/10.1038/nprot.2008.183>
- Tandon, N., Marsano, A., Maidhof, R., Wan, L., Park, H., & Vunjak-Novakovic, G. (2011). Optimization of electrical stimulation parameters for cardiac tissue engineering Nina. *Journal of Tissue Engineering and Regenerative Medicine*, 4(7), 524–531. <http://doi.org/10.1002/term>
- Terrovitis, J. V., Smith, R. R., & Marb??n, E. (2010). Assessment and optimization of

- cell engraftment after transplantation into the heart. *Circulation Research*, 106(3), 479–494. <http://doi.org/10.1161/CIRCRESAHA.109.208991>
- Thavandiran, N., Nunes, S. S., Xiao, Y., & Radisic, M. (2013). Topological and electrical control of cardiac differentiation and assembly. *Stem Cell Research & Therapy*, 4(1), 14. <http://doi.org/10.1186/scrt162>
- Thurston, A. J. (2007). Paré and Prosthetics: the Early History of Artificial Limbs. *ANZ Journal of Surgery*, 77(12), 1114–1119. <http://doi.org/10.1111/j.1445-2197.2007.04330.x>
- Thygesen, K., Alpert, J. S., Jaffe, A. S., Simoons, M. L., Chaitman, B. R., & White, H. D. (2012). Third Universal Definition of Myocardial Infarction. *Global Heart*, 7(4), 275–295. <http://doi.org/10.1016/j.gheart.2012.08.001>
- Tortora, G. J. & Derrickson, B. (2009). "Principles of anatomy and physiology". Wiley, 12th Ed.
- Tulloch, N. L., Muskheli, V., Razumova, M. V., Korte, F. S., Regnier, M., Hauch, K. D., ... Murry, C. E. (2011). Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circulation Research*, 109(1), 47–59. <http://doi.org/10.1161/CIRCRESAHA.110.237206>
- van Dalen, B. M., Kauer, F., Vletter, W. B., Soliman, O. I. I., van der Zwaan, H. B., Ten Cate, F. J., & Geleijnse, M. L. (2010). Influence of cardiac shape on left ventricular twist. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, 108(1), 146–51. <http://doi.org/10.1152/japplphysiol.00419.2009>
- Vunjak Novakovic, G., Eschenhagen, T., & Mummery, C. (2014). Myocardial tissue engineering: In vitro models. *Cold Spring Harbor Perspectives in Medicine*, 4(3), 1–16. <http://doi.org/10.1101/cshperspect.a014076>
- Wainwright, J. M., Czajka, C. a, Patel, U. B., Freytes, D. O., Tobita, K., Gilbert, T. W., & Badylak, S. F. (2010). Preparation of cardiac extracellular matrix from an intact porcine heart. *Tissue Engineering. Part C, Methods*, 16(3), 525–532. <http://doi.org/10.1089/ten.tec.2009.0392>
- Wang, B., Borazjani, A., Tahai, M., De Jongh Curry, A. L., Simionescu, D. T., Guan, J., ... Liao, J. (2010). Fabrication of cardiac patch with decellularized porcine myocardial scaffold and bone marrow mononuclear cells. *Journal of Biomedical Materials Research - Part A*, 94(4), 1100–1110.

<http://doi.org/10.1002/jbm.a.32781>

- Wang, B., Tedder, M. E., Perez, C. E., Wang, G., De Jongh Curry, A. L., To, F., ... Liao, J. (2012). Structural and biomechanical characterizations of porcine myocardial extracellular matrix. *Journal of Materials Science: Materials in Medicine*, 23(8), 1835–1847. <http://doi.org/10.1007/s10856-012-4660-0>
- Wang, B., Wang, G., To, F., Butler, J. R., Claude, A., Ronald, M., ... Liao, J. (2014). Myocardial scaffold-based cardiac tissue engineering apps of coordinated mechanical and electrical stimulations, 29(35). <http://doi.org/10.1021/la401702w>. Myocardial
- Watanabe, S., Shite, J., Takaoka, H., Shinke, T., Imuro, Y., Ozawa, T., ... Yokoyama, M. (2006). Myocardial stiffness is an important determinant of the plasma brain natriuretic peptide concentration in patients with both diastolic and systolic heart failure. *European Heart Journal*, 27(7), 832–8. <http://doi.org/10.1093/eurheartj/ehi772>
- Willems, E., Cabral-Teixeira, J., Schade, D., Cai, W., Reeves, P., Bushway, P. J., ... Mercola, M. (2012). Small molecule-mediated TGF- β type II receptor degradation promotes cardiomyogenesis in embryonic stem cells. *Cell Stem Cell*, 11(2), 242–252. <http://doi.org/10.1016/j.stem.2012.04.025>
- Wu, D.-Q., Qiu, F., Wang, T., Jiang, X.-J., Zhang, X.-Z., & Zhuo, R.-X. (2009). Toward the Development of Partially Biodegradable and Injectable Thermoresponsive Hydrogels for Potential Biomedical Applications. *ACS Applied Materials & Interfaces*, 1(2), 319–327. <http://doi.org/10.1021/am8000456>
- Wu, J., Zeng, F., Huang, X. P., Chung, J. C. Y., Konecny, F., Weisel, R. D., & Li, R. K. (2011). Infarct stabilization and cardiac repair with a VEGF-conjugated, injectable hydrogel. *Biomaterials*, 32(2), 579–586. <http://doi.org/10.1016/j.biomaterials.2010.08.098>
- Xi, J., Khalil, M., Shishechian, N., Hannes, T., Pfannkuche, K., Liang, H., ... Pillekamp, F. (2010). Comparison of contractile behavior of native murine ventricular tissue and cardiomyocytes derived from embryonic or induced pluripotent stem cells. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 24(8), 2739–2751. <http://doi.org/10.1096/fj.09-145177>
- Xia, Y., Buja, L. M., Scarpulla, R. C., & McMillin, J. B. (1997). Electrical stimulation of neonatal cardiomyocytes results in the sequential activation of nuclear genes

- governing mitochondrial proliferation and differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, 94(October), 11399–11404. <http://doi.org/10.1073/pnas.94.21.11399>
- Xin, M., Olson, E. N., & Bassel-Duby, R. (2013). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nature Reviews Molecular Cell Biology*, 14(8), 529–541. <http://doi.org/10.1038/nrm3619>
- Yamada, K. (2005). Distinct Pathways Regulate Expression of Cardiac Electrical and Mechanical Junction Proteins in Response to Stretch. *Circulation Research*, 97(4), 346–353. <http://doi.org/10.1161/01.RES.0000178788.76568.8a>
- Yamakawa, H., & Ieda, M. (2015). Strategies for Heart Regeneration. *International Heart Journal*, 56(1), 1–5. <http://doi.org/10.1536/ihj.14-344>
- Yamanaka, S., Activity, T., June, R., & Ebs, M. (2006). Topic A . Generation and characterization of human iPS cells.
- Yamanaka, S., Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., & Tomoda, K. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131(5), 861–72. <http://doi.org/10.1016/j.cell.2007.11.019>
- Yang, M., Baldwin, S. L., Marutyan, K. R., Wallace, K. D., Holland, M. R., & Miller, J. G. (2006). Elastic stiffness coefficients (c11, C33, and C13) for freshly excised and formalin-fixed myocardium from ultrasonic velocity measurements. *The Journal of the Acoustical Society of America*, 119, 1880–1887. <http://doi.org/10.1121/1.2168547>
- Zhang, D., Shadrin, I. Y., Lam, J., Xian, H.-Q., Snodgrass, H. R., & Bursac, N. (2013). Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials*, 34(23), 5813–20. <http://doi.org/10.1016/j.biomaterials.2013.04.026>
- Zhang, X., Haynes, P., Campbell, K. S., & Wenk, J. F. (2015). Numerical Evaluation of Myofiber Orientation and Transmural Contractile Strength on Left Ventricular Function. *Journal of Biomechanical Engineering*, 137(4), 044502. <http://doi.org/10.1115/1.4028990>
- Zhang, Y. (2009). Connexin43 Expression Levels Influence Intercellular Coupling and Cell Proliferation of Native Murine Cardiac Fibroblasts, 8(3), 425–429.

<http://doi.org/10.1037/1528-3542.8.3.425>.Beyond

Zhu, W.-Z., Hauch, K. D., Xu, C., & Laflamme, M. A. (2009). Human embryonic stem cells and cardiac repair. *Transplantation Reviews*, 23(1), 53–68.

<http://doi.org/10.1016/j.ttre.2008.05.005>

Zhuang, J., Yamada, K. a, Saffitz, J. E., & Kléber, a G. (2000). Pulsatile stretch remodels cell-to-cell communication in cultured myocytes. *Circulation Research*, 87(100001590507), 316–322. <http://doi.org/10.1161/01.RES.87.4.316>

Zimmermann, W.-H., & Eschenhagen, T. (2003). Cardiac tissue engineering for replacement therapy. *Heart Failure Reviews*, 8(3), 259–269.

<http://doi.org/10.1023/A:1024725818835>

Zimmermann, W.-H., & Eschenhagen, T. (2007). Embryonic stem cells for cardiac muscle engineering. *Trends in Cardiovascular Medicine*, 17(4), 134–40.

<http://doi.org/10.1016/j.tcm.2007.02.007>

Zimmermann, W.-H., Fink, C., Kralisch, D., Remmers, U., Weil, J., & Eschenhagen, T. (2000). Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. *Biotechnology and Bioengineering*, 68(1), 106–114.

[http://doi.org/10.1002/\(SICI\)1097-0290\(20000405\)6](http://doi.org/10.1002/(SICI)1097-0290(20000405)6)

Zimmermann, W.-H., Melnychenko, I., Wasmeier, G., Didié, M., Naito, H., Nixdorff, U., ... Eschenhagen, T. (2006). Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nature Medicine*, 12(4), 452–458.

<http://doi.org/10.1038/nm1394>

Zimmermann, W.-H., Schneiderbanger, K., Schubert, P., Didié, M., Münzel, F., Heubach, J. F., ... Eschenhagen, T. (2002). Tissue Engineering of a Differentiated Cardiac Muscle Construct. *Circulation Research*, 90(2), 223–230.

<http://doi.org/10.1161/hh0202.103644>