UNIVERSITÁ DEGLI STUDI DI NAPOLI "FEDERICO II"

Dipartimento di Sanità Pubblica

Scuola di Dottorato in Sanità Pubblica e Medicina Preventiva

XXX Ciclo

Coordinatore: Prof.ssa Stefania Montagnani

Tesi di Dottorato di Ricerca

CONSTRUCTION OF SELF-ASSEMBLING SCAFFOLD OF DECELLULARIZED CARDIAC ECM AND FIBRIN FOR THE TREATMENT OF MYOCARDIAL INFARCTION

RELATORE:

Prof.ssa: Franca Di Meglio

CANDIDATO:

Dott.ssa Mariagrazia Di Gennaro

CONTENTS

ABBREVIATIONS

3D	Three-dimensional
bFGF	Basic Fibroblast Growth Factor
$CaCl_2$	Calcium Chloride
CCT	Cardiac Cell Therapy
CHAPS	Sodium deoxycholate, sodium dodecylsulfate, 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate
CHD	Coronary Heart Disease
CPCs	Cardiac Primitive Cells
CPC-P	Cardiac Primitive Cells from Pathological Hearts
CS/DS	Chondroitin Sulfate/Dermatan Sulfate
CSCs	Cardiac Stem Cells
DAPI	4',6-diamidino-2-phenylindole
d-ECM	Decellularized Extracellular Matrix
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ESCs	Embryonic Stem Cells
F12K	Nutrient Mixture F-12 Ham
F:M	Fibrin to Matrix ratio
FBS	Fetal Bovine Serum
GAGs	Glycosaminoglycans
gDNA	Genomic DNA
H/E	Hematoxylin and Eosin
HA	Hyaluronan Acid

HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric Acid
HS	Heparin Sulfate/Heparin
IL	Interleukin
iPSc	Induced Pluripotent Cells
KS	Keratin Sulfate
LV	Left Ventricular
LVR	Left Ventricular Remodeling
MI	Myocardial Infarction
MMPs	Metalloproteinases
NaOH	Sodium Hydroxide
NF	Nuclear Factor
OPN	Osteopontin
PCL	Poly Caprolactone
PGA	Poly Glycolic Acid
PGs	Proteoglycans
PLA	Poly Lactic Acid
PLGA	Poly Lactic-co Glycolic Acid
PURs	Polyurethanes
SDS	Sodium Dodecyl Sulfate
SPARC	Secreted Protein, Acid and Rich in Cysteine
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TRL	Tall-Like Receptor
TSP	Thrombospondin
US	United States

ABSTRACT

Cardiac tissue engineering aims at restoring cell compartment along with myocardial extracellular microenvironment. Even though countless combinations of synthetic or biological scaffolds and stem/progenitor cells of various origin have been tested so far, the most suitable candidate is yet to be found. With the aim to develop a natural injectable self-assembling scaffold able to be serve as both three-dimensional platform and stem/progenitor cell delivery method, we assembled fibrin gels incorporating Cardiac Primitive Cells (CPC) and cardiac decellularized extracellular matrix (d-ECM). Cryosections of cardiac ECM were decellularized and d-ECM was lyophilized and solubilized. d-ECM solution was mixed with the fibrin solution carrying Cardiac Primitive Cells (CPC) isolated from adult heart and allowed to gel at 37°C. Several Fibrin to Matrix ratios (F: M) were tested to determine the ideal composition in terms of time of gelling and three-dimensional (3D) architecture. Gels were cultured for three days, then fixed and processed as tissues for histological study. Histochemistry revealed the presence of viable CPC in the gels whose architecture varied from densely packed to very loose. Due to time of gelling and to concentration of fibrin, the distribution of CPC in the scaffold was even only in the gel with F:M of 1:1. According to our results, the combination of CPC with fibrin and d-ECM at F:M of 1:1, being injectable and self-assembling at body temperature, provides an attracting alternate to bioconstructs.

1. INTRODUCTION

1.1 MYOCARDIAL INFARCTION AND HEART FAILURE

Coronary heart disease (CHD) is now the leading cause of death worldwide (1). In 2010, CHD caused ∼1 of every 6 deaths in the United States (US) (2). Recent data show that death rates from CHD have decreased in North America and in many countries in Western Europe (3). This decline has been due to improved prevention (reduced cigarette smoking among adults, lower average levels of blood pressure and blood cholesterol), diagnosis, and treatment (pharmacological or interventional).

Myocardial infarction (MI) is the most common manifestation of CHD, accounting for ~ 50% of all CHD cases in the US (4). Acute myocardial infarction (MI), usually known as a heart attack, is triggered by the occlusion of one or more of the blood vessels (coronary arteries) supplying blood to the heart. Consequently, the supply of nutrients and oxygen to the heart muscle (myocardium) is reduced, and leads to a remarkable loss of contractile muscle cells (cardiomyocytes). The tissue becomes necrotic and cardiac performance can be impaired. In addition, healthy myocytes close to the infarct may also die, causing an increase in size of the necrotic tissue area. Furthermore, since cardiomyocytes rarely divide, contractile cells cannot repopulate the infarcted area, while non-contractile cells (fibroblasts) gradually replace them (5, 6, 7), causing the formation of a densely collagenous non-contractile scar (8). All of these results in significant alterations in the structure and composition of the myocardial extracellular matrix (ECM) (9, 10), in addition to the loss of cellular

elements. The post-MI process, that commonly affects the mechanical properties of the left ventricle of myocardium, is called left ventricular remodeling (LVR). During LVR, the ventricle wall becomes thinner and structural changes, such as an increase in left-ventricular volume, occur in the heart (5, 6, 11-14). To block the cascade of unfavorable events of MI and restore the original structure of the lost cardiac tissue, new cardiomyocytes must replace the lost ones.

Unfortunately, to date, the problem of how to treat the thousands of patients per year worldwide, who survive an extensive myocardial infarction (MI) and develop advanced heart failure, has not been resolved, despite optimal medical therapy (15). Heart transplant, traditionally, is the best solution for the patients with end-stage heart failure. However, donor supply is declining, increasing the gap between supply and demand for heart replacement therapies (16). In addition, lifelong immune suppression often causes serious complications (17, 18).

Therefore, it is desirable to develop less invasive alternative therapeutic strategies which can promote rapid reconstruction of the affected tissue and efficient renewal of its contractile capacity, in order to ameliorate both patient prognosis and life quality.

Different approaches in cell transplantation and cardiac tissue engineering have emerged as potential treatments to restore cardiac function (19).

1.2 CARDIAC EXTRACELLULAR MATRIX (ECM)

1.2.1 COMPOSITION AND FUNCTION

Myocardial tissue is composed of cardiomyocytes, non-myocytes (e.g. fibroblasts, endothelial cells, vascular smooth muscle cells, etc.), and extracellular matrix (ECM) proteins (20-24). The ECM, that refers to the non-cellular component, constitutes a scaffold for myocytes, fibroblasts, and endothelial cells, providing mechanical stability, physical strength, stiffness, ductility, and energy absorption to tissues. It is essential for efficient cardiac function via myocyte alignment, regulating blood flow during contraction, and compliance. Moreover, the ECM is an important mediator of growth-related factor and plays an essential role in development, remodeling, and signaling in the cardiovascular system. Therefore, the disruption of ECM homeostasis is a key factor for the progression of cardiac dysfunction (20). Cellular interactions with the ECM are also essential to normal formation of the heart (25-28).

The ECM, whose composition and architecture varies depending on the native tissue, is a three-dimensionally (3D) arranged complex mixture of various molecules, such as collagens, glycoproteins (e.g fibronectins, laminins and elastins), proteoglycans (PGs) including glycosaminoglycans (GAGs), extracellular protease and ECM receptors (integrins) (29). Other important components of the extracellular matrix include a family of matrix metalloproteinases (MMPs) which guarantee constant extracellular matrix remodeling, and growth factors.

Although glycoproteins and proteoglycans are essential in proper cardiac geometry and for various functions of the ECM, the most abundant structural components of the ECM are collagens (21). The ability to synthesize the ECM components depends on cell types in the heart. Fibroblasts and smooth muscle cells synthesize collagen types I and III, and fibronectin, whereas cardiomyocytes and endothelial cell produce collagen type IV. Cardiomyocytes, smooth muscle cells, and endothelial cells produce laminin (29).

a) COLLAGENS

Collagen is the most abundant and ubiquitous ECM protein in mammals, which is not only critically important for the biomechanical stability of tissues, but is also intimately involved in cell adhesion and migration during growth, differentiation, morphogenesis and wound healing (30). To date, nearly 30 types of collagen have been identified (31). Each protein contains three polypeptide (α) chains, displaying an extended polyproline-II conformation, a right-handed supercoil and a one-residue stagger between adjacent chains (32). Each polypeptide chain has a repeating Gly-X-Y triplet in which glycine residues occupy first position and the X and Y positions are frequently occupied by proline and 4-hydroxyproline, respectively. These repeats allow the formation of a triple helix, which is the characteristic structural feature of the collagen superfamily. In the heart, collagen constitutes a relatively small fraction of myocardial mass. Nevertheless, the myocardial collagen network is essential to the mechanics of the heart (33). The myocardial collagen matrix mainly consists

of type I and III collagens, which form a structural continuum. Collagen type I fibers mainly provide structural support and give the heart properties that include stiffness and resistance to deformation. The collagen type III fibers seem to play an important role as a link between contractile elements of adjacent myocytes, carrying some information useful for cell function and contributing elasticity (34-36). The two type of collagens jointly support and tether myocytes in maintaining their alignment, tensile strength, shape and thickness in order to prevent rupture and contribute to the passive and active stiffness of the myocardium (37).

b) ELASTIN

While collagens provide tissue rigidity, elastin allows many tissues in the body to resume their shape after stretching or contracting.

Elastin is highly hydrophobic, composed primarily of aminoacids such as alanine, valine, leucine and glycine (38). It is an insoluble protein generated by lysyl oxidase crosslinking of soluble tropoelastin monomers. The elastin tends to be located in the inner core of the elastic fiber and is surrounded by a fine mesh of microfibrils. These microfibrils are predominantly fibrillin-1, but to a lesser extent Fibrillin-2 (39). Myocardium contains elastin, both in the walls of coronary blood vessels and in the interstitium, but it is unclear whether elastin contributes significantly to myocardial mechanics (40).

c) LAMININ

Cell adhesive molecules, such as laminin, are important non-collagenous glycoproteins (41, 42).

Laminin is one of the main components of the basement membrane. Its molecular weight is 200-400 kDa and it is composed of three disulphide-linked chains which form characteristic cross shape. Laminin is a heterotrimeric glycoprotein consisting in one α, one β, and one γ chain, in various combinations (five α, three β, and three γ chains). Twelve distinct laminin isoforms have been isolated and the trimers are named according to their trimeric composition of α, β, and γ chains (43). Laminin-1 (α1β1γ1) is the first extracellular matrix protein to be expressed during embryonic development, and it has been observed that the heart organogenesis does not proceed in the absence of this protein (44). The absence of laminin-2 (α2β1γ1), an isoform typical of muscle tissue, causes congenital muscular dystrophy with cardiac involvement (45). Moreover it has been showed that, in the adult heart, laminin-1 and laminin-2 protect from apoptosis and stimulate proliferation of CPCs in vitro (46). The complex adhesion protein - laminin mediates cell adhesion, migration, growth and differentiation. Analysis of the interaction between basement membrane proteins and cardiomyocytes has led to the recognition that laminin is important for the survival of these specialized cells *in vitro* (47).

d) FIBRONECTIN

Fibronectin is a dimeric glycoprotein (with the subunits that range in size from 230 kDa to 270 kDa) found in the extracellular matrix of most tissues. It is located within the basement membrane, that serves as a bridge between cells and the interstitial collagen meshwork and influences diverse processes including cell growth, adhesion, migration, and wound repair (48). The expression of fibronectin is a critical factor in cardiac extracellular matrix, particularly in developing myocardium and in response to injury. Fibronectin levels have been found to be higher in neonatal hearts when compared to adult. In the cardiovascular system, fibronectin is synthesized by many cell types but not by the cardiomyocytes. Recently, expression of fibronectin was shown to influence cardiac progenitor cell response after myocardial infarction in adult mice (49).

e) PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

Proteoglycans (PGs) and glycosaminoglycans (GAGs) perform numerous vital functions within human body. Accordingly, PGs can be considered as one of the most critical ECM components for normal cell function and tissue development (50). PGs regulate cartilage mechanics by controlling the flow of water in and out of the tissue during loading. Azeloglu et al. recently demonstrated that PGs are an important determinant of residual stress in arteries, where, as in myocardium, their presence had been largely ignored (51).

GAGs are polysaccharide chains consisting of repeating amino sugar and uronic acid disaccharide units. There are four classes of GAGs. The most abundant GAG in the developing heart is hyaluronan (HA) which consists of alternating UDP glucuronic acid and UDP-N-acetylglucosamine molecules, which alternate to combine in a chain-like fashion (52). HA is part of a pericellular matrix that provides a hydrated environment that facilitates cellular proliferation and motility (53). The other three classes of GAGs are chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS), and keratin sulfate (KS). These GAGs are found in the ECM of the heart linked to proteoglycan core proteins (54).

f) INTEGRINS

ECM in the heart is linked to cellular cytoskeleton by transmembrane molecules, mainly integrins, which provides a physical connection between cytoskeleton and ECM proteins (55, 56). They orchestrate multiple functions in the intact organism including organogenesis, regulation of gene expression, cell proliferation, differentiation, migration, and death (57). The integrin family consists of a large multi-adhesive extracellular matrix molecules that bind different proteins, including extracellular matrix molecules, growth factors, cytokines, and MMPs. Integrins, heterodimeric type I transmembrane proteins, consist of α and β subunits. The family is composed of 18 α subunits and 8 β subunits, that can assemble into 24 different heterodimers. The integrins can be grouped based on ligand-binding properties or on their subunit composition. In the cardiac myocytes, the integrin heterodimers most highly expressed are α1β1, α5β1 and

α7β1, which are predominantly collagen, fibronectin and laminin binding receptors, respectively (58). They are expressed in all cellular components of the cardiovascular system, including the vasculature, blood, cardiac myocytes, and non-muscle cardiac cells (25-28).

1.2.2 REMODELLING OF ECM AFTER MYOCARDIAL INFARCTION

The cardiac extracellular matrix is critical to maintain the structural integrity of the heart. The disruption of the matrix network results in alterations of the ventricular geometry (59-61). Perhaps, the most dramatic changes in the composition of the cardiac extracellular matrix occur in the setting of acute myocardial infarction. Myocardial infarction ultimately results in replacement of dead cardiomyocytes with a collagen-based scar. Cardiac wound repair after MI involves cellular and molecular events that can be divided in three overlapping phases: inflammatory, proliferative and maturation phase (62).

Immediately, after a myocardial infarction, cardiomyocytes death triggers activation of the complement system, generates free radicals and actives Tall-Like Receptor (TRL)-mediated pathway. These overlapping pathways activate Nuclear Factor (NF)-kB in resident myocardial cells inducing expression of cytokines (such as Tumor Necrosis Factor (TNF)-alfa and IL-1beta), chemokines and adhesion molecules (63, 64), initiating the *inflammatory phase*. The activation of the cytokines enhances Matrix Metalloproteinase (MMP) expression and activity, promoting cardiac matrix degradation (65, 66). During the inflammatory phase, increased permeability of the cardiac microvasculature

results in extravasation of fibrinogen and plasma fibronectin, forming a provisional matrix network that serves as a scaffold for migration and proliferation of infiltrating inflammatory cells (leukocyte), endothelial cells and fibroblasts (67). In addition, the fragmentation of extracellular matrix constituents during the early stages following infarction, as collagen and low molecular weight hyaluronan fragments, exerts potent pro-inflammatory effects (68-70). As the professional phagocytes clear the wound from dead cells and matrix debris, the activation of "stop signals" (such as Interleukin (IL)-10 and Transforming Growth Factor (TGF)-beta) suppress the inflammatory reaction leading to transition to the *proliferative phase* (62). During this phase, the plasma-derived provisional matrix is lysed by proteolytic enzymes produced by granulation tissue cells, and is replaced by an organized cell-derived provisional matrix containing cellular fibronectin and hyaluronan (69). When fibrin and plasma fibronectin are cleared from the infarcted area, the cellular fibronectin is secreted by fibroblasts and macrophages and serves as a "second order" provisional matrix (71). Fibronectin is essential for modulation of fibroblasts toward myofibroblasts, an important process for reparative response (72-74). Activated myofibroblasts play an important role in wound contraction and are the main source of collagen in the healing infarct (75).

During the proliferative phase, the matricellular proteins play an important role as regulators of the reparative response. These proteins are a family of structurally diverse extracellular matrix proteins that modulate cell function and activity; their induction is a prominent feature of the proliferative phase of

healing and appears to play an important role in regulating the dynamic cellular events and in promoting matrix organization, in cardiac repair. They include thrombospondin (TSP)-1 and -2, tenascin-C and –X, Osteonectin/SPARC (secreted protein, acid and rich in cysteine), osteopontin (OPN) and periostin. These proteins are not expressed in normal hearts (62).

Finally, *the maturation phase* occurs, promoting the formation of a mature scar comprised of dense cross-linked collagen, that enhances tensile strength of the infarct, and increases the passive stiffness contributing to diastolic dysfunction (76). Scar formation is an essential aspect of rapid wound healing without which the ischaemic region would be subject to rupture. However, scar tissue is largely acellular and lacks the normal biochemical properties of the host cells. This leads to electrical uncoupling, mechanical dysfunction, and loss of structural integrity (77, 78).

This post-MI remodeling process is a clinically significant problem in that it can lead to LV dilation, systolic and diastolic dysfunction, and the progression to heart failure (59).

The discovery of the proliferative capacity and plasticity of various stem cells populations has sparked much interest and debate regarding their use as a potential therapy.

1.3 CELL BASED THERAPY FOR CARDIAC REPAIR

Hopefully, the regeneration of myocardium after a major insult should involve new contracting cardiomyocytes into the infarct zone, which would empower the heart contractility after integration with the host tissue. Therefore, the Cardiac Cell Therapy (CCT) represents the first realistic strategy for reversing the deleterious effects of the terminal damage of the heart. The cell therapy, which involves the transplantation of stem cells to replace necrotic cardiomyocytes, could have the potential to restore cardiac function by inducing neovascularization, and regenerating and protecting cardiomyocytes (79). Stem cells are distinguished by two important characteristics: first, they are unspecialized cells capable of self-renewing through cell division; second, under certain physiologic or experimental conditions, they can be induced to become tissue-or organ-specific cells with special functions (80, 81). These two properties make stem cells unique and suitable for cardiac repair. The ideal source of cells to heart repair would: *a)* expand *in vitro* on a large scale *b)* integrate with damaged tissue, and *c)* differentiate into new cardiomyocytes electromechanically coupled with the host tissue (82). Thus, during the last decade, several cell types that might replace necrotic tissue and minimize scarring have been considered, including *skeletal myoblasts* (83), *bone marrow-derived haematopoietic stem cells* (84), *mesenchymal stem cells* (85), *cardiac stem cells* (CSCs) (86), *embryonic stem cells* (ESCs) (87-89), *endothelial progenitor cells* (90), and *induced pluripotent stem* (iPSc) *cells* (91).

More specifically, *skeletal myoblasts* have been implanted because they can be easily isolated and they have high rate of proliferation and are hypoxia-resistant (92, 93). Similarly, different cell populations residing in bone marrow have been tested because of their great plasticity towards cells of cardiogenic and endothelial lineage (94): *haematopoietic stem cells* (95), *mesenchymal stem cells* (96) and *endothelial progenitor cells* (97, 98).

Embryonic stem cells have been tested because of their strong capacity for expansion and subsequent differentiation into cardiomyocytes, endothelial cells and cardiac fibroblasts (99, 100). *Induced pluripotent cells* were artificially derived from non-pluripotent cells by specifically inducing the expression of genes involved in self-renewal and potency (101-103). These cells, as embryonic stem cells, have been tested because they have limited replication and ample capacity for differentiation.

Unfortunately, these approaches had limited success as revealed by several studies in animal models suggesting that cellular transplantation is feasible, safe and beneficial, but, nonetheless, the effective regeneration of infarcted myocardium and cardiac function improvement are weak. Modest results have also been obtained due to massive cell loss, low cellular survival or lack of cellular effect after administration. Hypoxic conditions in the host tissue, failure to establish electrical or mechanical heart coupling result, in fact, in arrhythmias and low rates of cell differentiation into a cardiac lineage. Furthermore, the status of indifferentiation of embryonic stem cells generates their uncontrolled proliferation, giving rise to the formation of teratomas (93, 104-106), whereas

obtaining induced pluripotent stem cells entails the use of viral vectors that could also promote unwanted oncogenic activity (107, 108).

However, the modest functional effects of transplanted stem cells stimulated further research into the natural regenerative mechanism of the cardiac tissue.

1.3.1 CARDIAC PRIMITIVE CELLS AS STEM CELLS

For several decades, the paradigm that the heart is an organ incapable of regenerating parenchymal cells has been questioned (15, 109-111). The belief that the heart is a fully differentiated organ, unable to synthesize DNA and undergo mitosis, has its basis on the anatomical studies of Karsner et al. (112) and profoundly limited investigation on myocardial regeneration potential (113). In the last decade, this notion has been almost completely invalidated, since several studies emphasized the capability of adult mammalian cardiac myocytes to synthesize DNA and reenter the cell cycle after a MI (114-117).

The most interesting revolution has been the discovery in the adult heart of stem cells (86), that can also be isolated and explanted from human myocardium samples obtained using a minimally invasive biopsy procedure (118, 119).

Stem cells and progenitor cells with the capacity to differentiate into three major cardiac cell types - cardiomyocytes, smooth muscle cells and endothelial cells have been described in both embryonic (120-122) and adult heart tissue (86, 123, 124). The adult human heart, in particular, hosts a population of cardiac stem cells, positive for CD117, the stem cell factor receptor, responsible for physiological tissue homeostasis and, presumably, regeneration in pathological

conditions (111, 125-127). These cells have been characterized and the expression of different markers could be associated with the degree of stem cell differentiation. When induced to differentiate, they adopt phenotype specific for myocytes, smooth muscle or endothelial cells, expressing markers typical for these cell lineages (Nkx 2.5 and α-sarcomeric actin, GATA6, and smooth muscle actin, Ets-I, and FVIII, respectively). These cells are identified as cardiac primitive cells (CPCs), defined as cells expressing stem cell markers only, or together with markers of commitment towards cardiac cell lineages. Moreover, it was observed (46, 128-131) that the number of CD-117-positive cells in the adult human heart increase significantly in ischemic cardiomyopathy and pressure overload (46, 128, 132, 133). In addition, it has also been shown that these cells (as well as stem cells from other tissue) appear to reside in specialized microenvironment, termed niches, which support growth and maintenance of cells (134-135). This specific microenvironment regulates stem cell function.

All these observations have prompted studies focusing on the regeneration of tissue damaged by diseases (136) or the aging process (137), as the cardiac-resident primitive cells (CPCs) seem to be a promising target for acute and chronic heart disease therapy in which cardiac regeneration may be accomplished by enhancing the normal turnover of myocardial cells (84, 138, 139).

Nevertheless, the recruitment and/or activation of these cells for cardiac repair is insufficient to significantly affect and prevent the deterioration in cardiac performance and adverse remodeling after an ischemic event. The physical

separation of niches from the site of injury, the formation of fibrotic scar tissue, or the lack of appropriate signaling are major causes of these limitations (140). In fact, some reports demonstrated that after myocardium infarction, new cardiomyocytes are produced in the border region of the infarcted area but not in the middle of the damaged zone (141), because dense fibrosis presents a formidable physical barrier to regenerating cell (142). This hostile microenvironment might therefore prevent the activation of resident cells and thus also reduce the success of exogenous cell therapies.

Therefore, the heart is unable to regenerate, due to replacement of cells with fibrous tissue, which is considered central to the evolution of adverse cardiac remodeling, and this condition leads to progressive cardiac dysfuction. Nowadays, to overcome these limitations, one of the most promising approaches for therapeutic regeneration is cardiac tissue engineering that uses biomaterials as vehicles for cell delivery and retention.

1.4 CARDIAC TISSUE ENGINEERING AND BIOMATERIALS

Tissue engineering is an interdisciplinary scientific area that attempts to restore or improve the biological functions of damaged tissues or no longer able to carry out their function (143). This therapeutic strategy is of particular interest for the treatment of heart diseases, where large portions of functional tissue are lost (i.e., after myocardial infarction (MI)) with very limited intrinsic regeneration ability of the heart. In general, the tissue-engineering paradigm relies on the use of

combinations of cells with regenerative capacity, biomaterial scaffolds, growth factors, differentiation factors and proangiogenic factors (144).

In the last decade, several strategies were developed under the concept of cardiac tissue engineering. One strategy uses biomaterials as vehicles for cell delivery and retention in the infarcted heart (145-147). The biomaterial vehicle, usually in the form of three-dimensional porous degradable scaffolds, providing mechanical support for the infarcted tissue, creates a favorable microenvironment for promoting transplanted cell survival and long-term action. A second strategy is the *in vitro* bioengineering of cardiac patches (144, 148) that are seeded with cardiac cells. The third strategy focuses on the use of biomaterials in acellular forms as structural restrainers and scar filling to attenuate heart remodeling and dysfunction (149, 150). The fourth strategy offers combination of biomaterials with bioactive molecules, in the form of local and controlled delivery system (151, 152).

The biomaterials used in each of the strategies for myocardial repair and regeneration not only need to be cell and tissue compatible and biodegradable, with no or minimal inflammatory response after implantation, but also need to support cell attachment, differentiation, and proliferation (153-156). Biodegradable biomaterials provide a high biocompatibility due to polymer backbone degradation or by dissolution of the matrix, and the degradation products must be non-toxic and readily eliminate from the body. The biomaterial of the scaffolds should be porous to facilitate mass transport, hydrophilic to enhance cell attachment, elastic to enable transmission of contractile forces,

resistant to stress and strain, sterilizable and match biomechanical characteristics of tissue. The scaffold should be able to release growth factors, gene signals and other proteins (157, 158). Hence, the ideal scaffolding should mimic the microenvironment found within native tissue.

1.4.1 BIOMATERIAL CLASSIFICATION

Many different biomaterials are be used for tissue engineering, and they can be classified as natural and synthetic. The first group generally includes proteins and polysaccharides, whereas the second one is composed of metallic, ceramic or polymeric materials. All of them have been used to manufacture scaffolds in tissue engineering (159, 160).

Natural biomaterials are usually biodegradable and biocompatible and better recreate the native myocardial microenvironment. The inclusion of growth factors and other proteins able to boost cellular functions allow their stimulating effects. Nevertheless, natural biomaterials can transfer pathogen associated with natural polymers (161).

Synthetic biomaterials are easy to handle and can be designed with versatile properties, such as mechanical strength and biodegradation rate, and can be tailored with functional groups. However, they do not transfer pathogens (162).

a) NATURAL BIOMATERIALS

The most popular natural biomaterials are collagen, gelatin, chitosan, alginate, hyaluronic acid, and fibrin.

Collagen is the most abundant and ubiquitous structural protein in the body. It can be fabricated in many forms, such as hydrogel or macroporous scaffold; its fabrication frequently requires chemical cross-linking. Several optimal properties, such as being biocompatible, cell-adhesive (163), suturable, porous and readily combinable with other materials, have made collagen appropriate for use as a natural scaffold in tissue engineering applications (164-169).

Gelatin is a natural polymer that can be obtained from bone, skin, or tendon by partial hydrolysis with acid or alkaline solutions. Gelatin is highly biocompatible and biodegradable, has low cost (170, 171) and it has been used in myocardial tissue engineering, especially in the format of a hydrogel prepared by chemical cross-linking. The gelatin under various conditions can provoke an unspecific inflammatory response (172).

Chitosan is a biodegradable and biocompatible cationic polymer. Its final degradation products are biocompatible chitosan oligosaccharides of variable length (173). This natural material has the capacity to combine with conductive materials to improve electrical signals transmission and/or with other biomaterials (174, 175). Additionally, chitosan was shown to be capable of high growth factor retention and strong cellular receptor adhesion due to its hydrophilicity (176, 177).

Alginate is a polysaccharide extracted from brown algae and can form a hydrogel through ionic crosslinking with divalent cations (mainly calcium) (178, 179). Alginate is not degradable in mammals; yet, the calcium cross-linked hydrogel is readily erodible with time due to exchange of calcium ions by sodium ions in physiological milieu, leading to hydrogel dissolution (180). This property also enables incorporation and retention proteins inside the hydrogel; thus, it can be used as a scaffold for tissue regeneration (181, 182). Interestingly, the implantation of highly purified alginate, free of protein contaminants, resulted in a complete absence of adverse host immune response (183, 184).

Hyaluronic acid (HA) is a linear un-sulfated polysaccharide and has versatile biological functions, such as a lubrificant material and numerous receptor-mediated roles in different cell processes (185, 186). It has a bioactive role in several regulatory processes, such as angiogenesis, inflammation, wound healing, and tissue regeneration (187, 188). Hyaluronic acid acts in the ECM as space filler and significantly contributes to the mechanical properties of a tissue, by linking proteoglycans and maintaining hydration (189).

Fibrin is a biocompatible polymer and plays an important role in injured tissue. It is endowed with fit properties for healing and angiogenesis (190). This polymer that mimics the last step of the coagulation mechanism is obtained through the enzymatic conversion of fibrinogen catalyzed by thrombin and followed by a spontaneous polymerization. Fibrinogen is a very large and long glycoprotein that is converted in fibrin through the action of thrombin and activated Factor XIIIa. This process depends largely on calcium ions and specifically consists of

two different stages: proteolysis of fibrinogen by thrombin, followed by the polymerization of fibrin monomers, and fibrin stabilization through the action of Factor XIIIa (191-195). When fibrin is used for engineering applications, it is important to understand how the formulation of the fibrin matrix affects the porosity, permeability, and stiffness and the subsequent effects on cells grown in fibrin. Several studies have shown that it is possible to control the stiffness and porosity by changing the concentration of fibrinogen used to make the three-dimensional (3D) fibrin matrix. Specifically, as the concentration of fibrinogen increases, the stiffness of 3D fibrin matrices increases too, and the porosity decreases (196-198). These findings are significant as porosity is important for nutrition uptake, gas exchange and waste removal. Fibrin scaffolds were prepared as hydrogels and in injectable forms, where its components (fibrinogen and thrombin) are mixed during injection into the tissue.

Fibrin is an excellent scaffold for tissue engineering because it has several advantages such as its components that can be autologously harvested and its degradation products are physiological and therefore nontoxic (199); they are not allergenic or inflammatory (200). Then, fibrin has the potential to incorporate both cells and cell mediators (heparin and/or growth factors), with the potential to replicate a specific microenvironment. Moreover, the fibrin network has a nanometric fibrous structure, with a high area/volume ratio, mimicking ECM (201). In fact, it provides a suitable matrix for cell growth, differentiation and function (202), and for these reasons, fibrin is also used for the construction of cellularized scaffolds or “patches” that are placed over the infarcted myocardium

to provide optimal cell retention and implantation at the sites of injury (203). In particular, the presence of arginine-glycine-aspartic acid motifs within the fibrin network allows cell adhesion and binding of growth factors (204). The high elasticity of fibrin gel in comparison to other characterized protein gels is another essential aspect for its efficiency as a cell matrix (190), because its fibers are extraordinarily extensible and elastic. Fibrin has also viscous properties that can vary greatly, depending on clot structure and biochemical properties (205).

Hence, fibrin scaffolds are good candidates for treating MI due to their high biocompatibility, biodegradability and capacity of incorporating different cell types. In addition, fibrin scaffolds can be assembled with other scaffolds materials (206-208). Due to its intrinsic properties, application of fibrin patch alone (without cells), in combination with other materials, or with cardiac ECM, have provided acceptable cell viability, and its administration was been feasible (209-211).

In fact, fibrin gels have been successfully used in cardiovascular system, as cell and growth factor delivery vehicles to reduce ischemia and improving their survival within ischemic tissues (212), representing a good and plausible possibility for regenerating infarcted myocardium.

b) SYNTHETIC BIOMATERIALS

Synthetic materials are widely used in myocardial tissue regeneration, due to some excellent characteristics. First of all, they generally show superior mechanical properties than natural polymers that make them suitable for

mechanical support and resistance to cyclic stress of force-generating contractile tissue (153, 213). In addition, these properties, together with hydrophilicity and degradability, can be controlled in a reproducible manner. Their drawbacks regard the poor interaction with cells and the biological environment and their capacity to induce higher inflammatory reaction (213).

The most popular synthetic biomaterials employed in cardiac tissue regeneration are degradable polyesters. Although their biocompatibility has been proved and several reports on their successful applications in regenerative medicine exist, synthetic polymers have not yet considered optimal scaffolding materials. They are basically hydrophobic, preventing a good cell adhesion, and tend to crumble rather than slowly degrade. In addition, their acid biodegradation products can induce high inflammatory responses, and a drop in local pH, upon degradation process, can affect the viability of the surrounding cells (213). Synthetic polyesters such as poly lactic acid (PLA), poly glycolic acid (PGA) (214), poly caprolactone (PCL) (215), and poly lactic-co glycolic acid (PLGA) have generated immense interest as tissue engineering materials.

PGA is a rigid thermoplastic material with high crystallinity and hydrophilicity; it has a simpler structure than PLA, but PLA is more hydrophobic and less crystallizable and, for this reason, it degrades slower than PGA. However, PLA dissolves easier in organic solvents (216). From the combination of glycolic acid and lactic acid, copolymers poly lactic-co-glycolic acid (PLGA) have been obtained. Like PGA and PLA, PLGA they have a long history in clinical

applications (217, 218). These materials provide rigid structures of medium mechanical strength, in which cells adhere and grow on a pseudo three-dimensional environment (219).

PCL, which is a non-toxic compound, is known to be compatible and to have a long-term degradability, if compared to PLA, PGA, and PLGA. From mechanical point of view, PCL seems to be the less stiff polyester with a remarkable high strain at failure (220).

These polymers have been mainly used as solid macroporus scaffold for cardiac patch reconstruction.

In addition to polyesters, polyurethanes (PURs), other extensively explored materials, are a large group of polymers that have been studied for a long time, because of their biocompatibility (221, 222) and elastomeric properties (capability to resist to cyclic stress without plastic deformation or failure) that fit the requirements of materials for cardiovascular applications (223).

Although synthetic materials seem to be promising, they lack some properties, as they provide a poor interaction with cells, therefore, the challenge is to find the "perfect" biomaterial with the proper three-dimensional structure and the appropriate environment for retention of stem cells after implantation.

1.5 BASIC SCAFFOLD FABBRICATION FORMS: HYDROGELS

Hydrogel is a network of polymer chains that are water-insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are superabsorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural ECM, due to their significant water content. The hydrogels can be prepared from natural and synthetic polymers by physical/ionic interactions (alginate) or via chemical cross-linking (collagen, HA, and others). Cells are incorporated/encapsulated in the hydrogel during fabrication. Due to their resemblance to ECM texture, hydrogels are extensively being investigated as ECM replacements for damaged ECM after MI. They are delivered either by intramyocardial injection or by catheter-based techniques via the intracoronary route (209).

1.6 ACELLULAR BIOMATERIALS FOR CARDIAC REPAIR

In parallel to the development of 3D matrices prepared from biological or synthetic materials, the emerging direction is the use of natural ECM as an appropriate matrix for cardiac tissue engineering through the process of organs and/or tissues decellularization.

The goal of tissue decellularization is to efficiently remove cells and cell remnants while retaining the three-dimensional ultrastructure and composition of the native ECM to the extent possible (224). Residual cellular material within ECM may contribute to cytocompatibility problems, *in vitro,* and adverse host

responses, *in vivo*, upon reintroduction of cells (225-228). Through the removal of cellular antigens, the matrix could be used without inducing a foreign body reaction, inflammation, and potential transplant rejection (229) and would be considered biocompatible. Although complete removal of all cell remnants is not possible, analyses suggested that the decellularization could be considered acceptable when the amount of DNA is less than 50 ng per mg of dry tissue of ECM, the remaining DNA fragments have a length less than 200 bp, and when there is no visible nuclear material in tissue sections stained with 4',6-diamidino-2-phenylindole DAPI or hematoxylin and eosin (H/E) (230).

Moreover, degradation products of decellularized matrix have angiogenic (231) and chemoattractive properties and promote cell migration and proliferation (231-234).

Therefore, about the importance of ECM, preservation of this complex structure is pivotal in the decellularization process (235, 236).

Several techniques for decellularization have been developed, depending on characteristics of each tissue, including its cellularity, density, lipid content, and thickness. Several physical, chemical or enzymatic treatments have been tested.

Examples of *physical* methods are: freeze-thaw cycles that produce minor disruptions in the tissue ultrastructure (224) and electroporation that induces the formation of micropores that cause loss of cell homeostasis and leads to cell death (237).

There are also various *chemical* treatments to achieve an ideal decellularization: acids and bases, that cause or catalyze hydrolytic degradation of biomolecules; hypotonic and hypertonic solutions**,** that cause cell lysis and dissociate DNA from proteins, respectively (238); alcohols such as isopropanol, ethanol, and methanol are more effective than lipase in removing lipids from tissues, but damage ECM and they can precipitate proteins; detergents that solubilize cell membranes and dissociate DNA from proteins, and they are therefore effective in removing cellular material from tissues (triton X-100, sodium deoxycholate, sodium dodecylsulfate, 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate (CHAPS)) (238). Triton X-100 can effectively remove cell residues from thicker tissues (239), but sodium dodecyl sulfate (SDS) appears more effective than Triton X-100 for removing nuclei from dense tissues and organs (230) and its addition to a decellularization protocol can make the difference between complete and incomplete cell nuclei removal (240).

B*iological* agents are also common: enzymes that include nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and α-galactosidase can provide high specificity for removal of cell residues or undesirable ECM constituents. However, complete cell removal by enzymatic treatment alone is difficult and enzyme residues may impair recellularization or evoke an adverse immune response (241); chelating agents as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) induce cell dissociation from ECM proteins by sequestering metal ions (242), but they can contribute to disruption in protein-protein interactions by the same mechanism (243).

To date, many organs and tissues have been completely decellularized, including heart valves, myocardium, pericardium, lung, pancreas, kidney, liver, mammary gland, and nerve (241, 244). Ideally, the best source of a decellularized matrix for myocardial repair should be the matrix derived from the myocardium, which potentially possesses the composition and structure required for the specific needs of reconstructing the heart (245). The use of decellularized matrices from natural ECM sources represents an attractive approach, as in this way the properties of the resulting scaffold may potentially be matched to the target tissue, including the heart. However, several technical issues are still to be solved, such as the need for effective decellularization protocols, possible immunogenicity, preservation/ storage.

2. HYPOTHESIS AND AIM OF THE STUDY

Microenvironment can strongly influence survival, differentiation, and control biological activity of stem cells and their progeny. Hence, for a successful transplantation, a suitable microenvironment into which CPCs can survive and differentiate is required.

The ECM obtained from the decellularized cardiac tissue is a promising construct and it can be utilized to repair damaged areas of myocardium by promoting its vascularization and cardiac stem cells proliferation and differentiation. It potentially possesses the composition and the architecture required for reconstructing the heart, but solubilizing ECM and reforming it into a hydrogel, gels have a limited range of mechanical properties that do not match the native myocardium, thus pure ECM hydrogels cannot be used as a three-dimensional scaffold for cells.

Since biomaterials can improve cell retention, survival and differentiation, they are widely used in cardiac tissue engineering, to support cell-based therapies and enhance their efficacy for cardiac diseases.

Many biocompatible biomaterials have been used and among the ones tested, fibrin is an ideal candidate.

Fibrin is a versatile, FDA-approved material, known to form hydrogels with tunable mechanical properties. Fibrin gels have many positive characteristics that qualify them as good scaffolds for tissue engineering. However, fibrin itself does not entirely mimic the complex microenvironment of the heart.

Therefore, the overall goal of this study was to develop cardiac hybrid-scaffold, by combining cardiac decellularized ECM with fibrin, to assess if the construct obtained could be used as a three-dimensional scaffold for CPCs, and if it was able to improve cell culture quality, *in vitro*, and to treat myocardial infarction *in vivo*.

More specific aims were:

- To isolate and characterize CPCs from pathological adult human hearts;
- To develop a novel and specific protocol of decellularization to obtain cardiac ECM;
- To characterize the resulting cardiac d-ECM;
- To solubilize cardiac d-ECM and incorporate it into fibrin gels made with different fibrin/ matrix ratios;
- To establish which ratio could better mimic the extracellular microenvironment;
- To characterize hybrid scaffold by histological analysis.

3. MATERIALS AND METHODS

3.1 CARDIAC TISSUE SAMPLES

Cardiac tissue samples were obtained from pathological adult human hearts of patients undergoing heart transplantation because of end-stage heart failure due to ischemic cardiomyopathy (n = 20, 14 males and 6 females, mean age 56 ± 5.5 years, mean ejection fraction 25 ± 1%). Samples were harvested from macroscopically uninjured areas of the free wall of the left ventricle of explanted hearts. Specimens were collected, without patient identifiers, following protocols approved by the Monaldi Hospital and in conformity with the principles outlined in the Declaration of Helsinki.

3.2 CELL CULTURES

3.2.1 ISOLATION OF CARDIAC PRIMITIVE CELLS

Cardiac tissue samples, isolated from pathological adult human hearts, were dissected, minced, and enzymatically disaggregated by incubation in 0.25% trypsin for 6 hours at 4°C and 0.1% (w/v) collagenase II (both from Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C. The digestion was stopped by adding a double volume of Hank's Balanced Salt Solution (HBSS) supplemented with 10% fetal bovine serum (FBS) (both from Sigma-Aldrich). This preparation was further disaggregated by pipetting and tissue debris and cardiomyocytes were removed by sequential centrifugation at 100g for 2 minutes, passage through 40 μm cell strainer (BD Biosciences, Franklin Lakes,

NJ, USA), and centrifugation at 400g for 5 minutes. Cell population was seeded on culture dishes in Nutrient Mixture F-12 Ham (F12K) (Sigma-Aldrich) supplemented with 10% FBS, 5% horse serum, 0.2 mM glutathione, 5U/L erythropoietin, 50 μg/ml porcine gelatine, 10 000 U penicillin and 10 mg/ml streptomycin (all from Sigma-Aldrich) and 10 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA). The cell population was cultured for a time ranging from one to 2 weeks. Once the adherent cells were more than 75% confluent, they were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and the cell suspension was used to isolate cardiac primitive cells (CPCs) by immunomagnetic cell sorting. In particular, cell suspension, previously depleted of fibroblasts, was incubated with anti-human-CD117 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and passage through columns placed in magnetic field, to purify the positive fraction of CD117-positive cardiac primitive cells (CPCs).

3.2.2 CULTURE OF CARDIAC PRIMITIVE CELLS

CD117-positive CPCs were plated at a density of $4x10^3$cells/cm^2 in F12K medium (Sigma-Aldrich) supplemented as reported above. In order to check the effectiveness of sorting, CPCs were fixed in 4% paraformaldehyde for 20 min at room temperature and incubated with 10% donkey serum to block unspecific binding of secondary antibodies. Cells were incubated with primary antibody against the human stem cell marker CD117 (mouse monoclonal, Merck Millipore, Billerica, Massachusetts, USA), followed by an incubation with

secondary antibodies conjugated with rhodamine (Jackson ImmunoResearch Europe, Newmarket, UK). Nuclei were counterstained with DAPI (Merck Millipore). Stained area of culture dish was mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems, Wetzlar, Germany) and connected to a digital camera DFC345FX (Leica Microsystems).

CD117-positive CPCs at passage 5 were then used to assemble fibrin gels.

3.3 CARDIAC TISSUE SECTIONING

Samples of left ventricle from explanted hearts were harvested, snap-frozen and stored at -80°C until use. Specimens were mounted on a cryostat (Leica Microsystems) chuck using filling medium (Bio-Optica, Milan, Italy) and sliced into 350-µm-thick sections. Sections obtained were used either for the decellularization or as control for molecular analysis. Three sections were fixed in formalin for paraffin embedding and histochemical analysis.

3.4 DECELLULARIZATION OF CARDIAC TISSUE

Cardiac tissue sections were decellularized using a combination of sodium dodecyl sulfate (SDS) and Triton X-100. Specifically, sections were decellularized in a 1% SDS and 1% Triton X-100 solution (both from Sigma-

Aldrich) in distilled water, for 24 hours. Cardiac decellularized ECM (d-ECM) was then transferred in PBS containing 100 U/ml of Penicillin, 50U/ml of Streptomycin and 0.25 μg/ml Amphotericin B (all from Sigma-Aldrich) for 24h, followed by wash in distilled water for 30 minutes. All processing steps were performed under constant mild agitation and at room temperature. Cardiac d-ECM was stored at 4° C in F12K medium until use. Additionally, three sections were fixed in formalin for paraffin embedding and histochemical analysis, while all other sections were stored at -80° C until their use for molecular analysis.

3.5 CHARACTERIZATION OF d-ECM

3.5.1 QUANTITATIVE MEASUREMENT OF DNA CONTENT

To assess the complete removal of cellular nucleic component after decellularization process, genomic DNA (gDNA) was extracted from frozen native (not decellularized) and decellularized cryosections, using All Prep DNA/RNA Mini kit (Qiagen, Hilden, Germany), following manufacturer's instructions.

Briefly, tissue samples were lysed and homogenized in a highly denaturing buffer containing guanidine-isothiocyanate, which immediately inactivates DNases, to ensure isolation of intact DNA. Lysates were then passed through an AllPrep DNA spin column, which, in combination with the high-salt buffer, allows selective and efficient binding of gDNA. The columns were washed and pure, ready-to-use, then DNA was eluted.

DNA concentration was quantified by measuring the absorbance at 260 nm using Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) and the gDNA band was visualized by electrophoresis in 0,8% agarose gel and photographed under UV-light exposure with FireReader XS D-55 imaging system equipped with 1D software (UVItec Limited, Cambridge, UK). Data were averaged and expressed as mean values ± SD of ng of DNA/mg of dry tissue.

3.5.2 HISTOLOGICAL ANALYSIS

Native and decellularized sections were fixed in 10% (v/v) neutral buffered formalin for 24 h, dehydrated in graded ethanol series, cleared in xylene, infiltrated and embedded in paraffin. Sections were sliced into 6.0 μm, mounted on poly-lysine-coated glass slides, deparaffinized, rehydrated and stained for Hematoxylin and Eosin (H&E), Sirius Red, Masson's Trichrome, and paraldehyde fuchsin Gomori, using specific staining Kits (all from Bio-Optica, Milan, Italy). H&E staining was used to evaluate the effectiveness of decellularization procedure. Masson's staining was used to evaluate the architecture of d-ECM, while Sirius Red and Gomori stain kits were employed to detect collagen and elastic fibers respectively in d-ECM sections. The microscopic analysis was performed by using a light microscope Leica DM2000LED (Leica Microsystems, Wetzlar, Germany) connected to the digital camera Leica ICC50 HD (Leica Microsystems), for microscopic photodocumentation.

3.6 LYOPHILIZATION AND SOLUBILIZATION OF d-ECM

Cardiac d-ECM, frozen at -80°C, was lyophilized overnight and became a milled powder. Then the lyophilized matrix underwent to enzymatic digestion by 0.5 mg/ml Pepsin (Sigma-Aldrich) dissolved in 0.1 M hydrochloric acid (HCl). A 20:1 ratio of d-ECM:Pepsin was digested on tube rotator at 37°C. After 24 hours, when completely solubilized, as indicated by the lack of particles in solution, d-ECM was neutralized to a pH 7.4, at room temperature, through the addition of 1M sodium hydroxide (NaOH) (Sigma-Aldrich), in order to cease pepsin activity. The solution containing cardiac d-ECM was used in combination with CPCs to assemble fibrin gels.

3.7 PREPARATION OF ECM-FIBRIN HYBRID-SCAFFOLDS

3.7.1 PREPARATION OF COMMERCIAL INJECTABLE FIBRIN SEALANT

Modified and optimized, for these experiments, according manufacture's protocol, commercial injectable TISSEEL fibrin sealant (Baxter, Los Angeles, CA, USA) was prepared, to create 3D scaffolds incorporating 20 mg/ml of cardiac d-ECM, obtained as described in section 3.6. TISSEEL consists of 2 components, one containing fibrinogen, factor XIII and aprotinin, and one containing a mixture of thrombin and calcium chloride ($CaCl_2$). The two components were taken, in sterile conditions, from a double-chamber syringe, and then gently sequentially mixed with d-ECM solution in each well of 8-well

glass chamber slides (Nunc, Thermo Scientific). Five different ratios of fibrin to matrix (F:M) were tested (1:1, 2:1, 3:1, 4:1, and 1:4) to obtain a well-structured scaffold with the highest possible content of d-ECM.

3.7.1.1 CHARACTERIZATION OF COMMERCIAL INJECTABLE FIBRIN SEALANT

To evaluate the histological structure of the ECM-fibrin hybrid-scaffolds, gels were fixed in 10% neutral-buffered formalin for paraffin embedding and sliced into serial 6 μm thick sections, as previously described in section 3.5.2. Then the sections were stained using Sirius Red, Mallory's Trichrome and PAS staining kits (all from Bio-Optica), according to the manufacturer's protocol.

Sirius Red and Mallory's Trichrome stainings were used to detect the presence of collagen within the gels, while Pas stain was employed to detect glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins within the gels. Microscope observation was performed by using a light microscope Leica DM2000LED (Leica Microsystems) connected to a Leica ICC50 HD digital camera connected to the microscope (Leica Microsystems).

3.7.2 PREPARATION OF FIBRIN GEL BY SINGLE COMPONENTS: FIBRONOGEN AND THROMBIN

Additionally, in order to obtain 3-D scaffolds of fibrin, cardiac d-ECM and CPCs, gels were prepared, using as a reference the TISSEEL but modifying the

concentration of each single component. In particular 100 mg/ml bovine fibrinogen, 40 IU thrombin from bovine (both from Sigma-Aldrich), cardiac d-ECM at final concentration of 20 mg/ml and $3x10^6$ CPCs were combined. The fibrinogen was first reconstituted in a solution containing 30012 KIU/ml of bovine aprotinin (Sigma-Aldrich) and then was prewarmed in a 37°C water bath for 45-90 minutes to allow the complete dissolution. Also thrombin was first reconstituted in 40μM of calcium chloride solution ($CaCl_2$) and then was warmed at 37°C. Briefly, reconstituted fibrinogen solution was added into each well of 8-well glass chamber slides (Nunc, Thermo Scientific) and sequentially mixed with the d-ECM, previously dissolved in pepsin solution as described in section 3.6. Successively, cell suspension (CPCs) and at the end thrombin solution were added and mixed into each well (final volume: 340 μl/well). In order to favor an homogenous distribution of cells and components of fibrin within the gels, all components were gently pipetted, and then the so obtained d-ECM-CPC fibrin gels were left to polymerize in incubator at 37°C in 5% CO2 for 8 h, before carrying out further experiments. The d-ECM fibrin gels were assembled with low ratios of F:M, precisely 1:1, 1:2 and 2:1.

After gelling, F12K medium (Sigma-Aldrich) supplemented as reported above, was added to each well, and constructs with incorporated cells were maintained in culture for 72 hours. During this period, the morphology of cells was evaluated by an inverted phase contrast microscope with Nikon Eclipse Ti-E DS-Qi2 Microscope (Nikon Instruments Europe, Holland).

3.7.2.1 CHARACTERIZATION OF FIBRIN GEL BY SINGLE COMPONENTS: FIBRONOGEN AND THROMBIN

After 72 hours, gels were fixed in 10% neutral-buffered formalin for paraffin embedding and sliced into serial 6 µm thick sections, as previously described in section 3.5.2. Serial sections of gels were stained with Hematoxylin and Eosin, Masson's Trichrome, Sirius Red and PAS Morel-Maronger modified stains using specific kits (all from Bio-Optica) and following manufacturer's protocol. Hematoxylin and Eosin stain served to determine the presence and the distribution of cellular component in the gels, while Masson's Trichrome and Sirius Red stain kits were employed to detect collagen in the gels. Finally, PAS stain kit was used to detect glycogen and mucosubstances, such as glycoproteins, glycolipids and mucins in the gels. Sections stained were then observed using a light microscope Leica DM2000Led (Leica Microsystems) equipped with the digital camera ICC50 HD (Leica Microsystems).

3.8 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, Ca, USA). All data were expressed as the mean ± SD. A value of $p<0.05$ was used to identify any statistically significant differences.

4. RESULTS

4.1 CELL CULTURES

The observation by phase contrast microscope provided image of primary cell culture obtained from pathological adult human hearts as shown in Fig. 1.

4.1.1 CHARACTERIZATION OF CARDIAC PRIMITIVE CELLS

CD117-positive cells isolated from pathological adult human hearts and sorted, as described in Materials and Methods (section 3.2.1), were stained by immunofluorescence to confirm the purity of cell population, which resulted equal to 98%, as shown in Fig. 2.

Phase contrast microscope image shows sorted population of CPC-P (Fig. 3).

4.2 DECELLULARIZATION OF CARDIAC TISSUE

Thick sections of native cardiac tissue of 350 μm are shown in Fig. 4 A.

After 24 h of decellularizing treatment (Table 1) with SDS (1%) and Triton-X100 (1%), macroscopic observation of cardiac tissue sections showed the typical color change from brown to translucid-white occurring during the process, as a result of a complete and effective decellularization procedure (Fig. 4 B)

4.2.1 CHARACTERIZATION OF d-ECM

4.2.1.1 QUANTITATIVE MEASUREMENT OF DNA CONTENT

To test the effectiveness of decellularization procedure, quantitative measurement of DNA content was performed both in decellularized and native cryosections. This analysis confirmed the presence of a lower amount of DNA content in decellularized sections (d-ECM) (3.5 ± 0.71 ng/mg dry tissue) compared with native sections (116, 2 ± 1,13 ng/mg dry tissue), as shown in Fig. 5 A.

To assess the quality of DNA extracted, electrophoresis was performed on 0.8% agarose gel. No DNA band was visualized for decellularized samples, while the band for native samples was clearly visible (Fig. 5 B)

4.2.1.2 HISTOLOGICAL ANALYSIS

The histological analysis confirmed the effectiveness of the decellularization procedure, in fact Hematoxylin and Eosin staining showed no evidence of whole cell or fragments in the decellularized sections when compared to native sections, which present nuclei and cellular components. Moreover, Sirius Red and Gomori stainings documented the retention, in d-ECM, of collagen, and the presence of elastic fibers, respectively; while Masson's Trichrome staining revealed a well preserved three-dimensional architecture of d-ECM (Fig. 6).

4.3 LYOPHILIZATION AND SOLUBILIZATION OF d-ECM

d-ECM, as shown in Fig. 7, was lyophilized, milled into a powder, and finally solubilized with a pepsin digestion. The pepsin concentration (0.5 mg/ml), the temperature (37°C) and the turning on tube rotator were all the conditions suitable to digest d-ECM; in fact, after approximately 24 h, d-ECM appeared completely solubilized as indicated by the lack of ECM particles in solution. Solution of d-ECM remained a viscous liquid on ice or at room temperature.

4.4 CHARACTERIZATION OF COMMERCIAL INJECTABLE FIBRIN SEALANT

It was attempted to make scaffolds composed solely of commercial injectable fibrin sealant and cardiac d-ECM. Five different ratios of F:M (1:1, 2:1, 3:1, 4:1, and 1:4) were tested, and it was found that fibrin sealant gelled within few seconds, and pipetting was tricky, thus resulting in an uneven incorporation of d-ECM in the gel. Furthermore, it was observed that when F:M ratio was too high (3:1; 4:1), the polymerization of fibrin was too rapid and the gels became stuck inside the micropipette tips during the mixing of d-ECM. In addition, Mallory's Trichrome and Sirius Red stainings revealed that collagen distribution and orientation were not homogenous, especially when F:M ratio was too high (3:1; 4:1). Also PAS staining revealed that the presence of glycoproteins in the gels appeared not consistent and uniform (Fig. 8). With F:M ratio at 1:4, the fibrin appeared loosely compacted, resulting slightly viscous and was fragmented after

detachment from each well and hence not properly gelling. With this ratio, it is not possible to visualize a proper distribution of the matrix, as shown in Fig. 8.

4.5 CHARACTERIZATION OF FIBRIN GEL BY SINGLE COMPONENTS: FIBRINOGEN AND THROMBIN

4.5.1 QUALITATIVE ANALYSIS

To prevent fast gelling, gels were prepared starting from single components of fibrin. In particular, three F:M ratios (1:1, 1:2 and 2:1) were tested to select those that met the incorporation into fibrin gels of d-ECM and CPCs.

Using F:M ratio of 1:2, the higher quantity of d-ECM inhibited a proper polymerization of fibrin, consequently fibrin gels were difficult to manipulate, very small, and they were fragmented after detachment from each well.

Whereas, with F:M at ratio of 2:1, d-ECM was not packed among fibrin's components. The higher quantity of fibrin, in fact, inhibited the homogenous incorporation of d-ECM in the gel: the gels were easier to handle, densely packed on the bottom of the well, with a small quantity of aqueous solution on the surface, and they resulted not fragmented after detachment from each well. Gelling time, in both ratios, varied from one to twenty hours.

With F:M at ratio of 1:1, gels gelled after 8 hours, they were easier to handle and densely packed. In addition, macroscopic observation of gels after 8h showed the typical uniform white color, as a result of a perfect gelling. They resulted not fragmented after incubation, and they were easily to manipulate.

4.5.2 HISTOLOGICAL ANALYSIS

Histochemical stainings revealed the presence of CPCs in the gels whose architecture varied from densely packed (F:M 2:1), very loose (F:M 1:2), to proper packed (F:M 1:1).

Related to the ratio of fibrin to matrix, homogeneous distribution of cells is showed by H&E only in the gels with F:M ratio of 1:1. With F:M ratios of 2:1 and 1:2, the cell distribution is visible around the perimeter of each gel as shown in Fig. 9.

Masson's trichrome staining indicated that collagen fibers were present in the gels with F:M ratio of 1:1 and they appear blue when compared to the ratios F:M ratios of 2:1 and 1:2, where d-ECM were not very visualized (Fig. 9)

PAS and Sirius Red stainings have shown the presence of glyproteins and collagen, respectively, in the gels with F:M ratio of 1:1, when compared to the ratio F:M ratios of 2:1 and 1:2 (Fig. 9)

Phase contrast microscope pictures showed CPCs embedded in fibrin gels at different F:M ratios (1:1, 1:2, and 2:1) (Fig. 10).

5. DISCUSSION AND CONCLUSIONS

Cardiovascular diseases are the leading cause of death worldwide and one of their most important clinical manifestations is represented by MI (4), which results in the irreversible loss of cardiomyocytes accompanied by scar formation, progressive alteration in the geometry of heart, ventricular remodeling and a pathologically modified extracellular matrix (82). Currently, the only successful treatment for end-stage heart failure post-MI is the total heart transplantation. Nonetheless, transplantation is highly limited by heart donor availability and host immunological response against the donated organ (82).

As a result, there is great interest in alternative therapeutic strategies to reverse this common and deadly disease. One of these strategies accounts the concept of increasing the number of contractile cells through the transplantation of viable cells, in the infarcted zone, that function as normal cardiac cells and integrate into the host tissue so that heart function can be restored.

In these circumstances, stem cell-based therapeutic option, generating new cardiomyocytes, has emerged as a promising treatment for both myocardial infarction and heart failure. Several cell types that might replace necrotic tissue and minimize scarring have been considered; among multiple stem cells tested, cardiac primitive cells (CPCs) have the most promising therapeutic potential (246). They can be isolated from normal and pathological human hearts, and as stem cells, CPCs are self-renewing, clonogenic, multipotent, and able to differentiate into the three cardiovascular lineages of the heart: endothelial cells,

vascular smooth muscle cells and cardiomyocytes (86, 120-124), arousing an increased interest about myocardium regenerative processes.

Despite encouraging results in stem cell-based therapy, the lack of an appropriate extracellular environment for cellular adhesion has limited cell retention, survival, and integration into the host tissue within the damaged infarct region (247-249).

All the cells, in fact, exist *in vivo* in a specialized environment in which their survival and function is assured, while their biological activity is controlled. This environment, mainly formed by the extracellular matrix (ECM) and supporting survival, proliferation, and differentiation of resident cell populations, provides the perfect, unique environment for every organ cell (250-251). Moreover, this environment has mechanical properties that can play a critical role in directing cell response (252-253).

Since, during a cardiac disease, including myocardial ischemia, qualitative and quantitative alterations in ECM proteins are ensured (254), it seems reasonable that the role of extracellular matrix and the effects of the modifications of its composition ongoing in pathological conditions could be associated with poor cell engraftment, survival, differentiation and retention in the infarct area. Hence, considering the influence of the microenvironment on cells, it is essential to restore the extracellular compartment along with the cellular compartment.

More recently, biomaterials with their own intrinsic biological activity, have offered a plausible solution to the drawbacks encountered previously. They are

desirable as delivery vehicles to help improve cell retention and to contribute to replace lost tissue and re-establish damaged connections after MI. Both synthetic and natural biomaterials have been investigated for use in cardiac tissue engineering applications. Many of these biomaterials have been used to form implantable scaffolds incorporating cells and mimicking an ECM-like structure (222). However, immune rejection, degradation, and mechanical integration problems have limited the success of many biomaterials in cardiac repair (222, 255), moreover the implantation of these scaffolds involves an invasive procedure.

Based on these premises, this work aimed to construct an injectable scaffold as potential cell deliver directly into the infarcted area replacing the damaged myocardial ECM, and, at the same time, offering a minimally invasive delivery approach.

Currently, d-ECM in cardiac regenerative medicine offered a neat solution to synthetic material issues. It enhances cellular functions, such as survival, maturation, differentiation and migration (256), in addition it possesses the composition and architecture required for reconstructing the heart, and its degradation products have been shown to be chemo-attractive (235, 257).

Thus, solubilizing d-ECM and reforming it into a hydrogel could be a promising method to develop an injectable material (258) capable of restoring the integrity of the myocardium. Unfortunately, ECM hydrogels have a limited range of mechanical properties that do not match the native myocardium, consequently, pure ECM hydrogels cannot be used for 3-D cell encapsulation (259-261).

To solve this problem, the final aim of this study was to develop a natural injectable self-assembling scaffold able to serve as both three-dimensional platform and cell delivery method, by combining two substrates:

- ECM from *ex vivo* decellularized heart tissue, which mimics the complex properties of the cardiac microenvironment;
- fibrin, which possesses tunable mechanical properties and elasticity of the native myocardium, that distinguish it from all other biomaterials (262, 263). Moreover, fibrin can be modified to incorporate biologically active peptides which facilitate cell adhesion, improve cell-cell interaction, support cell migration and differentiation inside the gel (161).

Based on these considerations, the first step of the work was to decellularize left ventricle of pathological human heart in order to obtain a cardiac ECM that could be tuned to mimic the native and ideal cardiac environment for CPCs.

A major limitation of decellularized materials is their immunogenicity and the risk of diseases transfer when derived from xenogeneic or allogeneic sources. Conversely, using the patient's own tissue to produce a tissue engineered scaffold could avoid or reduce similar endogenous consequences (264).

The ultimate goal of the tissue engineering is to design and fabricate natural-like functional human tissues suitable for regeneration, repair and replacement of damaged, injured or lost human organs (265-268). An ideal human engineered tissue must eliminate, dramatically reduce, or more realistically reinvent the

problem of biocompatibility (225-228) which is a critically important issue for any biomaterial-based approach.

The cardiac ECM comprises complex and specific mixture of proteins, and this composition changes throughout heart development and maturation (269). The ECM was, hence, obtained decellularizing pathological human ventricle samples by a previously developed and tested procedure (270). The protocol allowed to produce a d-ECM perfectly suited for the scope, as it was acellular, with a weak residual content of DNA that made it almost completely non immunogenic, limiting the eventual inflammatory response of the host (271). Furthermore the architecture was preserved and an optimal retention of structural ECM proteins occurred. Supporting the fact, H&E images has revealed empty spaces in diameter where cells may have been located prior to the decellularization procedure. Finally, d-ECM obtained showed a good preservation of collagen and elastic fibers, which are well known as critical elements for the structural integrity and biomechanical profile of tissue engineered constructs (272): the collagen network is a strong 3D structure, probably required for adequate cardiac functioning (33) while the elastic fibers contribute significantly to myocardial mechanics (40).

The second step was attempted to create hybrid-scaffolds composed solely of commercial injectable fibrin sealant incorporating previously solubilized cardiac d-ECM. Fibrin sealant was widely used as a helper tool in many surgical fields and consists primarily of two components: fibrinogen and thrombin (273). Changes in ratio of fibrinogen concentration and thrombin activity can generate

gels with different packing density that may influence proliferation, density and viability of the cells into the fibrin (273-276). Moreover, fibrin is also commonly served as a delivery vehicle in injectable cardiac tissue engineering (277). Christman and Lee (277) described an improved cell survival upon the transplantation of cells delivered by fibrin glue compared to the injection of cells alone. The fibrin glue was also shown to induce neovascularization within the ischemic myocardium and reduce infarct expansion (278).

It was demonstrated that even if the packing density of fibrin gels primarily depends on the fibrinogen concentration and thrombin activity (273-276), the ratio of F:M can significantly influence the packing density of the gels as well. In fact, results demonstrated that increasing fibrin content significantly increases the packing density, thus resulting in an uneven incorporation of d-ECM in the gels, especially using the two highest F:M ratio (3:1 and 4:1); while increasing the d-ECM content (F:M 1:4) produced loosely packed gels. Moreover, despite such difference, it was observed that fibrin sealant remained liquid for a few seconds, the reason why probably d-ECM incorporation was not homogenous in the gels and pipetting was tricky. Based on the results obtained, gel formulations with highest fibrin (F:M 3:1 and 4:1) and d-ECM content (F:M 1:4) were discarded.

Since the fibrin gels can be also created by mixing single solutions of fibrinogen and thrombin, to prevent fast gelling that inhibited a homogeneous incorporation of d-ECM, subsequent hybrid scaffolds were prepared reconstituting the single components of fibrin: bovine fibrinogen and thrombin.

For the construction of the d-ECM-CPCs incorporating gels, the above mentioned components were prepared and mixed using as a reference the TISSEEL but modifying the concentration of each single components. Based on the results of qualitative and histological analysis of hybrid-scaffolds obtained by mixing d-ECM and TISSEEL and considering the issues linked to the constructs with highest ratios of F:M or M:F that exhibited an abnormal polymerization, low ratios ranging from 1:2 to 2:1 F:M were chosen.

The choice of F:M ratio to create hybrid-scaffolds is crucial, as it can influence the packing density and gelling time. It was observed, in fact, that the density of the gels increased with increasing fibrin content and decreased with increasing d-ECM content.

Gels with F:M 1:1 ratio were easier to handle, densely packed with a gelling time of 8 hours, and these properties could be necessary to ensure consistent formation of the gel capable of incorporating accurately both d-ECM and CPCs, *in vitro*, and to treat myocardial infarction *in vivo*.

The constructs F:M 1:1 showed that the incorporated cells had a normal morphology and were uniformly distributed throughout the entire thickness of the gels. This cell distribution is probably due to the well-structured gels, with a desirable homogenization among fibrin and matrix that allow cells to migrate inside.

Conversely, the cells incorporated into the gels with F:M ratios of 2:1 and 1:2 were very often confined to approximately half of the construct. This may probably have been due to a high fibrin content that could have inhibited the cell

encapsulation (F:M 2:1) or to the high d-ECM content (F:M 1:2) that caused a retention of cells in the aqueous surface of the gels.

As the two main components of fibrin, fibrinogen and thrombin, can be isolated from human blood, a fibrin gel was used to create scaffolds. In the future, patient-specific scaffolds may be created from the blood of patients in order to reduce potential immunogenic response.

In conclusion, injectable fibrin gels could address the limitation of surgical implantation of a robustly engineered cardiac patch and replace the damaged myocardial ECM and/or deliver cells directly to the infarcted region. Thus, in the future, the use of an in situ injectable scaffold might offer a valuable therapeutic option with the advantages of a minimally invasive delivery method and an attracting alternate to bio-constructs.

6. ICONOGRAPHY

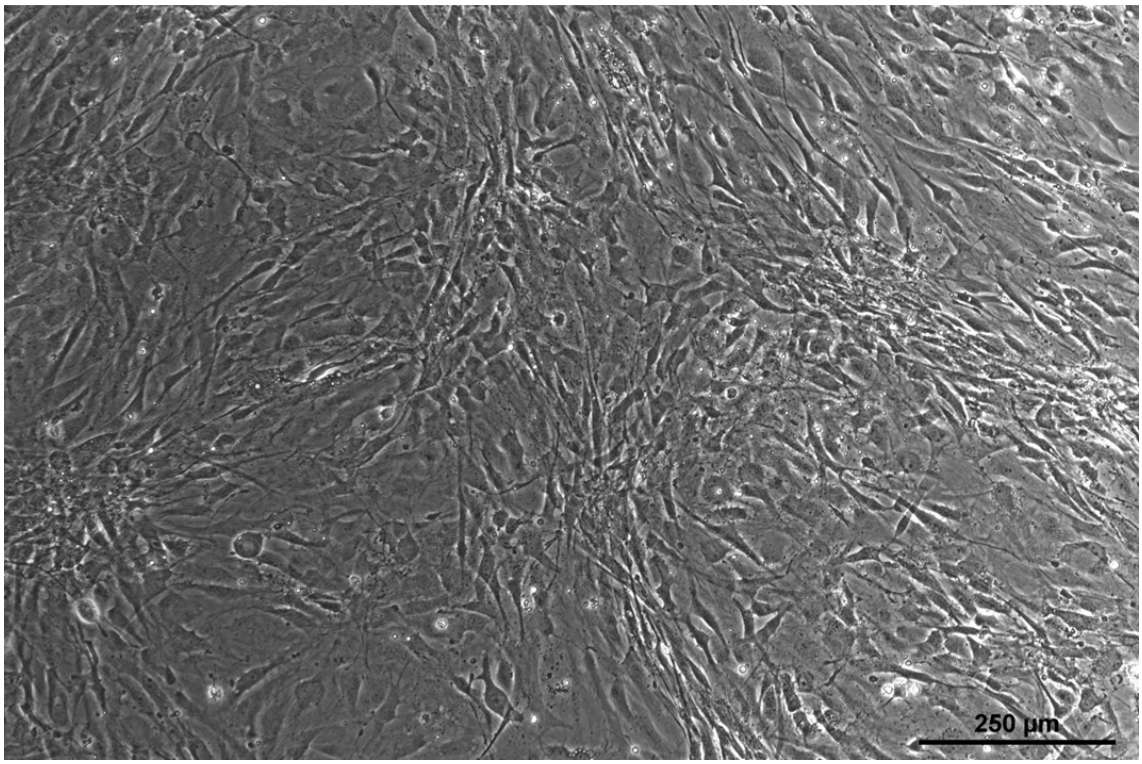


Fig.1

Phase contrast microscope image of primary cell culture obtained from pathological adult human hearts.

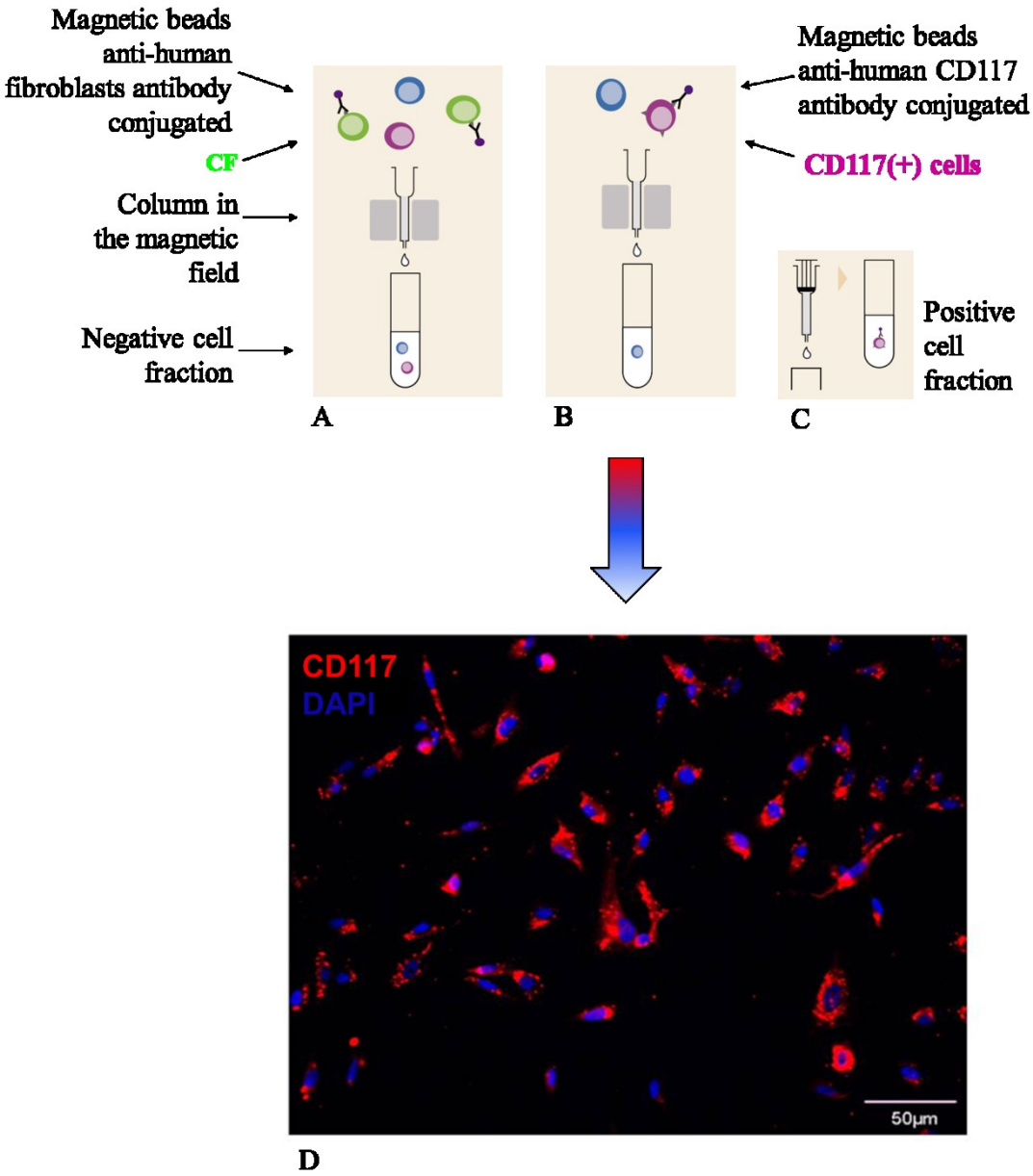


Fig. 2

Immunomagnetic cell sorting: preventive cardiac fibroblasts depletion from primary culture **(A)** and CD117(+) cardiac primitive cells isolation **(B-C)**. Fluorescence microscope image of CD117(+) cardiac primitive cells **(D)**.

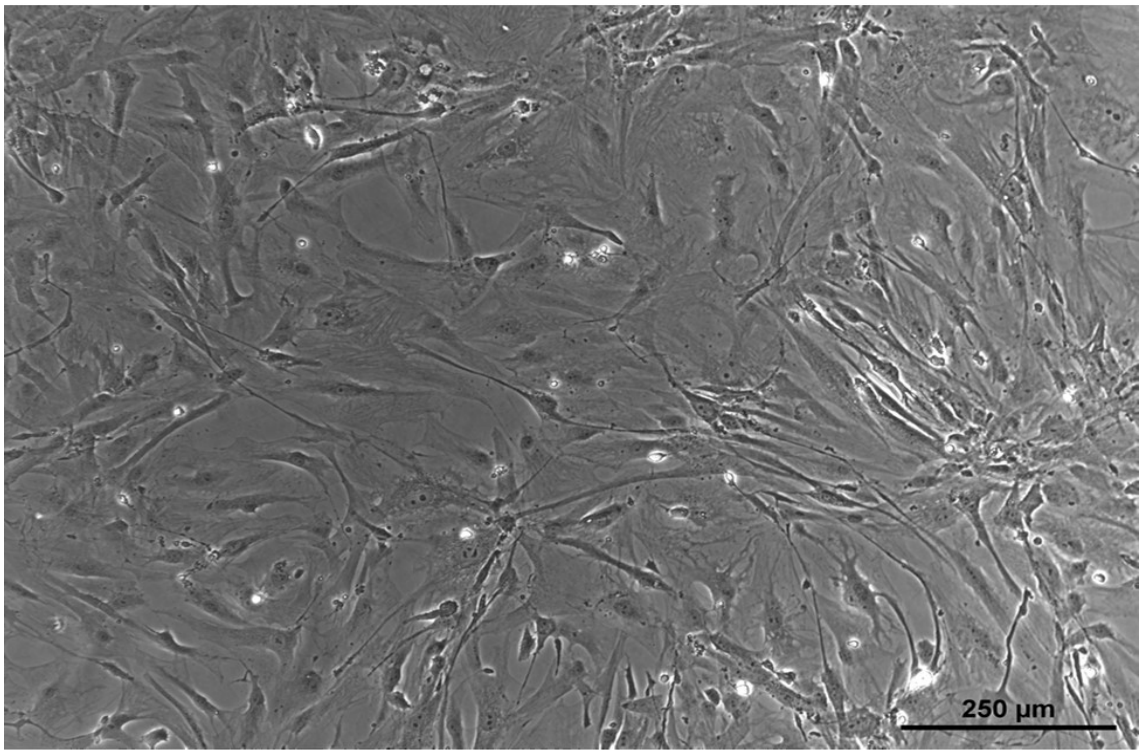
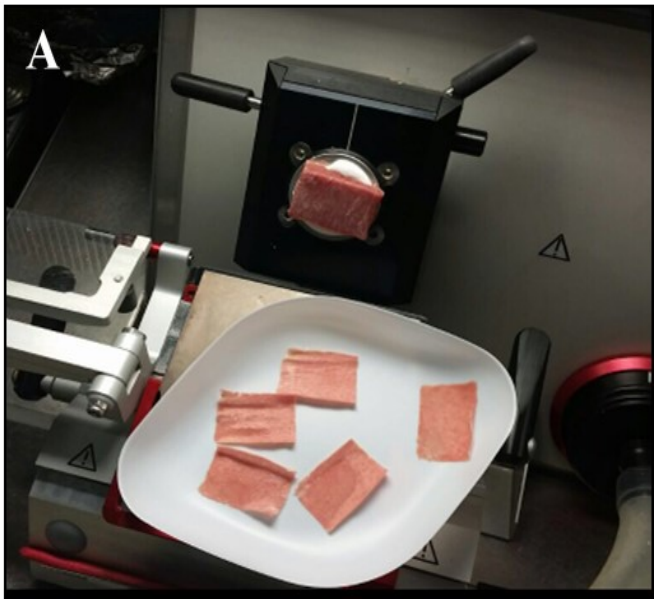


Fig. 3

Phase contrast microscope image of CPC-P in culture.

DAY 1			DAY 2			DAY 3		
T	TIME	BUFFER/ REAGENT	T	TIME	BUFFER/ REAGENT	T	TIME	BUFFER/ REAGENT
RT	24 hrs	1% SDS, 1% Triton X-100 in distilled water	RT	24hrs	0.25 µg/ml Amphotericin B, 100U/ml Penicillin, 50U/ml Streptomycin in 1xPBS	RT	30 min	Distilled water

Table 1. Overview of the decellularization protocol tested.



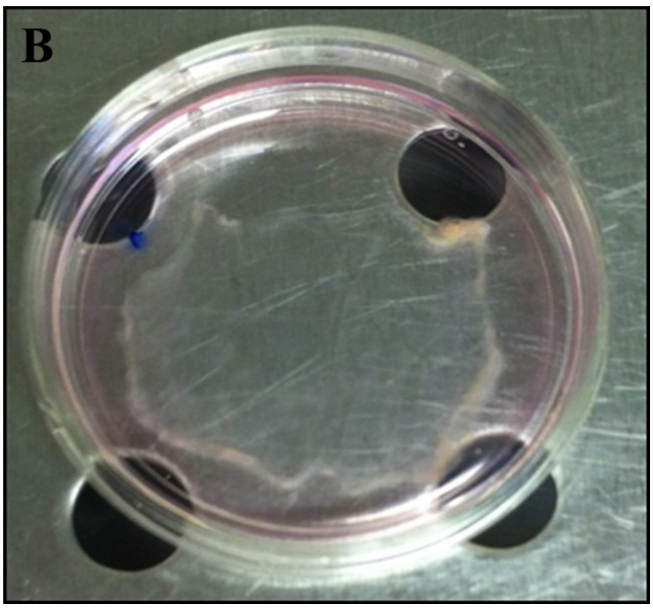


Fig. 4

Representative macroscopic images of cardiac tissue sectioned by microtome before **(A)** and after decellularization procedure **(B).**

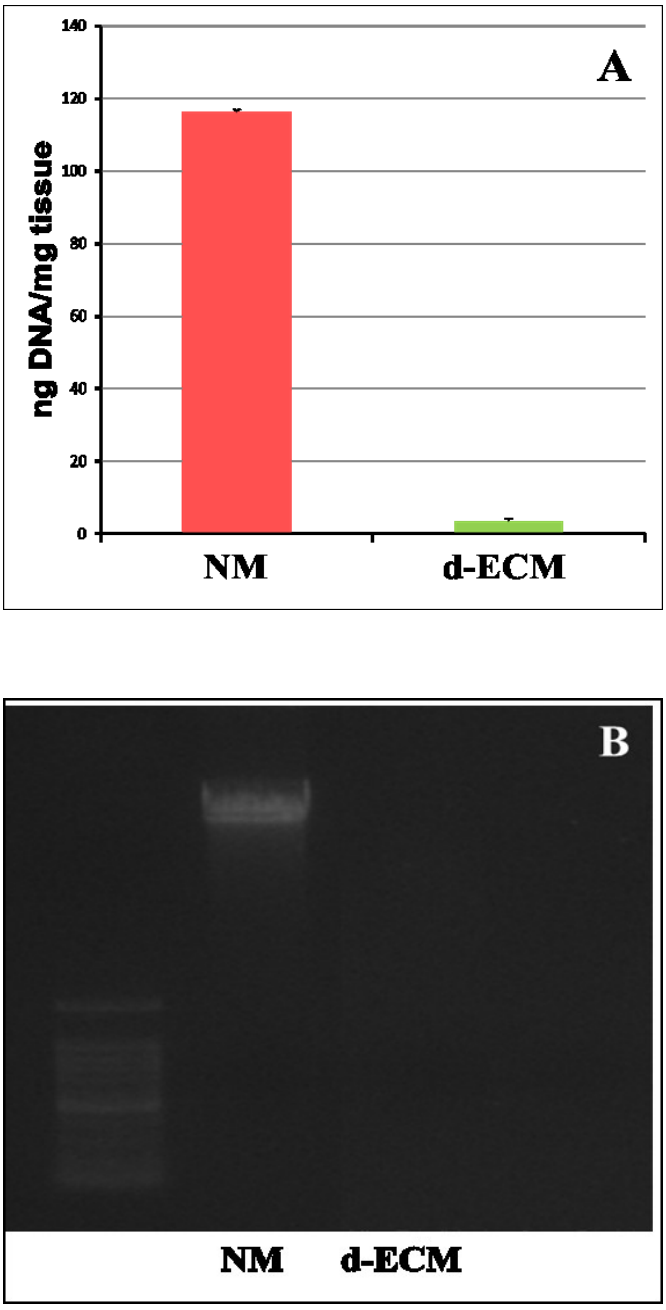


Fig. 5

Quantification **(A)** and qualitative analysis **(B)** of DNA content in sections of native (NM) and decellularized cardiac tissue (d-ECM).

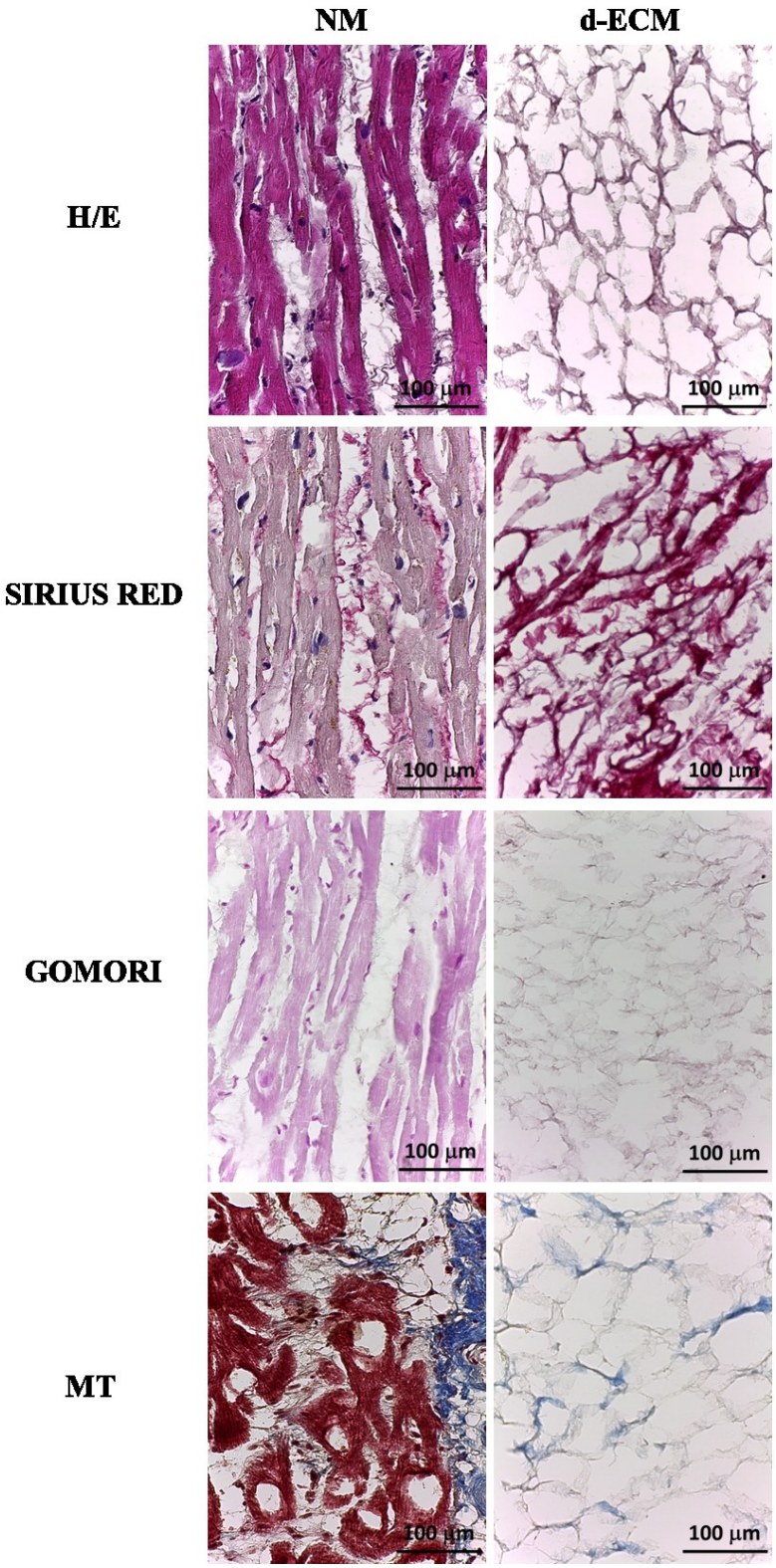


Fig. 6

Representative images of Hematoxylin and Eosin (H/E), Sirius Red, Gomori and Masson's Trichrome (MT) stainings on 350 um-thick sections of native (NM) and decellularized cardiac tissue (d-ECM).

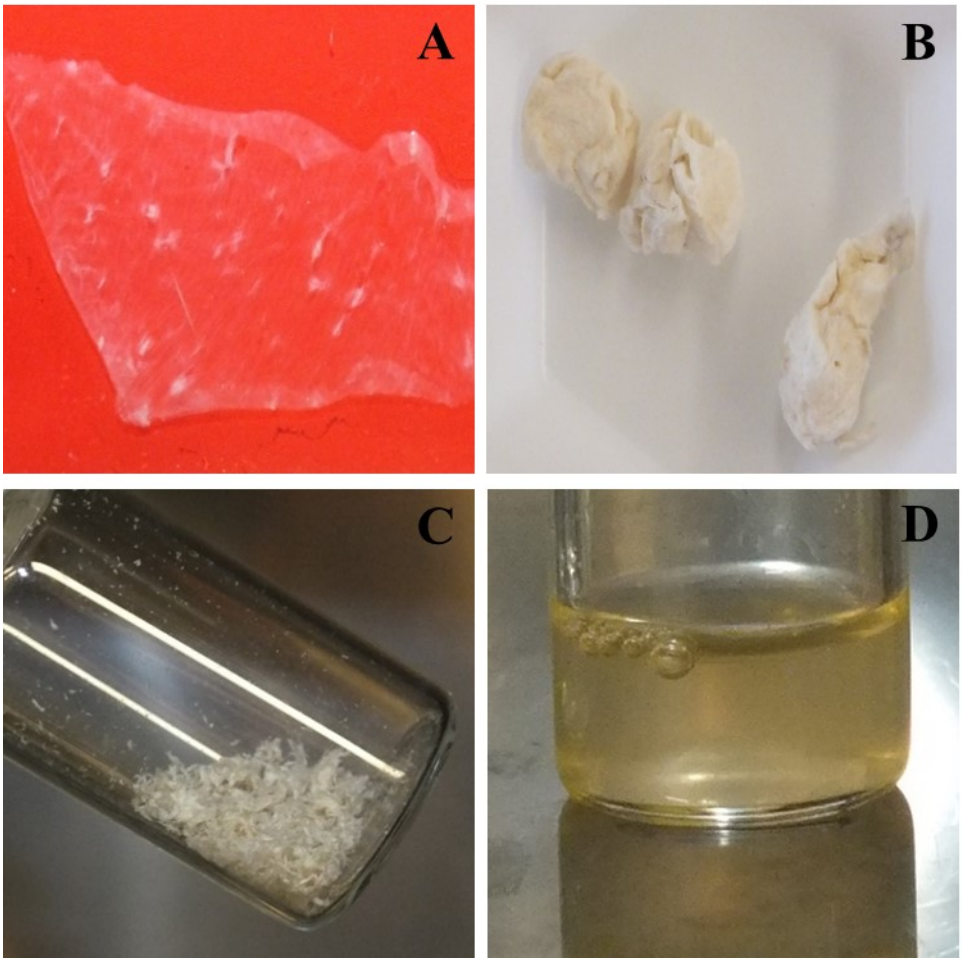


Fig. 7

Representative images of decellularized **(A)**, lyophilized **(B)**, milled into a powder **(C)**, and solubilized **(D)** myocardium.

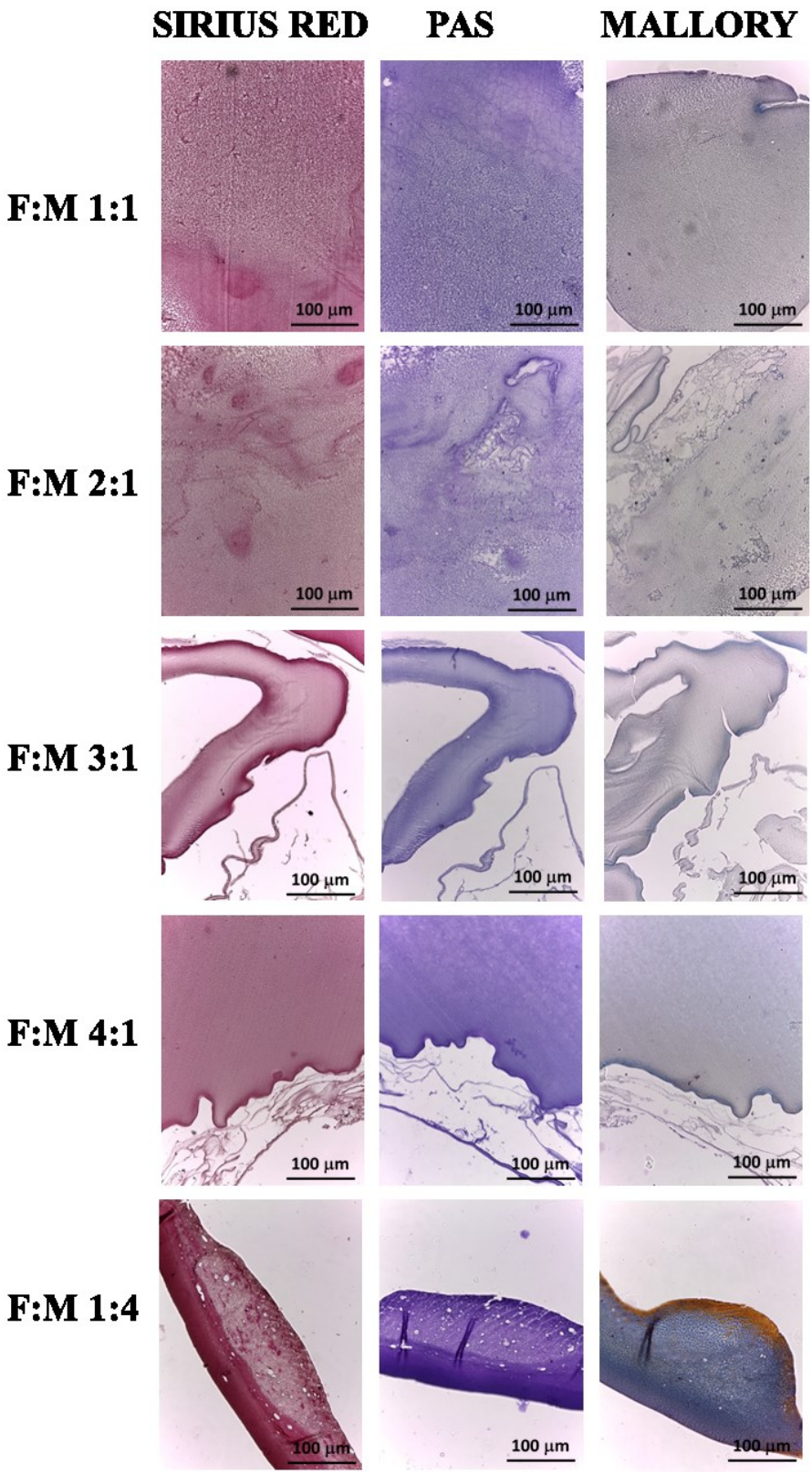


Fig. 8

Representative images of Sirius Red, PAS and Mallory stainings of commercial fibrin gels at different F:M ratios (1:1, 2:1, 3:1, 4:1 and 1:4).

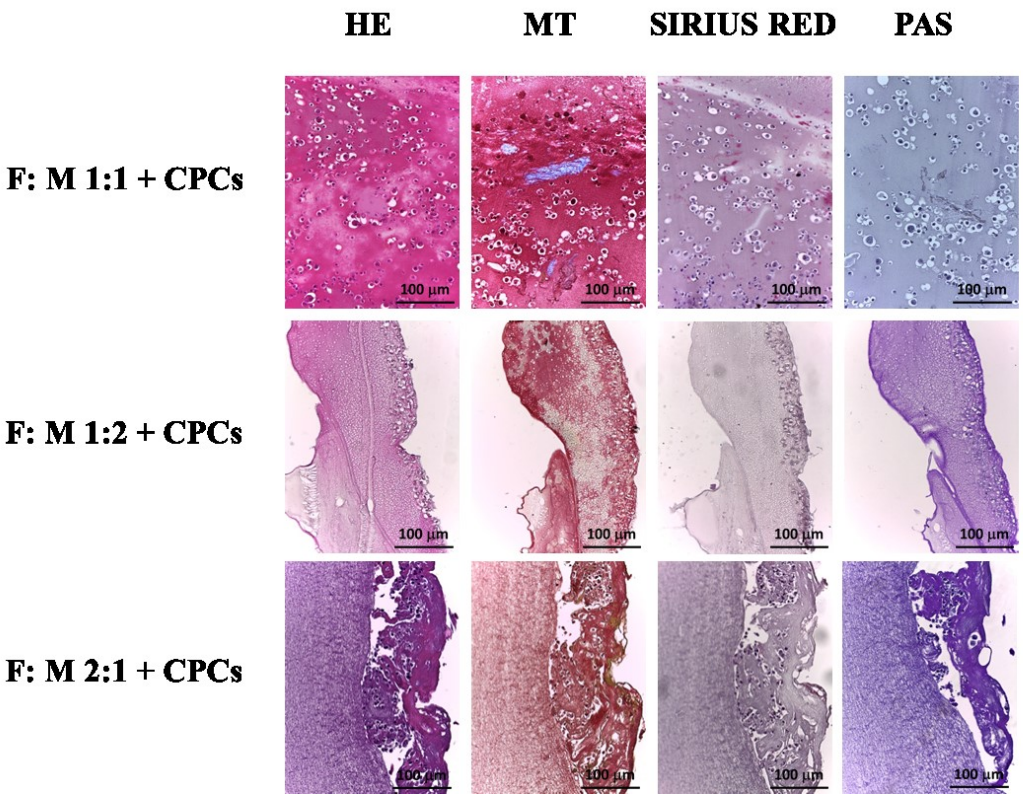


Fig. 9

Representative images of Hematoxylin and Eosin (HE), MT (Masson's Trichrome, Sirius Red and PAS stainings of fibrin gels by single components: fibrinogen and thrombin at different F:M ratios (1:1, 1:2, and 2:1).

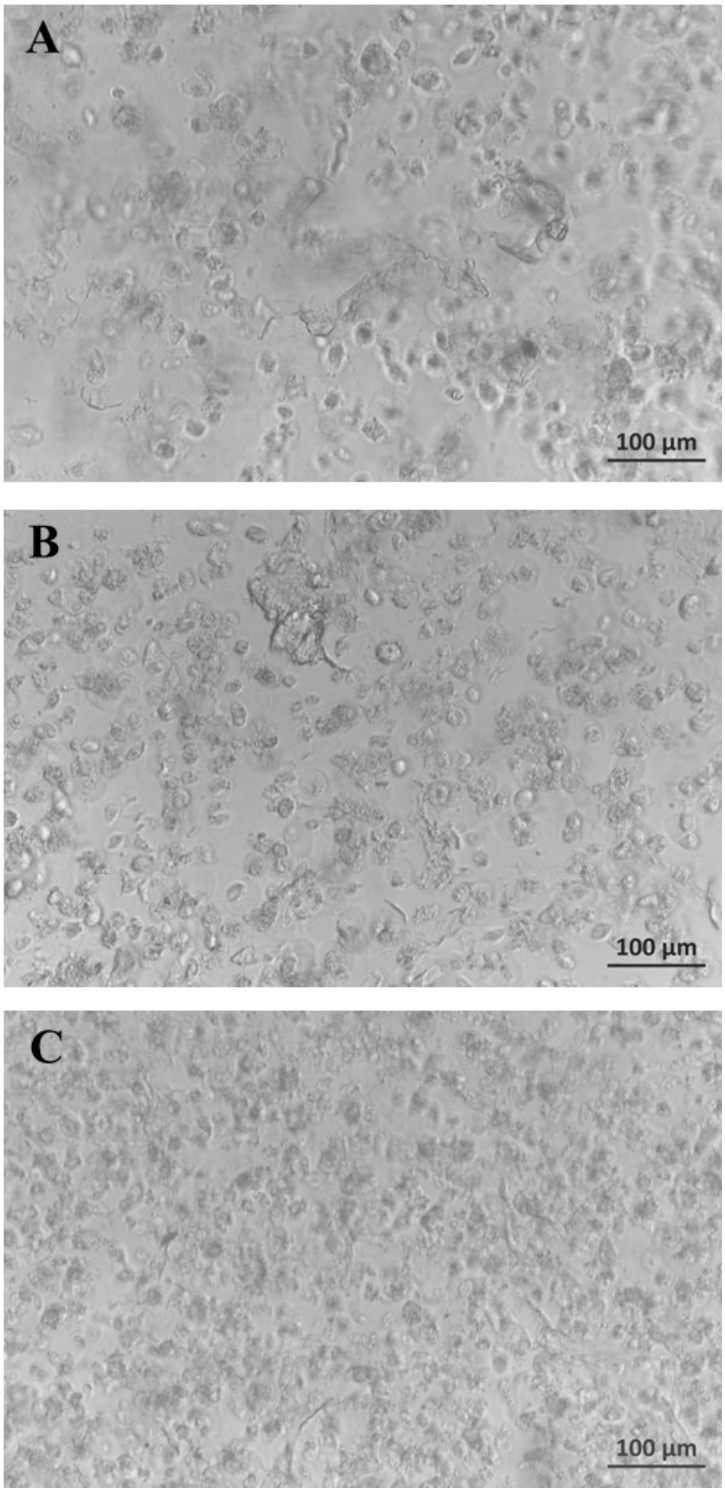


Fig. 10

Phase Contrast microscope pictures illustrating CPC embedded in fibrin gels at different F:M ratios. **A:** 2:1, **B:** 1:2, **C:** 1:1.

7. REFERENCES

1. Mackay J, Mensah G. *The Atlas of Heart Disease and Stroke* - World Health Organization in collaboration with the Centers for disease control and prevention; 2004.

2. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler III ER, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS ,Wong ND, Woo D, Turner MB. *Executive Summary: Heart Disease and Stroke Statistics—2014 Update A Report From the American Heart Association.* Circulation; 129:399-410; 2014.

3. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. *Heart Disease and Stroke Statistics–2012 Update: A Report from the American Heart Association*. Circulation; 125: 2-220; 2012.

4. Ruvinov E, Sapir Y, Cohen S. *Cardiac Tissue Engineering: Principles, Materials and Application.;* 2012.

5. Cohen S, Leor J. *Rebuilding broken hearts. Biologists and engineers working together in the fledgling field of tissue engineering are within reach of one of their greatest goals: constructing a living human heart patch.* Sci. Am.; 291(5):44-51; 2004.

6. Holmes JW, Borg TK, Covell JW. *Structure and mechanics of healing myocardial infarcts.* Annu. Rev. Biomed. Eng.; 7:223-53; 2005.

7. Sun Y, Weber KT. *Infarct scar: a dynamic tissue.* Cardiovasc. Res.; 46(2):250-6; 2000.

8. Olivetti G, Capasso JM, Meggs LG, Sonnenblick EH, Anversa P. *Cellular basis of chronic ventricular remodeling after myocardial infarction in rats*. Circ Res.; 68:856–69; 1991.

9. Spinale FG. *Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function.* Physiological reviews*;* 87:1285–342; 2007.

10. Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. *The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction.* Journal of Molecular and Cellular Cardiology; 48:504–11; 2010.

11. Pfeffer MA, Braunwald E. *Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications*. Circulation; 81(4):1161-72; 1990.

12. Zamilpa R, Lindsey ML. *Extracellular matrix turnover and signaling during cardiac remodeling following MI: causes and consequences.* J. Mol. Cell. Cardiol.; 48(3):558-63; 2010.

13. St. John Sutton MG, Sharpe N. *Left ventricular remodeling after myocardial infarction: pathophysiology and therapy*. Circulation; 101(25):2981-8; 2000.

14. Brower GL, Gardner JD, Forman MF, Murray DB, Voloshenyuk T, Levick SP, Janicki JS, Eur. J. *The relationship between myocardial extracellular matrix remodeling and ventricular function*. Eur. J. Cardiothorac. Surg.; 30(4):604-10; 2006.

15. Forrester JS, White AJ, Matsushita S, Chakravarty T, Makkar RR. *New paradigms of myocardial regeneration post-infarction: tissue preservation, cell environment, and pluripotent cell sources.* J. Am. Coll. Cardiol. Cardiovasc. Interv.; 2:1-8; 2009.

16. Leora J, Amsalema Y, Cohenb S. *Cells, scaffolds, and molecules for myocardial tissue engineering*. Pharmacology & Therapeutics; 105(2):151–163; 2005.

17. Taylor DO, Edwards LB, Aurora P, Christie JD, Dobbles F, Kirk R, Rahmel AO, Kucheryavaya AY, Hertz MI. *Registry of the International Society for Heart and Lung Transplantation: twenty-fifth official adult heart transplant report-2008.* J. Heart Lung Transplant; 27(9):943-944; 2008

18. Augoustides JG, Riha H. *Recent progress in heart failure treatment and heart transplantation.* J Cardiothorac Vasc Anesth; 23(5):738-48; 2009.

19. Mann BK, West JL. *Tissue engineering in the cardiovascular system: progress toward a tissue engineered heart*. Anat Rec; 263:367–371; 2001.

20. Baudino TA, Carver W, Giles W, Borg T. *Cardiac fibroblasts; friends or foe?*. Am J Physiol Heart Circ Physiol; 291:1015-1026; 2006.

21. Bowers SL, Baudino TA. *Cardiac myocyte-fibroblast interactions and the coronary vasculature.* J Cardiovasc Transl Res; 5:783-793; 2012

22. Camelliti P, Borg TK, Kohl P. *Structural and functional characterization of cardiac fibroblasts.* Cardiovasc Res; 65:40-51; 2005

23. Curtis MW, Russell B. *Micromechanical regulation in cardiac myocytes and fibroblasts: implications for tissue remodeling*. Pflugers Arch; 462:105-117; 2011.

24. Souders CA, Bowers SL, Baudino TA. *Cardiac fibroblast: the renaissance cell.* Circ Res; 105:1164-1176; 2009.

25. Farrell MJ, Kirby ML. *Cell biology of cardiac development*. Int Rev Cytol.; 202:99–158; 2001.

26. Hornberger LK, Singhroy S, Cavalle-Garrido T, Tsang W, Keeley F, Rabinovitch M. *Synthesis of extracellular matrix and adhesion through b1 integrins are critical for fetal ventricular myocyte proliferation*. Circ Res.; 87:508 –515; 2000.

27. Markwald RR. *The role of extracellular matrix in cardiogenesis*. Tex Rep Biol Med.; 39:249 –251; 1979.

28. Payne RM, Johnson MC, Grant JW, Strauss AW. *Toward a molecular understanding of congenital heart disease*. Circulation.; 91:494–504; 1995.

29. Corda S, Samuel JL, Rappaport L. *Extracellular matrix and growth factors during heart growth.* Heart Fail Rev; 5:119-130; 2000.

30. Layland KS. *Non-invasive multiphoton imaging of extracellular matrix structures.* J Biophotonics; 1(6):451-62; 2008.

31. Kadler KE, Baldock C, Bella J, Boot-Handford RP. *Collagens at a glance.* J Cell Sci.; 120:1955-1958; 2007.

32. Brodsky B, Persikov AV. *Molecular structure of the collagen triple helix.* Adv Protein Chem.; 70:301-339; 2005

33. Weber KT. *Cardiac interstitium in health and disease: the fibrillar collagen network*. J Am Coll Cardiol Jun; 13(7):1637–52; 1989.

34. Hein S, Schaper J. *The extracellular matrix in normal and diseased myocardium.* J. Nucl. Cardiol; 8:188–196; 2001.

35. Jane-Lise S, Corda S, Chassagne C, Rappaport L. *The extracellular matrix and the cytoskeleton in heart hypertrophy and failure.* Heart Fail. Rev.; 5:239–250; 2000.

36. Marijianowski MM, Teeling P, Mann J, Becker AE. *Dilated cardiomyopathy is associated with an increase in the type I/type III collagen ratio: a quantitative assessment.* J. Am. Coll. Cardiol.; 25:1263–1272; 1995.

37. Graham HK, Horn M, Trafford AW. *Extracellular matrix profile in the progression to heart failure.* Acta Physiol.; 194:3–21; 2008.

38. Ayad S, Boot-Handford RP, Humphries MJ, Kadler KE, Shuttleworth CA. *''The Extracellular Matrix''.* Second edition, Academic Press, San Diego; 1998.

39. Yip CYY, Chen JH, Zhao R, Simmons CA. *Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix.* Arteriosclerosis, Thrombosis, and Vascular Biology.; 29(6):936–42; 2009.

40. Fomovsky GM, Thomopoulos S, Holmes JW. *Contribution of Extracellular Matrix to the Mechanical Properties of the Heart.* J Mol Cell Cardiol.; 48(3): 490–496; 2010.

41. Borg TK. *Development of the connective tissue network in the neonatal hamster heart.* Anat. Rec; 165:435-444; 1982.

42. Borg TK, Terracio L. *Interaction of the extracellular matrix with cardiac myocytes during development and disease.* In Issue in Biomedicine (Robinson T, ed.) Karger Basel, pp.113-129; 1990.

43. Durbeej M. *Laminins.* Cell Tissue Res.; 339:259-268; 2010.

44. De Arcangelis A, Neuville P, Boukamel R, Lefebvre O, Kedinger M, Simon-Assmann P. *Inhibition of laminin alpha 1-chain expression leads to alteration of basement membrane assembly and cell differentiation.* J Cell Biol.; 133:417-430; 1996

45. Jones KJ, Morgan G, Johnston H, Tobiasd V**,** Ouvriere RA**,** Wilkinsonf I**,** Northa KN. *The expanding phenotype of laminin α2 chain (merosin) abnormalities: Case series and review.* J Med Genet.; 38:649- 657; 2001.

46. Castaldo C, Di Meglio F, Nurzynska D, Romano G, Maiello C, Bancone C, Műller P, Bőhm M, Cotrufo M, Montagnani S. *CD117-positive cells in adult human heart are localized in the subepicardium, and their activation is associated with laminin-1 and alpha6 integrin expression.* Stem Cells.; 26:1723-1731; 2008.

47. Ju H, Dixon IM. *Extracellular matrix and cardiovascular diseases.* Can J Cardiol.; 12(12):1259-67; 1996.

48. Jaspreet K, Shouvik B, Sharma RI. *The extracellular matrix: structure, composition, age-related differences, tools for analysis and applications for tissue engineering.* J Tissue Eng.; 5:1-17; 2014.

49. Goldsmith EC, Bradshaw AD, Zile MR., Spinale FG. *Myocardial fibroblast-matrix 98 interaction and potential therapeutic targets.* J Mol Cell Cardiol.; 70:92-99; 2014.

50. Badylak S, Gilbert T, Myers-Irvin J. "*The extracellular matrix as biological scaffold for tissue engineering"*. Tissue Engineering, 1st edition, C. van Blitterswijk, Ed., Academic Press; p.121; 2008.

51. Azeloglu EU, Albro MB, Thimmappa VA, Ateshian GA, Costa KD. *Heterogeneous transmural proteoglycan distribution provides a mechanism for regulating residual stresses in the aorta.* Am J Physiol Heart Circ Physiol Mar; 294(3):1197–205; 2008.

52. Toole BP. *Hyaluronan: from extracellular glue to pericellular cue.* Nat Rev Cancer.; 4(7):528–539; 2004.

53. Toole BP. *Hyaluronan in morphogenesis.* Semin Cell Dev Biol.; 12(2):79–87; 2001

54. Lockhart M, Wirrig E, Phelps A, Wessels A. *Extracellular Matrix and Heart Development.* Birth Defects Res A Clin Mol Teratol.; 91(6): 535–550; 2011.

55. Kjaer M. *Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading.* Physiol Rev.; 225:631-637; 2010.

56. Sarasa-Renedo A, Chiquet M. *Mechanical signals regulating extracellular matrix gene expression in fibroblasts.* Scand J Med Sci Sports; 15: 223-230; 2005.

57. Ross RS, Borg TK. *Integrins and the Myocardium*. *Circ Res.*; 88:1112-1119; 2001.

58. Israeli-Rosenberg S, Manso AM, Okada H, Ross RS. *Integrins and Integrin-Associated Proteins in the Cardiac Myocyte*. Circ Res.; 114(3):572-586; 2014.

59. Berk BC, Fujiwara K, Lehoux S. *ECM remodeling in hypertensive heart disease.* J Clin Invest.; 117(3):568-75; 2007.

60. Kim HE, Dalal SS, Young E, Legato MJ, Weisfeldt ML, D'Armiento J. *Disruption of the myocardial extracellular matrix leads to cardiac dysfunction*. J. Clin. Invest.; 106:857–866; 2000.

61. Senzaki H, Paolocci N, Gluzband YA, Lindsey ML, Janicki JS, Crow MT, Kass DA. *b-Blockade Prevents Sustained Metalloproteinase Activation and Diastolic Stiffening Induced by Angiotensin II Combined With Evolving Cardiac Dysfunction.* Circ Res.; 86:807-815; 2000.

62. Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. *The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction.* J Mol Cell Cardiol.; 48(3):504-11; 2010.

63. Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA. *Controversies in ventricular remodelling.* Lancet; 367:356–67; 2006.

64. Frangogiannis NG. T*he immune system and cardiac repair.* Pharmacol Res.; 58:88–111; 2008.

65. Siwik DA, Chang DL, Colucci WS. *Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro.* Circ Res.; 86:1259–65; 2000.

66. Bujak M, Dobaczewski M, Chatila K, Mendoza LH, Li N, Reddy A, Frangogiannis NG. *Interleukin-1 receptor type I signaling critically regulates infarct healing and cardiac remodeling*. Am J Pathol.; 173:57–67; 2008.

67. Clark, RA. Wound repair. Overview and general considerations. In: RA, C., editor. *The Molecular and Cellular Biology of Wound Repair*. New York: Plenum Press; p. 3-50; 1995.

68. Takahashi S, Barry AC, Factor SM. *Collagen degradation in ischemic rat hearts.* Biochem J; 265:233–41; 1990.

69. Dobaczewski M, Bujak M, Zymek P, Ren G, Entman ML, Frangogiannis NG. *Extracellular matrix remodeling in canine and mouse myocardial infarcts.* Cell Tissue Res.; 324:475–88; 2006.

70. Huebener P, Abou-Khamis T, Zymek P, Bujak M, Ying X, Chatila K, Haudek S, Thakker G, Frangogiannis NG. et al. *CD44 Is Critically Involved in Infarct Healing by Regulating the Inflammatory and Fibrotic Response*. J Immunol.; 180:2625–33; 2008.

71. Brown LF, Dubin D, Lavigne L, Logan B, Dvorak HF, Van de Water L. *Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing*. Am J Pathol.; 142:793–801; 1993.

72. Gabbiani G. *The myofibroblast in wound healing and fibrocontractive diseases. J Pathol.*; 200:500–3; 2003.

73. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. *The myofibroblast: one function, multiple origins.* Am J Pathol.; 170:1807–16; 2007.

74. Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L, et al. *The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1.* J Cell Biol; 142:873–81; 1998.

75. Cleutjens JP, Verluyten MJ, Smiths JF, Daemen MJ. *Collagen remodeling after myocardial infarction in the rat heart*. Am J Pathol.; 147:325–38; 1995.

76. Holmes JW, Borg TK, Covell JW. *Structure and mechanics of healing myocardial infarcts.* Annu Rev Biomed Eng.; 7:223–53; 2005.

77. Beltrami CA, Finato N, Rocco M, Feruglio GA, Puricelli C, Cigola E, Quaini F, Sonnenblick EH, Olivetti G, Anversa P. *Structural basis of end-stage failure in ischemic cardiomyopathy in humans.* Circulation.; 89(1):151-63; 1994.

78. Sun Y. *Myocardial repair/remodelling following infarction: roles of local factors.* Cardiovascular Research; 81:482-490; 2009.

79. Fraser JK, Schreiber RE, Zuk PA, Hedrick MH. *Adult stem cell therapy for the heart.* Int J Biochem Cell Biol; 36: 658–666; 2004.

80. Sell S. *Stem Cells Handbook.* Totowa, New Jersey: Humana Press; 2004.

81. Panno J. *Stem Cell Research: Medical Applications and Ethical Controversy*. New York, New York: Facts On File; 2005.

82. Gálvez-Montón C, Prat-Vidal C, Roura S, Soler-Botija C, Bayes-Genis A. *Cardiac Tissue Engineering and the Bioartificial Heart.* Rev Esp Cardiol.; 66(5):391–399; 2013.

83. Monton C, Prat-Vidal C, Roura S, Soler-Botija C, Bayes-Genisa A. *Cardiac Tissue Engineering and the Bioartificial Heart*; Rev Esp Cardiol.; 66(5):391–399; 2013.

84. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. *Skeletal myoblast transplantation for repair of myocardial necrosis*. J Clin Invest.; 98:2512–23; 1996.

85. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. *Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts*. Nature; 428: 664–8; 2004.

86. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ. *Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts*. Nat Med.; 9:1195–1201; 2003.

87. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P.

Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell.; 114: 763–76; 2003.

88. Doss MX, Koehler CI, Gissel C, Hescheler J, Sachinidis A. *Embryonic stem cells: a promising tool for cell replacement therapy*. J Cell Mol Med.; 8: 465–73; 2004.

89. Smits AM, van Vliet P, Hassink RJ, Goumans MJ, Doevendans PA. *The role of stem cells in cardiac regeneration*. J Cell Mol Med.; 9: 25–36; 2005.

90. He Q, Trindade PT, Stumm M, Li J, Zammaretti P, Bettiol E, DuboisDauphin M, Herrmann F, Kalangos A, Morel D, Jaconi ME. *Fate of undifferentiated mouse embryonic stem cells within the rat heart: role of myocardial infarction and immune suppression.* J Cell Mol Med.; 13:188–201; 2009.

91. Yoshioka T, Ageyama N, Shibata H, Yasu T, Misawa Y, Takeuchi K, Matsui K, Yamamoto K, Terao K, Shimada K, Ikeda U, Ozawa K, Hanazono Y. *Repair of infarcted myocardium mediated by transplanted bone marrow-derived CD34+ stem cells in a nonhuman primate model.* Stem Cells.; 23:355–64; 2005.

92. Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A. *Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation*.; 120: 408–16; 2009.

93. Jain M, DerSimonian H, Brenner DA, Ngoy S, Teller P, Edge AS, Zawadzka A, Wetzel K, Sawyer DB, Colucci WS, Apstein CS, Liao R. *Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction.* Circulation.; 103:1920–7; 2001.

94. Menasche´ P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, Lake S, Chatellier G, Solomon S, Desnos M, Hagège AA. *The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation.* Circulation.; 117:1189–200; 2008.

95. Bayes-Genis A, Roura S, Soler-Botija C, Farre´ J, Hove-Madsen L, Llach A, J. Cinca. *Identification of cardiomyogenic lineage markers in untreated human bone marrow–derived mesenchymal stem cells*. Transplant Proc.; 37:4077–9; 2005.

96. Suarez de Lezo, Herrera C, Romero MA, Pan M, Jime´nez R, Carmona D, Segura JM, Nogueras S, Mesa D, Suárez de Lezo J, Pavlovic D, Ojeda S, Torres A. *Recuperacion funcional tras infusion intracoronaria de celulas mononucleadas de medula osea autologa en pacientes con infarto cronico anterior y depresion severa de la funcion ventricular.* Rev Esp Cardiol.; 63:1127–35; 2010.

97. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. *HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells*. Exp Hematol.; 31:890–6; 2003.

98. Condorelli G, Borello U, De Angelis L, Latronico M, Sirabella D, Coletta M, Galli R, Balconi G, Follenzi A, Frati G, Cusella De Angelis MG, Gioglio L, Amuchastegui S, Adorini L, Naldini L, Vescovi A, Dejana E, Cossu G. et al. *Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration.* Proc Natl Acad Sci U S A.; 98:10733–8; 2001.

99. Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T. *Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia*. Circulation.; 103:634–7; 2001.

100. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. *Human cardiovascular progenitor cells develop from a b4 embryonic-stemcellderived population. Nature*; 53:524–8; 2008.

101. Caspi O, Lesman A, Basevitch Y, Gepstein A, Arbel G, Habib IH, Gepstein L, Levenberg S. *Tissue engineering of vascularized cardiac muscle from human embryonic stem cel*ls. Circ Res.; 100:263–72; 2007.

102. Takahashi K, Yamanaka S. *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors*. Cell.;126:663-676.112; 2006.

103. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. *Induced pluripotent stem cells generated without viral integration*. Science; 322:945-949; 2008.

104. Warren L, Manos PD, Ahfeldt T, et al. *Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA.* Cell Stem Cell.; 7:618-630; 2010.

105. Schachinger V, Erbs S, Elsa¨sser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Süselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM. *Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.* N Engl J Med.; 355:1210–21; 2006.

106. Siminiak T, Kalawski R, Fiszer D, Jerzykowska O, Rzezniczak J, Rozwadowska N, Kurpisz M. *Autologous skeletal myoblast transplantation for the treatment of, postinfarction myocardial injury: phase I clinical study with 12 months of follow-up.* Am Heart J.; 148:531–7; 2004.

107. Cao F, Van der Bogt KE, Sadrzadeh A, Xie X, Sheikh AY, Wang H, Connolly AJ, Robbins RC, Wu JC. *Spatial and temporal kinetics of teratoma formation from murine embryonic stem cell transplantation*. Stem Cells Dev.; 16:883–91; 2007.

108. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell.; 131:861–72; 2007.

109. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. *Induced pluripotent stem cell lines derived from human somatic cells.* Science.; 318:1917–20; 2007.

110. Anversa P, Kajstura J, Leri A, Bolli R. *Life and death of cardiac stem cells: a paradigm shift in cardiac biology.* Circulation.; 113(11):1451-63; 2006.

111. Nadal-G B, Kajstura J, Anversa P, Leri A. *A matter of life and death: cardiac myocyte apoptosis and regeneration.* J. Clin. Invest.; 111:1457–1459; 2003.

112. Bergmann O, R. Bhardwaj RD, Bernard S, Zdunek S, Barnab-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. *Evidence for cardiomyocyte renewal in humans. Science*; 324(5923):98-102; 2009.

113. Karsner HT, Saphir O, Todd TW. *The State of the Cardiac Muscle in Hypertrophy and Atrophy. Am J Pathol.;* 1(4):351-372.1; 1925.

114. Krupnick AS, Kreisel D, Riha M, Balsara KR, Rosengard BR. *Myocardial tissue engineering and regeneration as a therapeutic alternative to transplantation*. Curr. Top. Microbiol. Immunol.; 280:139-64; 2004.
115. Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P. *Myocyte proliferation in end-stage cardiac failure in humans*. Proc Natl Acad Sci U S A; 95(15):8801-5; 1998.

116. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. *Evidence that human cardiac myocytes divide after myocardial infarction.* New Engl. J. Med.; 344(23):1750-7; 2001.

117. Linzbach AJ. *Heart failure from the point of view of quantitative anatomy.* Am J Cardiol.; 5:370-82; 1960.

118. Anversa P, Nadal-Ginard B. *Myocyte renewal and ventricular remodelling. Nature.;* 415(6868):240-3; 2002.

119. Bearzi, C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. . *Human cardiac stem cells. Proc*. Natl Acad. Sci.; 104(35):14068–14073; 2007.

120. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E. *Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens*. Circulation; 115(7):896–908; 2007.

121. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR. *Multipotent embryonic Isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification.* Cell.; 127:1151– 1165; 2006.

122. Kattman S.J, Huber TL, Keller GM. *Multipotent Flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages.* Dev Cell.; 11:723–732; 2006.

123. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schiltheiss TM, Orkin SH. *Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart.* Cell.; 127:1137– 1150; 2006.

124. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. *Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction*. Proc Natl Acad Sci USA.; 100(21):12313-12318; 2003.

125. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F. *Isolation and expansion of adult cardiac stem cells from human and murine heart*. Circ Res.; 95(9):911-921; 2004.

126. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. *Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury.* Nat Med.; 13(8):970-4; 2007.

127. Nurzynska D, Castaldo C, Montagnani S, Di Meglio F. *Cardiac progenitor and stem cell biology and therapy*. In: Atala A (ed) Progenitor and stem cell technologies and therapies, 1 st edn. Woodhead Publishing, Cambridge, pp 418-442.

128. Sandstedt J, Jonsson M, Kajic K, Sandstedt M, Lindahl A, Dellgren G, Jeppsson A, Asp J. *Left atrium of the human adult heart contains a population of side population cells.* Basic Res Cardiol.; 107(2):255; 2012.

129. Di Meglio F, Castaldo C, Nurzynska D, Romano V, Miraglia R, Bancone C, Langella G, Vosa C, Montagnani S. *Epithelial-mesenchymal transition of epicardial mesothelium is a source of cardiac CD117-positive stem cells in adult human heart.* J Mol Cell Cardiol.; 49(5):719-27; 2010.

130. Altarche-Xifró W, Curato C, Kaschina E, Grzesiak A, Slavic S, Dong J, Kappert K, Steckelings M, Imboden H, Unger T, Li J. *Cardiac c-kit+AT2+ cell population is increased in response to ischemic injury and supports cardiomyocyte performance.* Stem Cells; 27:2488–2497; 2009.

131. Kubo H, Jaleel N, Kumarapeli A, Berretta RM, Bratinov G, Shan X, Wang H, Houser SR, Margulies KB. *Increased cardiac myocyte progenitors in failing human hearts.* Circulation; 118:649–657; 2008.

132. Rupp S, Bauer J, von Gerlach S, Fichtlscherer S, Zeiher AM, Dimmeler S, Schranz D. *Pressure overload leads to an increase of cardiac resident stem cells.* Basic Res Cardiol; 107(2):252; 2012.

133. Cesselli D, Beltrami AP, D'Aurizio F, Marcon P, Bergamin N, Toffoletto B, Pandolfi M, Puppato E, Marino L, Signore S, Livi U, Verardo R, Piazza S, Marchionni L, Fiorini C, Schneider C, Hosoda T, Rota M, Kajstura J, Anversa P, Beltrami CA, Leri A. *Effects of age and heart failure on human cardiac stem cell function*. Am. J. Pathol.; 179, 349–366; 2011.

134. D'Amario D, Fiorini C, Campbell PM, Goichberg P, Sanada F, Zheng H, Hosoda T, Rota M, Connell JM, Gallegos RP, Welt FG, Givertz MM, Mitchell RN, Leri A, Kajstura J, Pfeffer MA, Anversa P. *Functionally competent cardiac stem cells can be isolated from endomyocardial biopsies of patients with advanced cardiomyopathies*. Circ. Res.; 108: 857–86; 2011.

135. Fuchs, E., Tumbar, T. and Guasch, G. *Socializing with the neighbors: stem cells and their niche.* Cell; 116:769-778; 2004.

136. Moore, KA. and Lemischka, IR. *Stem cells and their niches*. Science; 311:1880-1885; 2006.

137. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. *Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy*. Proc Natl Acad Sci USA; 100:10440-5; 2003.

138. Anversa P, Rota M, Urbanek K, Hosoda T, Sonnenblick EH, Leri A, Kajstura J. and Bolli R. *Myocardial aging: a stem cell problem*. Basic Res Cardiol; 100:482-93; 2005.

139. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A. *Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival*. Circ Res; 97:663-73; 2005.

140. Oh H, Hidemasa Ohab H, Bradfutecd SB, Gallardoe TD, Nakamuraab T, Gaussinf V, Mishinag Y, Pociusbh J, Michaelbh LH, Behringeri RR , Garrye DJ, Entmanbh ML, Schneiderab MD. *Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction*. Proceedings of the National Academy of Sciences of the United States of America; 100:12313-1231; 2003.

141. Zilberman M. *Active Implants and Scaffolds for Tissue Regeneration.* Technology & Engineering; 2011.

142. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. *Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury.* Nat Med; 13(8):970-4; 2007.

143. Poss KD, Wilson LG, Keating MT. *Heart regeneration in zebrafish*. Science; 298:2188–2190; 2002.

144. Langer R, Vacanti JP. *Tissue engineering*. Science; 260:920–6; 1993.

145. Vunjak-Novakovic G, Lui KO, Tandon N, Chien KR. *Bioengineering heart muscle: a paradigm for regenerative medicine*. Annu Pev Biomed Eng.; 13:245–67; 2011.

146. Nunes SS, Song H, Chiang CK, Radisic M. *Stem Cell-Based Cardiac Tissue Engineering.* J Cardiovasc Transl Res.; 2011.

147. Martinez EC, Kofidis T. *Adult stem cells for cardiac tissue engineering.* J Mol Cell Cardiol.; 50:312–9; 2011.

148. Segers VF, Lee RT. *Biomaterials to enhance stem cell function in the heart*. Circ Res.; 109:910–22; 2011.

149. Ye KY, Black LD, 3rd. *Strategies for Tissue Engineering Cardiac Constructs to Affect Functional Repair Following Myocardial Infarction.* J Cardiovasc Transl Res.; 2011.

150. Tous E, Purcell B, Ifkovits JL, Burdick JA. *Injectable acellular hydrogels for cardiac repair.* J Cardiovasc Transl Res.; 4:528–42; 2011.

151. Singelyn JM, Christman KL. *Injectable materials for the treatment of myocardial infarction and heart failure: the promise of decellularized matrices*. J Cardiovasc Transl Res.; 3:478–86; 2010.

152. Segers VF, Lee RT. *Local delivery of proteins and the use of self-assembling peptides.* Drug discovery today.; 12:561–8; 2007.

153. Ruvinov E, Harel-Adar T, Cohen S. *Bioengineering the infarcted heart by applying bioinspired materials.* J Cardiovasc Transl Res.; 4:559–74; 2011.

154. Chen QZ, Harding SE, Ali NN, Lyon AR, Boccaccini AR. *Biomaterials in cardiac tissue engineering: Ten years of research survey.* Mater. Sci. Eng. R; 59:1-37; 2008.

155. Chang HI, Wang Y. *Cell Responses to Surface and Architecture of Tissue Engineering Scaffolds.* InTech, Rijeka; Chapter 27; 2011.

156. Hubbell JA. *Biomaterials in tissue engineering***.** Nat. Biotechnol.; 13(6):565-76; 1995.

157. Bouten CVC, Dankers PYW, Driessen-Mol A, Pedron S, Brizard AMA. *Substrates for cardiovascular tissue engineering***.** Adv. Drug Delivery Rev.; 63(4-5):221-41; 2011.

158. Park H, Radisic M, Lim JO, Chang, BH, Vunjak-Novakovic G. *A novel composite scaffold for cardiac tissue engineering.* In Vitro Cell Dev. BiolAnim.; 41:188–196; 2005.

159. Chachques JC, Trainini, JC, Lago N, Masoli OH, Barisani JL, Carpentier A. *Myocardial assistance by grafting a new bioartificial upgraded myocardium (MAGNUM clinical trial): one year followup*. Cell Transplant.; 16:927–934; 2007.

160. Liu C, Xia Z, Czernuszka JT. *Design and development of three- dimensional scaffolds for tissue engineering. Trans*. IChemE, Part A: Chem. Eng. Res. Des; 85(2007)1051–1064; 2007.

161. Ahmed TA, Dare EV, Hincke M. *Fibrin: a versatile scaffold for tissue engineering applications*. Tissue Eng. Part B 14:199–215; 2008.

162. Sarig U, Machluf M. *Engineering cell platforms for myocardial regeneration.* Expert Opin Biol Ther.; 11:1055–77; 2011.

163. Ng KW, Khor HL, Hutmacher DW. *In vitro characterization of natural and synthetic dermal matrices cultured with human dermal fibroblasts*. Biomaterials; 25:2807–2818; 2004.

164. Venugopal JR, Prabhakaran MP, Mukherjee S, Ravichandran R, Dan K, Ramakrishna S. *Biomaterial strategies for alleviation of myocardial infarction*. J R Soc Interface.; 9:1–19; 2012.

165. Xu Y, Dong S, Zhou Q, Mo X, Song L, Hou T, Wu J, Li S, Li Y, Li P, Gan Y, Xu J. *The effect of mechanical stimulation on the maturation of TDSCs-poly(L-lactide-co-e-caprolactone)/ collagen scaffold constructs for tendon tissue engineering*. Biomaterials.; 35:2760–72; 2014.

166. Gautam S, Chou CF, Dinda AK, Potdar PD, Mishra NC. *Surface modification of nanofibrous polycaprolactone/gelatin composite scaffold by collagen type I grafting for skin tissue engineering.* Mater Sci Eng C Mater Biol Appl.; 34:402–9; 2014.

167. Tedder ME, Simionescu A, Chen J, Liao J, Simionescu DT. *Assembly and testing of stem cell-seeded layered collagen constructs for heart valve tissue engineering.* Tissue Eng Part A.; 17:25–36; 2011.

168. Zhao Y, Xu Y, Zhang B, Wu X, Xu F, Liang W, , Du X, Li R. *In vivo generation of thick, vascularized hepatic tissue from collagen hydrogel-based hepatic units.* Tissue Eng Part C Methods.; 16:653–9; 2010.

169. Kijeńska E, Prabhakaran MP, Swieszkowski W, Kurzydlowski KJ, Ramakrishna S. *Electrospun bio-composite P(LLA-CL)/collagen I/collagen III scaffolds for nerve tissue engineering*. J Biomed Mater Res B Appl Biomater.; 100:1093–102; 2012.

170. Chiu LL, Reis LA, Momen A, Radisic M. *Controlled release of thymosin β4 from injected collagen-chitosan hydrogels promotes angiogenesis and prevents tissue loss after myocardial infarction.* Regen Med.; 7:523–33; 2012.

171. Elzoghby AO. *Gelatin-based nanoparticles as drug and gene delivery systems: reviewing three decades of research.* J Control Release.; 172:1075–91; 2013.

172. Van Vlierberghe S, Dubruel P, Schacht E. *Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review.* Biomacromolecules.; 9:1387–408; 2011.

173. Akhyari P, Kamiya H, Haverich A, Karck M, Lichtenberg A. *Myocardial tissue engineering: the extracellular matrix.* Eur J Cardiothorac Surg.; 34:229–41; 2008.

174. Kim IY, Seo SJ, Moon HS, Yoo MK, Park IY, Kim BC, Cho CS. *Chitosan and its derivatives for tissue engineering applications.* Biotechnol Adv.; 26: 1–21; 2008.

175. Ceccaldi C, Bushkalova R, Alfarano C, Lairez O, Calise D, Bourin P, Frugier C, Rouzaud-Laborde C, Cussac D, Parini A, Sallerin B, Fullana SG. *Evaluation of polyelectrolyte complex-based scaffolds for mesenchymal stem cell therapy in cardiac ischemia treatment*. Acta Biomater.; 10:901–11; 2014.

176. Martins AM, Eng G, Caridade SG, Mano JF, Reis RL, Vunjak-Novakovic G. *Electrically conductive chitosan/carbon scaffolds for cardiac tissue engineering.* Biomacromolecules.; 15:635–43; 2014.

177. Tsuchiya N, Sato S, Kigami R, Kawano E, Takane M, Arai Y, et al. *Effect of a chitosan sponge impregnated with platelet-derived growth factor on bone augmentation beyond the skeletal envelope in rat calvaria.* J Oral Sci.; 56:23-8; 2014.

178. Hussain A, Collins G, Yip D, Cho CH. *Functional 3-D cardiac co-culture model using bioactive chitosan nanofiber scaffolds*. Biotechnol Bioeng.; 110:637–47; 2013.

179. Bidarra SJ, Barrias CC, Granja PL. *Injectable alginate hydrogels for cell delivery in tissue engineering.* Acta Biomater.; 10:1646–62; 2014.

180. Wee S, Gombotz WR. *Protein release from alginate matrices*. Adv Drug Deliv Rev.; 4:267–85; 1998.

181. Al-Shamkhani A,Duncan, R. *Radioiodination of alginate via covalently-bound tyrosinamide allows monitoring of its fate in vivo.* Journal of Bioactive and Compatible Polymers; 10:4–13; 1995.

182. Moshaverinia A, Xu X, Chen C, Akiyama K, Snead ML, Shi S. *Dental mesenchymal stem cells encapsulated in an alginate hydrogel co-delivery microencapsulation system for cartilage regeneration.* Acta Biomater.; 9:9343–50; 2013.

183. Wang L, Shansky J, Borselli C, Mooney D, Vandenburgh D. *Design and fabrication of a biodegradable, covalently crosslinked shape-memory alginate scaffold for cell and growth factor delivery.* Tissue Eng Part A.; 18: 2000–7; 2012.

184. Ménard M, Dusseault J, Langlois G, Baille WE, Tam SK, Yahia L'H, Zhu XX, Hallé JP. *Role of protein contaminants in the immunogenicity of alginates*. J Biomed Mater Res B Appl Biomater.; 93:333–40; 2010.

185. Orive G, Ponce S, Hernández RM, Gascón AR, Igartua M, Pedraz JL. *Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates*. Biomaterials.; 23:3825–31; 2002.

186. Prestwich GD, Kuo JW. *Chemically-modified HA for therapy and regenerative medicine.* Current pharmaceutical biotechnology; 9:242–5; 2008.

187. Prestwich GD. *Hyaluronic acid-based clinical biomaterials derived for cell and molecule delivery in regenerative medicine*. J Control Release.; 155:193–9; 2011.

188. Stern R, Asari AA, Sugahara KN, Eur. J. Cell Biol.; 85:699; 2006.

189. Zhu H, Mitsuhashi N, Klein A, Barsky LW, Weinberg K, Barr ML, Demetriou A, Wu GD. *The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix.* Stem Cells; 24(4):928-35; 2006.

190. Laurent TC, Fraser J. *Hyaluronan.* FASEB J.; 6(7):2397-404; 1992.

191. Janmey PA, Winer JP, Weisel, JW. *Fibrin gels and their clinical and bioengineering applications*. J. R. Soc. Interface; 6(30):1–10; 2009.

192. Mosesson MW. *Fibrinogen and fibrin polymerization and functions*. Blood Coagul. Fibrinolysis 10 (Suppl.1) S45–S48; 1999.

193. Lauricella AM. *Fibrin network variability*. Acta Bioquim Clin Latinoam; 41:7–19; 2007.

194. Weisel JW. *Fibrinogen and fibrin*. Adv. Protein Chem; 70:247–299; 2005.

195. Siebenlist KR, Meh DA, Mosesson MW. *Plasma factor XIII binds specifically to fibrinogen molecules containing gamma chains.* Biochemistry; 35:10448–10453; 1996.

196. Mosesson MW. *Fibrinogen and fibrin structure and functions.* J. Thromb. Haemost; 3:1894–1904; 2005.

197. Chiu CL, Hecht V, Duong H, Wu B, Tawil B. *Permeability of three-dimensional fibrin constructs corresponds to fibrinogen and thrombin concentrations.* Biores Open Access.; 1: 34-40; 2012.

198. Duong H, Wu B, Tawil B. *Modulation of 3D fibrin matrix stiffness by intrinsic fibrinogen-thrombin compositions and by extrinsic cellular activity*. Tissue Eng Part A.; 15: 1865-1876; 2009.

199. Tawil BJ, Reinertsen E, Skinner M, Wu B. *Concentration of Fibrin and Presence of Plasminogen Affect Proliferation, Fibrinolytic Activity, and Morphology of Human Fibroblasts and Keratinocytes in 3D Fibrin Constructs*. Tissue Eng Part A. 2014.

200. Passaretti D, Silverman RP, Huang W, Kirchhoff CH, Ashiku S, Randolph MA, Yaremchuk MJ. *Cultured chondrocytes produce injectable tissue engineered cartilage in hydrogel polymer.* Tissue Eng; 7:805-815; 2001.

201. Wozniak G. *Fibrin sealants in supporting surgical techniques: The importance of individual components.* Cardiovasc. Surg. 11(Suppl 1); 17–21; 2003.

202. Barsotti MC, Magera A, Armani C, Chiellini F, Felice F, Dinucci D, Piras AM, Minnocci A, Solaro R, Soldani G, Balbarini A, Di Stefano R. *Fibrin acts as biomimetic niche inducing both differentiation and stem cell marker expression of early human endothelial progenitor cells.* Cell Prolif; 44(1):33–48; 2011.

203. Barsotti MC, Felice F, Balbarini A, Di Stefano R. *Fibrin as a scaffold for cardiac tissue engineering.* International Union of Biochemistry and Molecular Biology; 58(5):301-310; 2011.

204. Amrani DL, Diorio JP, Delmotte Y. *Wound healing. Role of commercial fibrin sealants.* Ann N Y Acad Sci.; 936:566-79; 2001.

205. Hunt NC, Grover LM. *Cell encapsulation using biopolymer gels for regenerative medicine.* Biotechnol. Lett.; 32(6):733–742; 2010

206. Weisel JW. *The mechanical properties of fibrin for basic scientists and clinicians.* Biophys. Chem*;* 112(2–3):267–276; 2004.

207. Wiesel JW. *Structure of fibrin: impact on clot stability.* J Thromb Haemost; 5:116–24; 2007.

208. Ahmed TAE, Dare EV, Hincke M. *Fibrin: a versatile scaffold for tissue engineering applications*. Tissue Eng Part B Rev.; 14:199–215; 2008.

209. Bensaïd W, Triffitt JT, Blanchat C, Oudina K, Sedel L, Petite H. *A biodegradable fibrin scaffold for mesenchymal stem cell transplantation.* Biomaterials; 24:2497–502; 2003.

210. Christman KL, Fok HH, Sievers RE, Fang Q, Lee RJ. *Fibrin glue alone and skeletal myoblasts in a fibrin scaffold preserve cardiac function after myocardial infarction*. Tissue Eng.; 10:403–9; 2004

211. Linnes M, Ratner BD, Giachelli CM. *A fibrinogen based precision microporous scaffold for tissue engineering.* Biomaterials.; 28:5298–306; 2007.

212. Liu J, Hu Q, Wang Z, Xu C, Wang X, Gong G, et al. *Autologous stem cell transplantation for myocardial repair.* Am J Physiol Heart Circ Physiol.; 287:501–11; 2004.

213. Nie, SP, Wang X, Qiao SB, Zeng QT, Jiang JQ, Liu XQ, Zhu XM, Cao GX, Ma CS. *Improved myocardial perfusion and cardiac function by controlled-release basic fibroblast growth factor using fibrin glue in a canine infarct model.* J. Zhejiang Univ. Sci. B*;* 11(12):895–904; 2010.

214. Sui R, Liao X, Zhou X, Tan Q. *The current status of engineering myocardial tissue.* Stem Cell Rev; 7(1):172-80; 2011.

215. Lavik E, Teng YD, Snyder E, Langer R. *Seeding neural stem cells on scaffolds of PGA, PLA, and their copolymers.* Methods Mol Biol; 198:89-97; 2002.

216. Hutmacher DW, Schantz T, Zein I, Ng KW, Teoh SH, Tan KC. *Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling*. J Biomed Mater Res; 55(2):203-16; 2001.

217. Seal BL, Otero TC, Panitch A. *Polymeric biomaterials for tissue and organ regeration.* Materials Science and Engineering; 34:147-230; 2001.

218. McDevitt TC, Angello JC, Whitney ML, Reinecke H, Hauschka SD, Murry CE, Stayton PS. *In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces.* J. Biomed. Mater. Res.; 60(3):472-9; 2002.

219. Zong X, Bien H, Chung CY, Yin L, Fang D, Hsiao BS, Chu B, Entcheva EX. *Electrospun fine-textured scaffolds for heart tissue constructs.* Biomaterials; 26(26):5330-8; 2005.

220. Semino CE. C*an We Build Artificial Stem Cell Compartments?.* J Biomed Biotechnol.; 2003(3):164-169; 2003.

221. Van de Velde PKK. *Biopolymers: overview of several properties and consequences on their applications.* Polym. Test; 21:433–442; 2002.

222. Jawad H, Lyon AR, Harding SE, Ali NN, Boccaccini AR. *Myocardial tissue engineering.* Br. Med. Bull.; 87:31-47; 2008.

223. Jawad H, Ali NN, Lyon AR, Chen QZ, Harding SE, Boccaccini AR. *Myocardial tissue engineering: a review.* J. Tissue Eng. Regen. Med; 1(5):327-42; 2007.

224. Guelcher SA. *Biodegradable polyurethanes: synthesis and applications in regenerative medicine.* Tissue Eng. Part B; 14(1):3-17; 2008.

225. S Keane TJ, Swineheart IT, Badylak SF. *Methods of tissue decellularization used for preparation of biologic scaffolds an in vivo relevance*. Methods; 84:25-34; 2015.

226. Nagata S, Hanayama R, Kawane K. *Autoimmunity and the clearance of dead cells. Cell.*; 140(5):619–630; 2010.

227. Manfredi AA, Capobianco A, Bianchi ME, Rovere-Querini P. *Regulation of dendriticand T-cell fate by injury-associated endogenous signals.* Crit Rev Immunol.; 29(1):69–86; 2009.

228. Brown BN, Valentin JE, Stewart-Akers AM, McCabe GP, Badylak SF. *Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component.* Biomaterials; 30(8):1482–1491; 2009.

229. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. *Circulating mitochondrial DAMPs cause inflammatory responses to injury.* Nature; 464(7285):104–107; 2010.

230. Gilbert TW, Sellaro TL, Badylak SF. *Decellularization of tissues and organs*. Biomaterials; 27(19):3675-83; 2006.

231. Nakayama KH, Batchelder CA, Lee CI, Trantal AF. *Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering.* Tissue Eng Part A; 16(7):2207-16; 2010.

232. Li F, Li W, Johnson S, Ingram D, Yoder M, Badylak S. *Low-molecular weight peptides derived from extracellular matrix as chemoattractants for primary endothelial cells.* Endothelium; 11(3-4):199-206; 2004.

233. Badylak SF, Park K, Peppas N, McCabe G, Yoder M. *Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix*. Exp Hematol; 29(11):1310-8; 2001.

234. Zantop T, Gilbert TW, Yoder MC, Badylak SF. *Extracellular matrix scaffolds are repopulated by bone marrow-derived cells in a mouse model of achilles tendon reconstruction.* J Orthop Res; 24(6):1299-309; 2006

235. Beattie AJ, Gilbert TW, Guyot JP, Yates AJ, Badylak SF. *Chemoattraction of Progenitor Cells by Remodeling Extracellular Matrix Scaffolds.* Tissue Eng Part A; 2008.

236. Badylak SF, Freytes DO, Gilbert TW. *Extracellular matrix as a biological scaffold material: Structure and function.* Acta Biomater.; 5(1):1-13; 2009.

237. Kawecki M, Łabuś W, Klama-Baryla A, Kitala D, Kraut M, Glik J, Misiuga M, Nowak M, Bielecki T, Kasperczyk A. *A review of decellurization methods caused by an urgent need for quality control of cell-free extracellular matrix' scaffolds and their role in regenerative medicine***.** J Biomed Mater Res B Appl Biomater.; 2017.

238. Lee RC. *Cell injury by electric forces*. Ann N Y Acad Sci.; 1066:85–91; 2005.

239. Cox B, Emili A. *Tissue subcellular fractionation and protein extraction for use in massspectrometry-based proteomics.* Nat Protoc.; 1(4):1872–1878; 2006.

240. Meyer SR, Chiu B, Churchill TA, Zhu L, Lakey JR, Ross DB. *Comparison of aortic valve allograft decellularization techniques in the rat.* J Biomed Mater Res A; 79(2):254–262; 2006.

241. Yang B, Zhang Y, Zhou L, Sun Z, Zheng J, Chen Y, Dai Y. *Development of a porcine bladder acellular matrix with well-preserved extracellular bioactive factors for tissue engineering*. Tissue Eng Part C Methods.; 16(5):1201–1211; 2010.

242. Crapo PM, Gilbert TW, Badylak SF. *An overview of tissue and whole organ decellularization Processes.* Biomaterials; 32(12): 3233–3243; 2011.

243. Klebe RJ. *Isolation of a collagen-dependent cell attachment factor.* Nature; 250(463):248–251; 1971.

244. Maurer P, Hohenester E. *Structural and functional aspects of calcium binding in extracellular matrix proteins*. Matrix Biol.; 15(8–9):569–580; 1997.

245. Song JJ, Ott HC. *Organ engineering based on decellularized matrix scaffolds*. Trends Mol Med.; 17:424–32; 2011.

246. Li TS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, Matsushita N, Blusztajn A, Terrovitis J, Kusuoka H, Marbán L, Marbán E. *Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells.* J Am Coll Cardiol ; 59:942–53; 2012.

247. Clifford DM, Fisher SA, Brunskill SJ, Doree C, Mathur A, Watt S, Martin-Rendon E. *Stem cell treatment for acute myocardial infarction. Cochrane Database Syst Rev*.; 2: CD006536; 2012.

248. Nowbar AN, Mielewczik M, Karavassilis M, Mielewczik M, Karavassilis M, Dehbi, H-M, Shun-Shin, MJ, Jones, S, Howard JP, Cole GD, Francis DP. *Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis.* BMJ.; 48: 2688; 2014.

249. Sanganalmath SK, Bolli R. *Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions.* Circ Res.; 113: 810–34; 2013.

250. Roediger M, Miosge N, Gersdorff N. *"Tissue distribution of the laminin β1 and β2 chain during embryonic and fetal human development".* Journal ofMolecularHistology; vol. 41,no. 2-3, pp. 177–184; 2010.

251. Imanaka-Yoshida K, Matsumoto KI, Hara M, SakakuraT, Yoshida T. *"The dynamic expression of tenascin-C and tenascin-X during early heart development in the mouse"* .Differentiation; vol. 71, no. 4-5, pp. 291–298; 2003.

252. Engler AJ, Sen S, Sweeney HL, Discher DE. *Matrix elasticity directs stem cell lineage specification.* Cell; 126:677-89; 2006.

253. Jacot JG, McCulloch AD, Omens JH. *Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes*. Biophys J; 95:3479-87; 2008.

254. Barallobre-Barreiro J1, Didangelos A, Schoendube FA, Drozdov I, Yin X, Fernández-Caggiano M, Willeit P, Puntmann VO, Aldama-López G, Shah AM, Doménech N, Mayr M. *"Proteomics analysis of cardiac extracellular matrix remodeling in a porcine model of ischemia/reperfusion injury* ". Circulation; vol. 125, pp. 789–802; 2012.

255. Laflamme M.A., Murry C.E. *Heart regeneration*; Nature, 473:326; 2011.

256. Jang J, Kim TG, Kim BS, Kim SW, Know SM, Cho DW. *Tailoring mechanical properties of decellularized matrix bionk by vitamin B2- induced photo-crosslinking. Acta Biomater*; 33:88-95; 2016.

257. Reing J.E, Zhang L, Myers-Irvin J, Cordero K.E, Freytes D.O, Heber-Katz E, Bedelbaeva K, McIntosh D, Dewilde A, Braunhut S.J, Badylak S.F. *Degradation products of extracellular matrix affect cell migration and proliferation.* Tissue Eng Part A; 15:605; 2009.

258. Singelyn JM, DeQuach JA, Seif-Naraghi SB, Littlefield RB, Schup-Magoffin PJ, Christman KL. *Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering*. Biomaterials; 30:5409–16; 2009.

259. Singelyn JM, Christman KL. *Modulation of material properties of a decellularized myocardial matrix scaffold*. Macromol Biosci; 11(6):731–8; 2011.

260. Grover GN, Rao N, Christman KL. Myocardial matrix-polyethylene glycol hybrid hydrogels for tissue engineering. Nanotechnology; 25:014011; 2014.

261. Jacot JG, Martin JC, Hunt DL. *Mechanobiology of cardiomyocyte development. J Biomech*; 43:93–8; 2010.

262. Guthold M, Liu W, Sparks EA, Jawerth LM, Peng L, Falvo M, Superfine R, Hantgan RR, Lord ST. *A comparison of the mechanical and structural properties of fibrin fibers with other protein fibers*. Cell Biochem. Biophys. 49,165–181; 2007.

263. Williams C, Budina E, Stoppel WL, Sullivan KE, Emani S, Emani SM, Black LD. *Cardiac extracellular matrix–fibrin hybrid scaffolds with tunable properties for cardiovascular tissue engineering.* Acta Biomaterialia; 14:84–95; 2015

264. Seif-Naraghi SB, Salvatore MA, Schup-Magoffin PJ, Hu DP, Christman KL. *Design and Characterization of an Injectable Pericardial Matrix Gel: A Potentially Autologous Scaffold for Cardiac Tissue Engineering*. Tissue Eng Part A.; 16(6): 2017–2027; 2010.

265. Mironov V, Markwald RR. Anatomy of tissue engineering. Anat. Rec; 263:335; 201.

266. Wilson HV. *On some phenomena of coalescence and regeneration in sponges*. J. Exp. Zool; 5:245-258; 1907.

267. Just EE. *The biology of Cell Surface*. Blakiston.

268. Townes PL, Holtfreter J. *Directed movements and selective adhesion of embryonic amphibian cells.* J. Exp. Zool.; 128: 53-120; 1955.

269. Young JL, Engler AJ. *Hydrogels with time-dependent material properties enhance cardiomyocyte differentiation in vitro.* Biomaterials; 32:1002-9; 2011.

270. Di Meglio F, Nurzynska D, Romano V, Miraglia R, Belviso I, Sacco AM, Barbato V, Di Gennaro M, Granato G, Maiello C, Montagnani S, Castaldo C. *Optimization of Human Myocardium Decellularization Method for the Construction of ImplantablePatches.* Tissue Eng Part C Methods; 2017.

271. Kajitani M, Wadia Y, Hinds MT, Teach J, Swartz KR, and Gregory KW. *Successful repair of esophageal injury using an elastin based biomaterial patch*. ASAIO Journal, vol. 47, no. 4,pp. 342–345; 2001.

272. Bader A, Schilling T, Teebken OE, Brandes G, Herden T, Steinhoff G, Haverich A. *Tissue engineering of heart valves--human endothelial cell seeding of detergent acellularized porcine valves*. Eur J Cardiothorac Surg; 14: 279–284; 1998.

273. Ahmed TA, Dare EV, Hincke M. *Fibrin: A Versatile Scaffold for Tissue Engineering Application.* Tissue Eng Part B Rev.; 14(2):199-215; 2008.

274. Sidelmann JJ, Gram J, Jespersen J, Kluft C. *Fibrin clot formation and lysis: basic mechanisms.* Semin. Thromb. Hemost.; 26, 605– 618; 2000.

275. Ho W, Tawil B, Dunn JC, Wu BM. *The behavior of human mesenchymal stem cells in 3D fibrin clots: dependence on fibrinogen concentration and clot structure.* Tissue Eng.; 12, 1587–1595; 2006.

276. Eyrich D, Gopferich A, Blunk T. *Fibrin in tissue engineering*. Adv. Exp. Med. Biol.; 585, 379–392; 2006.

277. Christman KL, Lee RJ. *Biomaterials for the treatment of myocardial infarction.* J Am Coll Cardiol; 48:907-13; 2006.

278. Huang NF, Lam A, Fang Q, Sievers RE, Li S, Lee RJ. *Bone marrow-derived mesenchymal stem cells in fibrin augment angiogenesis in the chronically infarcted myocardium.* Regen Med; 4:527-38; 2009.