Departamento de Tecnología y Química Farmacéuticas

Facultad de Farmacia y Nutrición

Universidad de Navarra

TESIS DOCTORAL

NOVEL TISSUE ENGINEERING STRATEGIES FOR CARDIAC REPAIR AFTER A MYOCARDIAL INFARCTION

Laura Saludas Echauri

Pamplona, 2020

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Trabajo presentado por Laura Saludas Echauri para

obtener el grado de Doctor en Farmacia

Fdo. Laura Saludas Echauri

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UNIVERSIDAD DE NAVARRA
FACULTAD DE FARMACIA Y NUTRICIÓN
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DÑA. MARÍA BLANCO PRIETO, Catedrática de la Universidad de Navarra y Profesora Investigadora del Departamento de Tecnología y Química Farmacéuticas y **D. FELIPE PRÓSPER CARDOSO**, director del Área de Terapia Celular y codirector del Servicio de Hematología de la Cínica Universidad de Navarra

Certifican:

Que el presente trabajo, titulado "**Novel tissue engineering strategies for cardiac repair after a myocardial infarction**", presentado por **DÑA. LAURA SALUDAS ECHAURI** para optar al grado de Doctor en Farmacia, ha sido realizado bajo su dirección en el Departamento de Tecnología y Química Farmacéuticas de la Universidad de Navarra y en el Área de Terapia Celular del Centro de Investigación Médica Aplicada (CIMA). Considerando finalizado el trabajo autorizan su presentación a fin de que pueda ser juzgado y calificado por el Tribunal correspondiente.

Y para que así conste, firman la presente:

Dra. María Blanco Prieto

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A mis padres,

Joaquín y Tomy

“Courage is like -it’s a habitus, a habit, a virtue: you get it by courageous acts. It is like you learn to swim by swimming. You learn courage by couraging.”

Marie M. Daly (1921-2003)

Bioquímica y primera mujer afroamericana en obtener el grado de Doctor en Química en Estados Unidos. Realizó importantes contribuciones acerca de las causas de un ataque al corazón.

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ABBREVIATIONS

Ade	Adenosine
ADSCs	Adipose-derived stem cells
Cav	Caveolin
CPCs	Cardiac progenitor cells
cTnT	Cardiac troponin T
CVD	Cardiovascular diseases
Cx43	Connexin-43
Dex	Dextrose
EDV	End-diastolic volume
EF	Ejection fraction
EPR	Enhanced permeability and retention effect
ESV	End-systolic volume
EVs	Extracellular vesicles
FAC	Fractional area change
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FS	Fractional shortening
GFP	Green fluorescent protein
hiPSC	Human induced pluripotent stem cells
hiPSC-CMs	Human induced pluripotent stem cell-derived cardiomyocytes
HSA	Human serum albumin
LAD	Left anterior descending coronary artery
LDL	Low-density lipoprotein
LV	Left ventricle
MI	Myocardial infarction
MPs	Microparticles
MSCs	Mesenchymal stem cells
MVs	Microvesicles
NPs	Nanoparticles

NRG	Neuregulin
NTA	Nanoparticle Tracking Analysis
PBS	Phosphate buffered saline
PEG	Poly(ethylene glycol)
PDI	Polydispersity index
PDL	Poly-D-lysine
PLGA	Poly(lactic-co-glycolic acid)
PVA	Poly(vinyl alcohol)
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
SMA	Smooth muscle actin
SQ	Squalenic acid
SQAd	Squalene-adenosine
SQAd/VitE	Squalene-adenosine/Vitamin E
TE	Tissue engineering
TEM	Transmission electron microscopy
TROMS	Total Recirculation One Machine System
UC	Ultracentrifugation
VitE	Vitamin E

FOREWORD

DRUG DELIVERY SYSTEMS FOR MENDING AN INFARCTED HEART

DRUG DELIVERY SYSTEMS FOR MENDING AN INFARCTED HEART

Tissue engineering, as one branch of regenerative medicine, is experiencing one of its most exciting eras. After years of continuous evolution and progress, it is starting to integrate into and revolutionize entire clinical areas [1,2]. Importantly, cell and protein therapies had repeatedly proved in previous years to play a key solid role in those areas aiming to regenerate a damaged organ by modulating several signalling pathways [3,4]. However, the combination of these elements with biomaterials, which is known as tissue engineering, has opened a whole world of new therapeutic options and avenues promising the future recovery of impaired organs [5,6].

The success of biomaterial implantation in regenerative medicine proves their capacity to address important existing challenges in the field. Cell therapy has evolved greatly due to advances in materials science. Administration of cells directly in an inflamed tissue is usually a poor option for therapeutic cells, as this tends to lead to cell death and lack of organ replenishment [7,8]. This is of special importance in the heart, where the incessant contraction of this organ also originates the washout of cells to off-target organs, raising challenging issues affecting clinical outcomes [9]. By contrast, delivery of cells in combination with drug delivery systems (i.e. hydrogels, patches, microcapsules, etc.) provides a favourable microenvironment for cell biological functions, protecting them from pro-inflammatory cytokines and providing them with a physical support for attachment [10–12]. Recent research has led to a consensus that the beneficial effects of cell transplantation are the result of the paracrine secretion of cells rather than cardiac differentiation and tissue integration of cells [13]. Therefore, front-line studies are moving towards a new generation of therapies based on acellular therapies. This involves the substitution of cells by their secreted extracellular vesicles (EVs), which promises to bring some advantages to the field [14,15]. Here, drug delivery systems have also much to offer as they provide protection, precise localization and controlled release.

Recent advances in biomaterials have also led to significant improvements in the delivery of pharmacologically active compounds with the potential to stimulate endogenous regeneration. Traditionally, therapeutic proteins, cytokines, growth factors and other molecules have been injected directly into the desired zone or, depending on the pathology, intravenously infused [16–18]. At first, this approach had to face serious limitations and side effects. Most of the tested therapeutic molecules experience a short half-life *in vivo*, which is insufficient to produce clinical benefits [19]. In order to resolve the issue of rapid metabolization, high doses are usually administered, which along with the poor specificity that characterises many of them, leads to undesired effects and toxicity [20,21]. By combining these fragile molecules with drug delivery systems, it is possible to positively modulate their metabolization, biodistribution and increase the therapeutic index [22–24]. In this sense,

delivery systems give molecules protection, prolonging their half-life and pharmacological action, and may deliver agents to their therapeutic target by passive or active targeting [25]. Furthermore, a tight adjustment of biomaterial properties allows for controlled release kinetics.

In this PhD thesis, we aimed at applying a powerful therapeutic tool, namely tissue engineering and drug delivery systems (microparticles (MPs), nanoparticles (NPs) and hydrogels), to bring significant improvements to the main cause of mortality and morbidity worldwide, i.e. myocardial infarction (MI). According to the World Health Organization, there are 32.4 million MI and strokes in the world every year [26]. Survivors of a MI are at increased risk of recurrent infarctions and morbid events that are costly to treat and that may culminate in heart failure [27]. Therapeutic solutions for patients undergoing an ischemic event are highly limited and ineffective in the long term. Current protocols after hospital admittance aim at reducing the size of infarct by immediate restoration of blood flow using fibrinolytic therapy and percutaneous coronary intervention [28]. Unfortunately, reperfusion therapy is the most effective option to prevent an exponential increase of the infarct, but it also entails a severe adverse tissue response that results in serious damage, accounting for 50% of final infarct size [29]. Certainly, in all cases a large number of cardiomyocytes die, leading to an important part of the myocardium becoming dysfunctional, and there is no approach to revert this detrimental situation and repair the irreversible damage [30].

Bearing this situation in mind, in this thesis I address heart injury after a MI by the application of drug delivery systems to two different purposes:

i) To repair or regenerate the dysfunctional myocardium after the loss of a large number of cardiomyocytes due to the ischemic insult and the following inflammatory process (**Chapters 1, 2 and 3**).

ii) To protect the infarcted heart from severe cardiomyocyte death and infarct expansion after reperfusion therapy ameliorating adverse mechanisms (**Chapter 4**).

In the **Introduction**, some of the most promising drug delivery systems for cardiac application are reviewed and discussed. First, we provide insights into the different particulate systems that have been investigated so far for the administration of potential drugs for cardiac repair/cardioprotection, including therapeutic molecules and stem cells. We place a special emphasis on the delivery route, the manufacturing material and the therapeutic agent. Finally, we discuss some advances, challenges and future perspectives in the cardiovascular field. In the second part, we briefly outline the pathophysiology of MI and address the limitations of current treatments before highlighting the

potential of novel therapies. We then summarize the latest hydrogels developed as therapeutic tools and delivery vehicles for cardiac repair.

The experimental section is divided into four chapters (Fig. 1):

1. In the **first experimental section**, we describe a novel strategy for overcoming the main limitation associated with cell therapy in clinical trials, i.e. cell engraftment and survival in the ischemic tissue. Although MPs are traditionally used for encapsulation of therapeutic proteins, in this chapter we propose their use as potential delivery vehicles for cells. In these experiments, cells were adhered to the surface of biomimetic MPs and evaluated in murine models of MI. In our first approach, adipose-derived stem cells (ADSCs) were administered in combination with neuregulin-loaded MPs. Although cardiac repair was achieved, lack of differentiation to cardiac cells along with research trends suggesting the use of cardiac-guided cells led to a second study based on the transplantation of cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs), a cell type more similar to myocardium native cells. In both studies, MPs increased cell survival dramatically, and this was the first time that cells were found in the cardiac tissue three months after injection. Consequently, cardiac repair was greatly enhanced.

2. The finding that cell beneficial effects are due to a paracrine effect meant that recent trends have moved towards acellular therapies, and in particular towards the therapeutic use of EVs. Therefore, in the **second chapter**, several methodologies were explored for an efficient and reproducible isolation of EVs. In this part, we compared the isolation of EVs from cardiac progenitor cells cultured in different conditions and using two of the main isolation protocols: ultracentrifugation and size-exclusion chromatography. Although many similarities were observed, our findings indicate that the latter method could lead to improved outcomes.

3. In the **third chapter**, we developed and characterized a novel alginate-collagen hydrogel for the retention and sustained delivery of EVs. Hydrogel possessed a low viscosity and good injectability, suggesting a possible non-invasive administration. Finally, EVs were incorporated and homogenously distributed in the developed hydrogel.

4. In the **fourth experimental chapter**, we address the major challenge associated with current clinical intervention after a MI, i.e. reperfusion injury. Nowadays, reperfusion is the most effective option for infarcted patients and it is routinely performed. However, it is a double-edged sword, being responsible for an increase in infarct size. In this section, we tested the cardioprotective efficacy of squalene-adenosine (SQAd) NPs and the multidrug SQAd/Vitamin E (VitE) NPs as well as the optimal timing of treatment administration in a rat ischemia/reperfusion MI model. After intravenous injection, SQAd NPs accumulated in the infarcted tissue. As a result, cardiac functional performance

was improved and left ventricular negative remodelling was prevented. By contrast, SQAd/VitE NPs did not reduce infarct size or adverse remodelling. Although several authors suggest that therapy for reperfusion injury should be administered prior to reperfusion, we found no differences between pre- or post-administration.

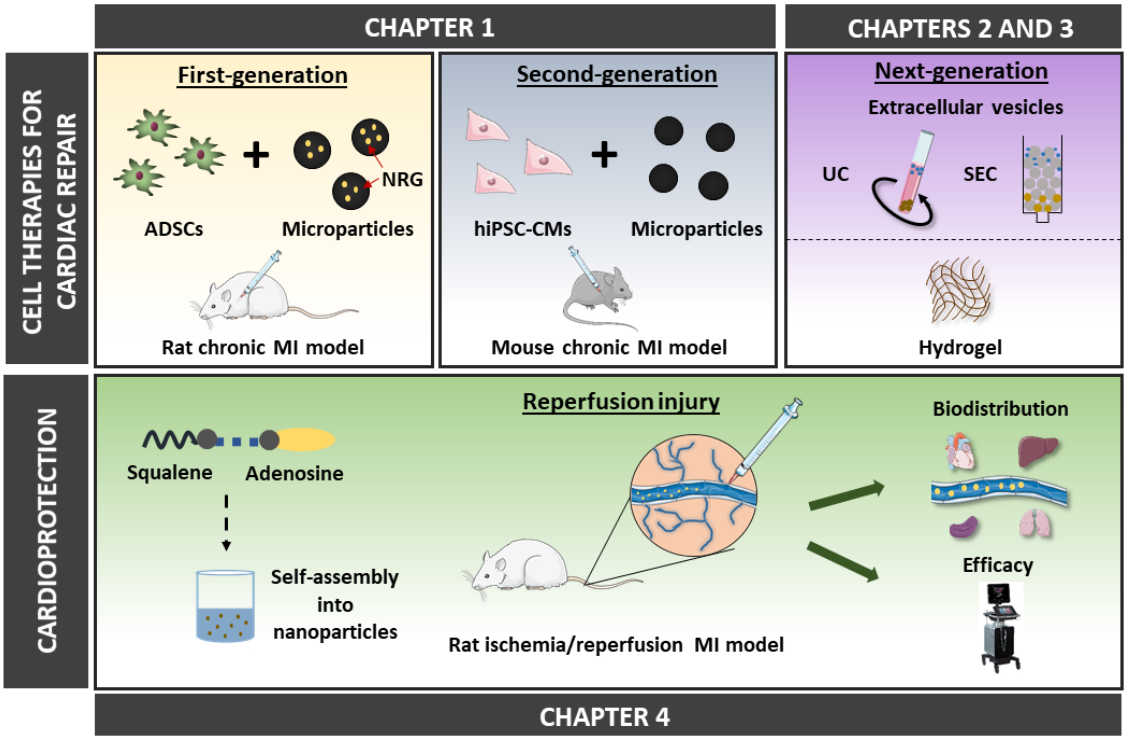

Figure 1. Therapeutic strategies for MI addressed in the different chapters of this thesis. Chapters 1, 2 and 3 focused on the combination of cell therapies and microparticles/hydrogels for cardiac repair. Chapter 4 aimed at ameliorating reperfusion injury by administration of encapsulated adenosine. MI: myocardial infarction, ADSCs: adipose-derived stem cells, NRG: neuregulin, hiPSC-CMs: human induced pluripotent stem cells-derived cardiomyocytes, UC: ultracentrifugation, SEC: size-exclusion chromatography.

Finally, a global view of the different strategies proposed in this thesis as well as their contribution to cardiac regenerative field is provided in the **General Discussion**. Overall, this thesis provides cutting-edge therapeutic approaches for cardiac regeneration and cardioprotection after a MI by exploiting the tremendous potential of drug delivery systems to overcome the current challenges in the field.

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INTRODUCTION

HEART TISSUE REPAIR AND CARDIOPROTECTION USING DRUG DELIVERY SYSTEMS

HYDROGEL BASED APPROACHES FOR CARDIAC TISSUE ENGINEERING

HEART TISSUE REPAIR AND CARDIOPROTECTION USING DRUG DELIVERY SYSTEMS

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ABSTRACT

Regenerative capacity of the heart to heal after suffering a myocardial infarction is extremely limited and not enough to restore normal cardiac function. Fortunately, delivery of therapeutics such as stem cells, growth factors, exosomes and small interfering ribonucleic acid (siRNA), among other bioactive molecules, has been shown to enhance heart repair and improve cardiac function. Furthermore, the emergence of delivery systems and their combination with these therapeutic agents have significantly fostered the regenerative and cardioprotective potential of these novel approaches. In particular, nano- and microparticles (NPs and MPs) have demonstrated to be promising for these applications. These systems may be administered directly in the infarcted myocardium or reach the heart after intravenous injection due to the enhanced permeability and retention effect or active targeting. Thus, NPs and MPs have made it possible to administer a wide number of potential drugs, including therapeutic molecules and/or stem cells, and evidence in favor of their use has been reported in several preclinical studies. Here, we review the studies from the last 5 years using NPs and MPs loaded with therapeutics for repairing cardiac tissue after a myocardial infarction, and discuss some of the advances, challenges and future prospects in this field. In addition, we address the application of NPs and MPs for cardioprotective purposes.

KEYWORDS

Myocardial infarction, repair, cardioprotection, nanoparticle, microparticle

1. Introduction

Myocardial infarction (MI) represents the major cause of death worldwide, with an increasing burden over the last decade [1]. Therefore, understanding how cardiac tissue is injured and how it regenerates is of prime importance to global health. In this sense, model organisms such as zebrafish and neonatal mice have been used to gain a better understanding of the endogenous regenerative capacity of the heart [2], revealing mechanistic insights into the roles of cardiomyocyte proliferation, neovascularization, extracellular matrix and, particularly, the immune system [3]. Although these mechanisms have proved to be directly related to final heart function outcomes and tissue remodeling, only now we are starting to understand how they must be addressed in order to achieve better heart repair after MI [4–6]. Interestingly, stem cells [7], exosomes [8] and growth factors [9], among other small therapeutic agents such as small interfering ribonucleic acid (siRNA) [10], are emerging as the most appropriate candidates to interact with such processes, acting as potential therapeutic agents. However, although these novel approaches are receiving significant attention, efforts to translate them into clinical practice have largely failed [11,12]. This highlights the need to optimize the new strategies and find new and safer ways to deliver therapeutic agents in order to enhance their bioavailability [11].

From a clinical point of view, particle based systems are considered a suitable choice for the delivery of an arsenal of therapeutic agents to patients that have experienced a cardiac ischemic event. Among particulate systems, nanoparticles (NPs) and microparticles (MPs) have already proved in preclinical studies to be efficient for the administration of both cells [13] and bioactive molecules [14–16] in cardiovascular disorders, reporting evidence in favor of their use as delivery systems for cardiac repair and cardioprotection (Fig. 1A). They protect labile molecules against degradation, allow their controlled release over time and work as a matrix support for cells. Interestingly, the ability of NPs and MPs to deliver therapeutic agents to the site of action minimizes side effects and toxicity in off-target organs. Moreover, the possibility to obtain controlled delivery is correlated to the maintenance of active molecules' bioactive concentration, what reduces the need of consecutive treatment administration. Furthermore, particles offers the possibility of administration by non-invasive approaches. In this sense, they may be injected in the myocardium using current catheter technology or may be systemically injected. All this finally leads to a higher patient compliance to the treatment. The implementation to the routine of these novel systems as drug delivery vehicles for therapeutic agents could make possible for the first time to repair cardiac tissue.

Here, we review the latest studies concerning the use of NPs and MPs for the administration of therapeutic agents aimed at enhancing cardiac repair or providing cardioprotection after a MI. A detailed review of the features of the particles used, as well as of the final treatment efficacy, is provided. Finally, an expert opinion is offered on how the latest developments in particles and therapeutic agents should influence current treatment paradigms

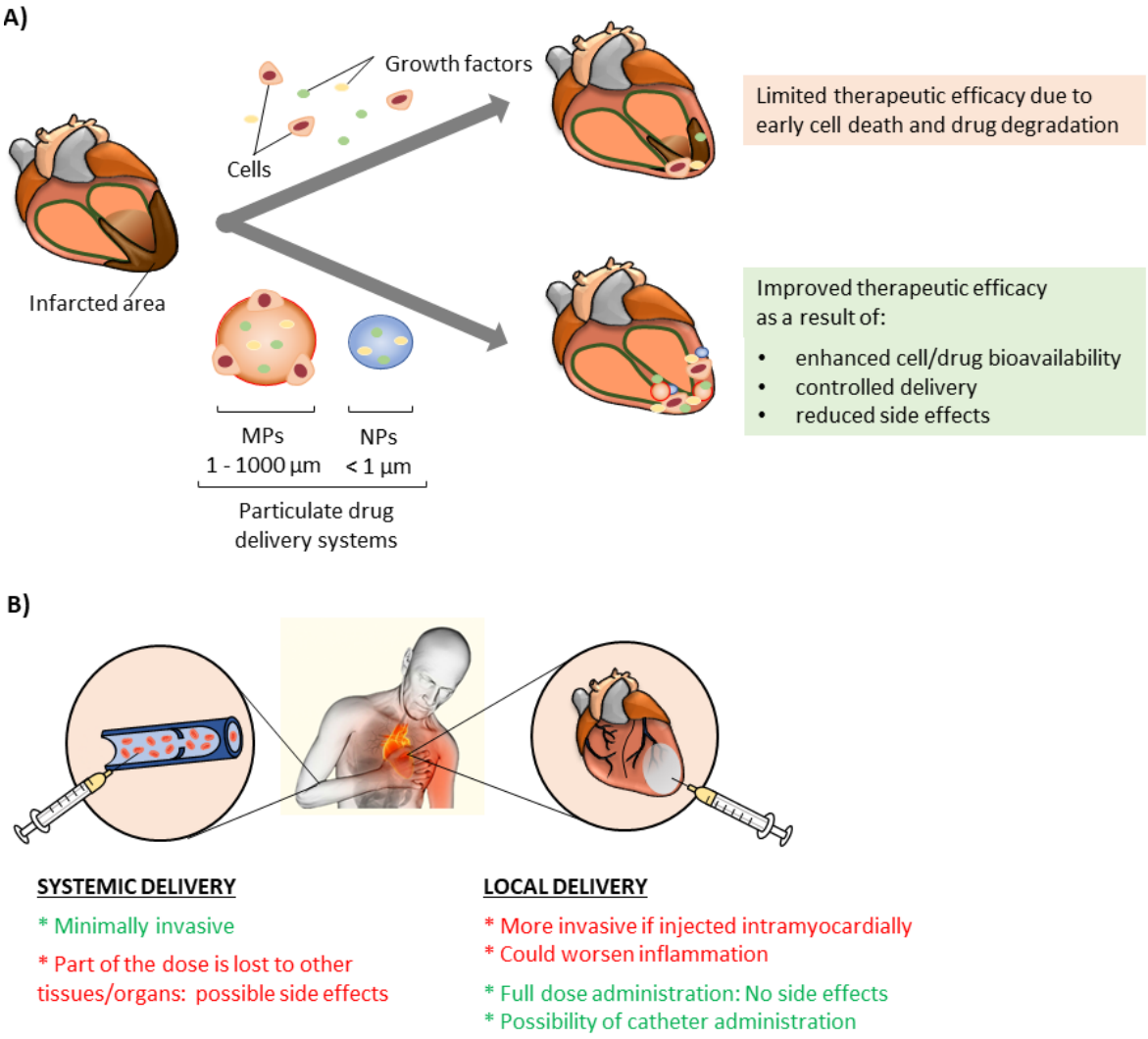

Figure 1. Administration of particulate systems combined with cells/bioactive molecules for cardiac repair and cardioprotection. A) Therapeutic efficacy limitations of cell and protein therapies and benefits obtained after combination with particulate systems, B) Advantages and disadvantages of systemic *versus* local cardiac delivery of particulate systems.

2. Methods

A systematic review of the literature was conducted in Scopus, Web of Science, and PubMed databases using the following keywords: heart attack, myocardial infarction, repair, cardioprotection, nanoparticle and microparticle. Articles included in this review: 1) were published between January 2012 and November 2017, 2) study the cardioprotective or reparative effects of NPs and/or MPs loaded with therapeutic agents as treatment for MI and 3) include an *in vivo* efficacy experiment.

3. Systemic administration of particles for treating myocardial infarction

Systemic delivery of therapeutics into the heart (Table 1) is far less invasive than direct injection, and thus intravenous (IV) administration is considered one of the most important features in

developing new delivery systems (Fig. 1B). Concerning IV administration, NPs are much more likely to be taken up by cells and less likely to cause embolization once in the blood stream than MPs, due to their smaller size [17]. In any case, it is important to note that relevant particle size variations may affect particle distribution and uptake [18]. Generally speaking, NPs make use of passive or active targeting for reaching infarcted heart tissue. Passive targeting is based on the heart's enhanced permeability and retention effect (EPR) after MI [19], whereas in active targeting strategies, NPs are functionalized with infarction targeting moieties [20] such as cluster of differentiation 34 (CD34) or the angiotensin II receptor (AT1), which are expressed by cardiomyocytes [21]. Special attention must be paid to the protein layer that is formed around NPs in contact with biological fluids [21]. This effect, known as protein corona, has not been adequately addressed in the field of cardiac nanotechnology and may affect the biological fate, drug release and immunogenicity of NPs.

3.1. Systemic administration of particles for cardiac repair

3.1.1. Silicon

The most interesting feature of this material, apart from its being biodegradable and biocompatible, is that it can be detected using near-infrared imaging technology or confocal microscopy, which means that the theranostic concept can be applied to the treatment [22,23]. Taking advantage of this, photoluminiscent silicon NPs were loaded with siRNA for C-C chemokine receptor type 2 (CCR2) and tested in a chronic MI model in mice [22]. In parallel, mesenchymal stem cells (MSCs) were intramyocardially injected. After IV injection, silicon NPs were deposited into the infarcted cardiac tissue using the enhanced EPR effect. This allowed an increased MSC survival and a reduced monocyte influx. These observations led to improved heart function after three weeks of treatment [22].

3.1.2. Lipid

Lipid NPs have also been broadly considered as promising candidates for the delivery of therapeutics into the infarcted heart, since they possess a similar morphology to cellular membranes and can incorporate both lipophilic and hydrophilic substances [24]. Zhou *et al*. loaded lipid NPs with collapsin response mediator protein 2 (CRMP2) siRNA and injected them via tail vein in a mice model of chronic MI [25]. Treatment resulted in a switch of wound macrophages from M1 to M2 phenotype, marked decrease in inflammation and fibrosis, and significant attenuation of post-MI heart failure and mortality. Cyclosporine A (CyA) lipid NPs were IV administered alone or in combination with intramyocardially injected adipose tissue-derived stem cells (ADSCs) in a swine model of chronic MI [26]. CyA inhibited mitochondrial permeability transition pore, thus reducing necrosis/apoptosis of cardiomyocytes in the early phase of ischemia/reperfusion (I/R) injury. This is

one of the few strategies that have reached larger preclinical animal models, and in fact, the interest in clinical uses of CyA has recently been boosted by a report demonstrating CyA effectiveness in limiting the infarct size in MI patients [27]. CyA NPs enhanced therapeutic cell effects, and the combinatory treatment resulted in an improved left ventricular function, higher neovascularization, reduced infarct size and decreased cardiomyocyte apoptosis [26].

3.2. Systemic administration of particles for cardioprotection

3.2.1. Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid) (PLGA) offers attracting characteristics such as biocompatibility, biodegradability, tunable mechanical properties and Food and Drug Administration approval [20]. PLGA NPs have proved to deliver different small bioactive molecules into the infarcted heart after IV administration [28–32]. CyA [28], irbesartan [29] and pitavastatin [30–32] were encapsulated in polymeric NPs, which were able to maintain the bioactivity of the encapsulated molecules. The three molecules have cardioprotective effects. Irbesartan blocked angiotensin receptor II and activated peroxisome proliferator-activated receptor (PPAR)γ, which reduced hypertension and the expression of pro-inflammatory cytokines. Pitavastatin activated the PI3K-Akt pathway and reduced inflammation. All this led to improvements in echocardiogram parameters, reduction of inflammation and decrease of oxidative stress after injection in rodent and pig models of I/R. In all these cases, NPs accumulated in the heart due to the EPR effect, which is augmented in I/R models [33]. Al Kindi *et al*. synthesized milrinone-loaded MPs and injected them via tail vein in rats after inducing chronic MI. Milrinone is known for its benefits for heart contractibility and vasodilation. This treatment resulted in improved left ventricular ejection fraction and decreased pro-inflammatory cytokines and neutrophil influx in the heart in the hours after treatment administration [34].

3.2.2. Polyethyleneimine (PEI)

In a very interesting approach, low molecular weight PEI-polyethylene glycol (PEG)-coated NPs were loaded with five siRNA (Icam1, Icam1, Vcam1, Sele and Selp), thus creating a multigene silencing strategy [35]. PEGylation strategy is used to enhance the hydrodynamic size of the vehicles, which prolongs their circulatory time by reducing renal clearance. After IV administration in a chronic MI model in mice, NPs were efficient in decreasing inflammation through suppressing leukocyte recruitment to ischemic myocardium. This was translated into better outcomes after acute MI [35].

Table 1. Studies using NPs or MPs for the delivery of therapeutics for cardiac repair and cardioprotection by IV administration. (ADSCs: adipose-derived stem cells, I/R: ischemia/reperfusion, siRNA: small interfering RNA, LV: left ventricle, MI: myocardial infarction, MSCs: mesenchymal stem cells, PEG: polyethylene glycol, PEI: polyethyleneimine, PLGA: poly(lactic-co-glycolic acid)).

Biomaterial	Therapeutic agent	Dose	Size	MI Model	Results	Ref
Heart repair						
Silicon	siRNA CCR2 MSCs	25 mg/kg $1x10^5$ cells	100-200 nm	Chronic, mouse	Reduced monocyte accumulation in the heart, amelioration of LV remodeling, increased vascular density	[22]
Lipid	siRNA CRMP2	70 µg/kg	50 nm	Chronic, mouse	Switch of wound macrophages from M1 to M2 phenotype, marked decrease in inflammation and fibrosis, significant attenuation of post-MI heart failure and mortality	[25]
Lipid	Cyclosporine A, ADSCs	2 mg/kg $4x10^7$ cells	<160 nm	Chronic, pig	Improved heart function and neovascularization, reduced infarct size and cardiomyocyte apoptosis	[26]
Cardioprotection						
Silicon	ERK1/2 inhibitor	33 µg	180 nm	Isoprenaline MI, rat	Attenuated hypertrophic signaling (ERK signaling cascade)	[23]
PLGA	Cyclosporine A	1 mg/kg	100 nm	I/R, mouse	LV remodeling was ameliorated, as well as oxidative stress. Inhibition of mPTP opening	[28]
PLGA	Irbesartan	3 mg/kg	200 nm	I/R, mouse	Inhibited recruitment of monocytes to the heart, reduced infarct size and ameliorated LV remodeling	[29]
PLGA	Pitavastatin	1 mg/kg	160 nm	I/R, rat	Inhibited inflammation and cardiomyocyte apoptosis	[30]
PLGA	Pitavastatin	1 mg/kg	160 nm	I/R, mouse	Attenuated post-infarct LV remodeling accompanied by a reduction of monocytes/macrophages in the heart	[31]
PLGA	Pitavastatin	8-32 mg/kg	160 nm	I/R, pig	Reduced the extent of LV remodeling: reduced the MI size, LV end systolic volume was significantly decreased, less apoptotic cardiomyocytes	[32]
PLGA	Milrinone	50 mg/kg	4-5 µm	Chronic, rat	Improved LV ejection fraction, reduced neutrophil influx.	[34]
PEI	siRNA Icam1, Icam1, Vcam1, Sele and Selp	1 mg/kg	45 nm	Chronic, mouse	Reduced neutrophil and monocyte recruitment, decreased matrix-degrading plaque protease activity	[35]
Gold	Pro-anthocyanidin	9 mg/kg	24 nm	Chronic, rat	Reduced diagnostic marker enzymes levels	[36]
Lipid	Puerarin	50 mg/kg	110 nm	Chronic, rat	Reduced infarct size and oxidative stress	[37]
Lipid	Baicalin	10 mg/kg	84 nm	Chronic, rat	Reduced infarct size and oxidative stress	[38]
Lipid	Schisandrin B	10 mg/kg	130 nm	Chronic, rat	Reduced infarct size	[39]
Lipid	Hemin	2 mg/kg	< 1000 nm	Chronic, mouse	Switched macrophages into a regenerative phenotype and improved infarct healing and repair	[40]
Lipid	Flavonoid extract from *Dracocephalum moldavica L.*	0.29 drug/lipid	105 nm	I/R, rat	Reduced infarct size and expression of pro-inflammatory cytokines, improved integrity of the myocardial membrane and myocardial fibers	[41]
PEG-poly oxymethyl estyrene	2,2,6,6-tetramethyl piperidine-1-oxyl	3 mg/kg	40 nm	I/R, dog	Reduced infarct size, apoptosis, ventricular fibrillation and decreased levels of coronary venous end-products of nitric oxide	[42]

3.2.3. Silicon

Ferreira *et al.* identified a novel inhibitor of ERK1/2 phosphorylation cascade, called trisubstituted-3,4,5-Isoxazole, with potential to improve heart remodeling. Authors encapsulated it into atrial natriuretic peptide-functionalized silicon NPs and studied their efficacy in a chronic rat MI model. Cardioprotective action of loaded NPs was confirmed by improvements in heart function as well as attenuated hypertrophic signaling in the endocardium [23].

3.2.4. Gold

Concerning gold NPs, they have been used for encapsulating proanthocyanidin, a natural polyphenolic antioxidant with anti-inflammatory and vasodilatory actions [36]. The cardioprotective action of this system was tested in a rat MI model, where loaded gold NPs showed a significant decrease of MI diagnostic marker enzymes including plasma homocysteine, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatine phosphokinase, total heart reduced glutathione and total cholesterol [36].

3.2.5. Lipid

Dong *et al*. used RGD modified and PEGylated NPs loaded with puerarin and administered them in a rat chronic MI model. Targeted NPs showed increased puerarin concentration in the heart compared to non-targeted lipid NPs, which resulted in a significant reduced infarct size. This is explained by the antioxidant and ROS scavenger properties of the therapeutic agent [37]. Similar results were observed by Zhang *et al*., who encapsulated baicalin into PEGylated lipid NPs, a molecule with protective functions against myocardial ischemia due to antioxidant effects and free radical scavenging activity [38]. Targeted NPs were also developed by Mingfeng *et al.* who used matrix metalloproteinase-sensitive peptide-modified lipid NPs. Such vehicles were PEGylated and loaded with schisandrin B. The cardioprotective effects of this molecule are due to its antioxidant properties and its ability to downregulate inflammation, activate eNOS pathway, inhibit cell apoptosis, and enhance cell proliferation. After IV administration in a chronic rat MI model, treatment resulted in reduced infarct size [39]. Interestingly, heart inflammatory response can be used as the perfect mark for developing heart targeting vehicles after MI. This approach was used to develop infarct-macrophages targeted lipid NPs, by modifying them with hyaluronic acid at their surface [40]. These NPs were loaded with hemin, an iron-containing porphyrin that activates heme oxygenase-1 (HO-1), an enzyme with anti-inflammatory and cytoprotective properties. In a chronic MI model in mice, IV administration of hemin NPs switched macrophages into a regenerative phenotype and improved infarct healing and repair [40]. In other approach, lipid NPs proved to efficiently deliver to the heart a flavonoid extract from *Dracocephalum moldavica L*, which has myocardial protective function [41]. Cardioprotective effects were confirmed in I/R infarcted rats treated with loaded NPs, which

when compared to control groups showed reduced infarct size, improved integrity of the myocardial membrane and myocardial fibers and reduced expression of pro-inflammatory cytokines in the heart [41].

3.2.6. PEG

Very recently, PEG-poly oxymethylestyrene copolymer was used to synthesize NPs and encapsulate 2,2,6,6-tetramethyl piperidine-1-oxyl, a nitroxyl radical with strong antioxidant properties [42]. In a canine model of I/R MI, IV administration of radical containing NPs proved to reduce infarct size, apoptosis, ventricular fibrillation and decrease levels of coronary venous end-products of nitric oxide [42].

3.2.7. Squalene

Within the framework of the European project 'NanoHeart', our group is currently evaluating the potential of squalene-adenosine NPs for heart ischemia injuries treatment. This is an interesting approach in which particles are formed by spontaneous self-assembly of squalene-adenosine bioconjugates into NPs. Adenosine is a myocardial protectant, due to its capacity to inhibit neutrophils adhesion, to promote vascular relaxation, to inhibit the production of oxygen free radical species and to restore calcium homeostasis.

4. Local administration of particles for treating myocardial infarction

More conventional approaches focused on heart repair and cardioprotection involve the administration of the therapeutic agents directly into the infarcted or peri-infarcted myocardium (Table 2). This strategy makes it possible to reduce the doses and ease side effects since therapeutic agents are localized in the infarcted heart region (targeted area) (Fig. 1B). Furthermore, local treatment application allows administration of particles in the microscale range without inducing embolization. Notably, local particle delivery may also be performed by percutaneous catheter-based technology, which would facilitate the implementation of this strategy in clinical practice [43].

4.1. Local administration of particles for cardiac repair

4.1.1. PLGA

Regarding biomaterials, in recent years PLGA has centered researchers' attention when it comes to the local administration of therapeutics for cardiac repair in the infarcted myocardium. Our group has long experience in the application of MPs loaded with growth factors for cardiac repair. In our first approach, we proved the beneficial effects of delivering microencapsulated vascular endothelial growth factor (VEGF) to the infarcted myocardium of rats, which improved vasculogenesis and

tissue remodeling one month after treatment [44]. After three months, cardiac function was also significantly enhanced in animals receiving VEGF-MPs treatment compared to the control group [15]. Building on this work, we also encapsulated fibroblast growth factor-1 (FGF1) and neuregulin-1 (NRG1), largely described as potential cardiac regenerative therapeutics, in PLGA MPs and injected them in the ischemic cardiac area in rats. Interestingly, we were able to prove that MPs remained in the tissue for up to three months with no particle migration toward other solid organs [14,45]. In addition, it was demonstrated that NRG1 remained bioactive after three months of intramyocardial administration [14]. The sustained release over time and localized delivery of these biomolecules resulted in an improved cardiac function after three months. This was accompanied by a smaller infarct size, lower fibrosis and revascularization of the peri-infarcted tissue [16]. Subsequently, these results were completed and validated in a study comparing the delivery of these growth factors encapsulated in PLGA or PEG-PLGA MPs in a rat MI model [46]. After three months, both systems were successful at improving cardiac function, with no significant differences among them. Interestingly, both types of MPs were equally phagocyted in the heart, revealing that PEGylation process could only prevent phagocytosis in the blood stream but not in the heart tissue [46]. The optimistic results obtained in murine MI models led to translation to a clinically relevant preclinical I/R porcine model. MPs were effectively administered through a minimally invasive approach using current catheter technology, which would facilitate clinical application. In consonance with previous studies, both FGF1-MPs and NRG1-MPs improved cardiac function, reduced infarct size and induced revascularization [43].

Apart from MPs, other researchers have explored the use of PLGA NPs as drug delivery systems and injected them locally in the damaged myocardium. Recently, liraglutide, approved for clinical use as type 2 diabetes treatment was postulated as an effective drug to stimulate cardiac regeneration. Liraglutide was encapsulated in PEG-PLGA NPs and intramyocardially injected in a rat chronic MI model. NPs remained in the myocardium for at least one month releasing the encapsulated protein. At the end of this period, infarct size was attenuated, wall thickness was preserved, angiogenesis was stimulated and cardiomyocyte apoptosis prevented. Altogether, these observations explained the improved cardiac function. It should be noted that although liraglutide is used for diabetes treatment, the delivery of this protein in the heart did not induce hypoglycemia [47]. Similar results were obtained in another approach using PLGA NPs to deliver VEGF to the ischemic heart of mice. One month after treatment, the sustained VEGF release resulted in increased vasculogenesis, reduced infarct size and improved cardiac function [48].

Based on the paradigm that the beneficial effects of cell therapy are a result of the secretion of paracrine factors [8], the group of Cheng *et al*. encapsulated the secretome of cardiac stem cells (CSCs) [49] and bone marrow-derived MSCs [50] in PLGA MPs. Furthermore, these MPs were functionalized on the surface with cell membrane fragments. When administered in a mouse model

of chronic MI, these synthetic cell-mimicking MPs preserved viable myocardium, mitigated left ventricular remodeling and improved cardiac function. Although these results were comparable to those obtained with cell therapy, this strategy overcomes the challenges associated with stem cell transplantation such as immunogenicity and tumorigenicity risks [49,50].

In a different approach, the three-dimensional structure of MPs has led researchers to use them as supportive scaffolds for cell delivery. For example, the entrapment of human MSCs into MPs formulated with porous PEI blended with PLGA showed beneficial effects in a rat MI model after intramyocardial administration. After one month, the preserved cardiac geometry, decreased cardiomyocyte loss and increased angiogenesis resulted in an augmented coronary blood flow and functional cardiac improvement [51]. Going a step further, Madonna *et al.* provided MPs a dual function: tridimensional scaffold for cell delivery and reservoir depot for the localized and sustained growth factor release. MSCs were attached to the surface of VEGF PLGA MPs. Particles were previously coated with fibronectin as a biomimetic molecule. Cellular engraftment was enhanced after transplantation of the system into the infarcted myocardium of mice, compared to the delivery of cells alone or cells adhered to empty MPs, proving the efficacy of using this scaffold as structural support for cells. Moreover, after 21 days fibrosis had decreased, arteriogenesis was stimulated, the number of proliferative cardiac resident cells was increased and left ventricular diameters were reduced. These findings correlated with an improved cardiac function [52]. As a proof-of-concept of the crucial role of the paracrine effect, similar results were obtained when the concentrated medium of MSCs was injected except for the fractional shortening that was more increased after cell transplantation [52]. Our group has also studied the repair potential of transplantation of ADSCs attached to NRG1 PLGA MPs on a rat chronic MI model. Particles were previously coated with collagen and poly-D-lysine to facilitate cell adhesion to the surface. Interestingly, the *in situ* controlled release of NRG1 induced a significant increase in the number of proliferative cardiomyocytes after one week. Also at this time point, macrophage phenotype was shifted towards an anti-inflammatory pro-healing phenotype demonstrating active induction of the immune response. In addition, this strategy made it possible for the first time to increase cell survival in the infarcted myocardium for up to three months, which correlated with positive outcomes after that period: reduced infarct size, thicker left ventricular wall and increased number of capillaries and arterioles [13]. In another study, the delivery of ADSCs adhered to the surface of PLGA MPs loaded with hepatocyte growth factor and insulin growth factor (IGF1) to the rat infarcted myocardium also enhanced cell retention. Treatment stimulated cardiac healing, which was reflected in higher density of blood vessels and a slight improved left ventricular remodeling and hemodynamics. However, infarct size and left ventricular wall thickness were not improved compared to control groups. Furthermore, the authors warned that both ADSCs and MPs fostered the appearance of arrhythmias that would require further investigations [53].

Table 2. Studies using NPs or MPs for the delivery of therapeutics for cardiac repair and cardioprotection by local administration. (ADSCs: adipose-derived stem cells, FGF1: fibroblast growth factor 1, hCSCs: human cardiac stem cells, HGF: hepatocyte growth factor, I/R: ischemia/reperfusion, IGF1: insulin growth factor 1, LV: left ventricle, miRNA: microRNA, MSC: mesenchymal stem cells, NRG1: neuregulin 1, PEG: polyethylene glycol, PEI: polyethyleneimine, PLGA: poly(lactic-co-glycolic acid), PLL: poly-L-lysine, siRNA: small interfering RNA, VEGF: vascular endothelial growth factor).

Biomaterial	Therapeutic agent	Dose	Size	MI Model	Results	Ref
Heart repair						
PLGA	VEGF	510 ng or 1360 ng	5 μm	Chronic, rat	Improved cardiac function and angiogenesis	[15]
PLGA	FGF1 or NRG1	1740 ng FGF1; 1300 ng NRG1	5 μm	Chronic, rat	Improved cardiac function, smaller infarct size, lower fibrosis and revascularization. Reduced cardiomyocyte apoptosis and induced proliferation of these cells.	[16]
PLGA	FGF1 or NRG1	200 μg	7 μm	I/R, pig	Improved cardiac function, increased vascularization and reduced fibrosis	[43]
PLGA, PEG-PLGA	FGF1, NRG1	1675 ng	5 μm	Chronic, rat	Increased ejection fraction, angiogenesis and arteriogenesis	[46]
PEG-PLGA	Liraglutide	380 μg	350 nm	Chronic, rat	Attenuated infarct size, preserved wall thickness, stimulated angiogenesis, cardiomyocyte apoptosis prevented and improved cardiac function	[47]
PLGA	VEGF	0.06, 2.6 or 0.6 pg	113 nm	Chronic,mouse	Improved cardiac function, increased wall thickness, reduced infarct size and vasculogenesis	[48]
PLGA	hCSC-conditioned media	-	20 μm	Chronic, mouse	Preservation of viable myocardium and increased cardiac function	[49]
PLGA	MSC-conditioned media	-	20 μm	Chronic, mouse	Mitigated LV remodeling and angiogenesis	[50]
PLGA	NRG1 and ADSCs	1294 ng NRG1; $5x10^5$ ADSCs	20 μm	Chronic, rat	Increased proliferation of cardiomyocytes, shift of macrophage phenotype from M1 to M2, reduced infarct size, increased LV wall thickness and angiogenesis	[13]
PLGA/PEI	hMSCs	$20x10^5$, $10x10^5$ or $5x10^5$	290 μm	I/R, rat	Improved LV systolic function, preserved cardiac geometry, augmented coronary blood flow, decreased cardiomyocyte apoptosis, angiogenesis, reduced infarct size and fibrosis	[51]
PLGA	VEGF and MSCs	300 ng VEGF; $2.5x10^5$ MSCs	60 μm	Chronic, mouse	Reduced fibrosis, increased arteriogenesis and number of proliferative cardiac resident cells. Improved LV diameters and fractional shortening.	[52]
PLGA	HGF, IGF1, ADSCs	480 ng (HGF); 500 ng (IGF); $1x10^5$ ADSCs	60 μm	Chronic, rat	Improved LV remodeling and hemodynamics. Higher density of blood vessels.	[53]
Alginate-PLL-Alginate	ADSCs	$1.5x10^6$	400 μm	I/R, pig	Failed to improve infarct size and LV ejection fraction	[54]
Cardioprotection						
PLGA	IGF1	20 ng	75 nm	Chronic, mouse	Prevented cardiomyocyte apoptosis, reduced infarct size, improved LV ejection fraction and cardiac geometry	[55]
PK3	Nox2-siRNA	5 μg/kg	500 nm	Chronic, mouse	Improved fractional shortening	[56]
PK3	Nox2-miRNA	5 μg/kg	500 nm	Chronic, mouse	Improved fractional shortening, ejection fraction and reduced infarct size	[57]

4.1.2. Alginate-poly-L-lysine-alginate

In another approach, ADSCs were encapsulated in alginate-poly-L-lysine-alginate microcapsules and injected in the ischemic myocardium of pigs. Microencapsulation allowed for increased cell retention in the cardiac tissue. However, treatment failed to improve heart rate, cardiac output and infarct size. An increase in cell loading of the particles may help to enhance therapeutic outcomes [54].

4.2. Local administration of particles for cardioprotection

4.2.1 PLGA

Particle-based drug delivery systems have also been locally injected with the aim of providing cardioprotection to the damaged heart and halt progressive organ dysfunction. IGF1 was administered in the infarcted heart of mice after being successfully encapsulated in PLGA NPs. The enhanced IGF1 retention in the cardiac tissue compared to free IGF1 delivery led to the Akt phosphorylation. This resulted in cardiomyocyte apoptosis prevention, infarct size reduction and improved cardiac function and left ventricular geometry 21 days after treatment [55].

4.2.2. Polyketal

PK3 polyketal NPs were used to deliver Nox2 specific siRNA to the post-MI environment in mice aiming to reduce the oxidative stress in the infarcted myocardium. After three days of intramyocardial administration of NPs, authors described that cardiac function had improved up to normal levels [56]. Later, polyketal NPs prepared following the same manufacturing method were separately loaded with three different microRNAs targeting Nox2. Local administration in a chronic MI mouse model led to the inhibition of Nox2 expression and activity, which resulted in improved ejection fraction, fractional shortening and reduced infarct size [57].

5. Concluding remarks

The development and innovation in fields such as delivery systems, biomaterials and regenerative strategies over the last few years has led to significant advances towards complete repair of the damaged myocardium after a MI. Stem cells and their secretome including micro-RNA packed exosomes, microvesicles and therapeutic proteins are considered as the ideal agents to modulate heart remodeling. Particulate systems such as NPs and MPs have already proved their efficacy in improving the bioavailability of therapeutics and thus their great potential as delivery systems in preclinical studies. Nevertheless, clinical translation remains tricky and more work is mandatory before finding clinically relevant solutions. Future efforts should be focused on deeper understanding of heart repair mechanisms and how delivery systems may interfere with them. Therefore, new

treatments must be designed as integrative strategies where heart repair is understood as the complex process it is and thus addressed globally: from the first inflammatory response to the final scar resolution.

Apart from heart repair mechanisms, other issues must be addressed regarding NPs and MPs. For instance, NPs injected by IV administration may reach other tissue compartments different from the heart, since heart EPR effects do not secure 100% accumulation. Although the targeting of NPs helps to reduce secondary accumulation, more efficient strategies are needed in order to totally avoid possible side effects. Targeting NPs may also be affected by protein corona, which could mask the attached biological moieties, reducing their targeting properties. In particular, the protein corona effect changes particle surface properties, which are responsible for particle interaction with the environment. This must therefore be considered a priority for NP characterization. Regarding MPs, the main concern with these systems is that they are more suitable to be directly injected on the ischemic myocardium. If administered via IV injection, they may cause the occlusion of blood vessels. Our group has shown that MPs could be administered by noninvasive approaches using percutaneous cardiac catheters in a relevant preclinical animal model. Moreover, the size and degradation products of MPs are factors that must be totally controlled in order to avoid increasing the pro-inflammatory heart response. On the other hand, there is very little consistency regarding the animal models used, the characteristics of the particles, the selection of the therapeutic agents, and the evaluation of cardiac repair among the aforementioned works. Unification of criteria would be highly beneficial for joining efforts and facilitating future research that could be conducted under the same conditions. In this sense, special attention must be paid to particles' size. Whereas other parameters such as dose of therapeutic agents present huge inherent variability due to different therapeutic doses among drugs, a universal particle size could be defined in order to avoid blood clearance or enhance tissue integration after IV or local administrations, respectively. For instance, NPs of a mean size between 10 and 100 nm show the lowest elimination rates compared to particles of a larger size. On the other hand, MPs between 5 and 20 μm remain at the site of injection in the myocardium without diffusing to other body compartments without causing excessive inflammation. This would lead to more comparable results that could more easily be extrapolated to clinical practice. For instance, only a few strategies using NPs or MPs for cardiac delivery of therapeutics have been tested in larger preclinical animal models.

In short, the application of drug delivery systems to repair the cardiac tissue or provide cardioprotection after a MI has revolutionized the search for therapeutic and regenerative alternatives to restore cardiac function. In this review, we have given evidence about some promising drug delivery systems, which are sound candidates likely to progress to clinical trials.

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Competing interest

We declare no financial conflicts of interest.

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HYDROGEL BASED APPROACHES FOR CARDIAC TISSUE ENGINEERING

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ABSTRACT

Heart failure still represents the leading cause of death worldwide. Novel strategies using stem cells and growth factors have been investigated for effective cardiac tissue regeneration and heart function recovery. However, some major challenges limit their translation to the clinic. Recently, biomaterials have emerged as a promising approach to improve delivery and viability of therapeutic cells and proteins for the regeneration of the damaged heart. In particular, hydrogels are considered one of the most promising vehicles. They can be administered through minimally invasive techniques while maintaining all the desirable characteristics of drug delivery systems. This review discusses recent advances made in the field of hydrogels for cardiac tissue regeneration in detail, focusing on the type of hydrogel (conventional, injectable, smart or nano- and micro-gel), the biomaterials used for its manufacture (natural, synthetic or hybrid) and the therapeutic agent encapsulated (stem cells or proteins). We expect that these novel hydrogel-based approaches will open up new possibilities in drug delivery and cell therapies.

Keywords: myocardial infarction, cell therapy, protein therapy, hydrogel, biomaterial, tissue engineering.

1. Introduction

1.1. Myocardial infarction and current treatments

In the history of myocardial infarction (MI), the limited regenerative capacity of the heart has been understood as a key restrictive factor when treating the damage caused after an ischemic event, which ultimately results in heart failure and death (Leor *et al*., 2016; Rubin *et al*., 2016; Heng Zhang *et al*., 2016). In fact, if we take a look at the incidence of this disease in the global death rate, MI is responsible for almost 8 million deaths each year (Mendis *et al*., 2011), making it the most deadly cardiovascular disease and the principal cause of death worldwide.

Going deeper into the physiopathology of the disease, MI is initiated when a coronary artery is blocked by a blood clot. As a consequence, the heart region irrigated by this artery loses blood supply and the affected cardiomyocytes (CMs) start dying within minutes to hours of the onset of ischemia, generating an infarcted area. At this stage, progressive morphological and functional changes in the heart muscle are triggered due to the replacement of lost cardiac muscle by a fibrous scar. This scar is unable to contract rhythmically and is not as efficient conductor of electrical signals as CMs (Ongstad and Gourdie, 2016; Pascual-Gil et al., 2015a). As a result, an increase in left ventricular (LV) volume and a thinning of the LV wall take place, which finally leads to relevant deterioration of LV performance, cardiac global function and a high risk of heart failure and death (Kurrelmeyer *et al*., 1998; Ongstad and Gourdie, 2016).

Considering that the lack of functional heart muscle recovery seems to be the most important drawback after a MI, the ideal treatment should address both palliative and regenerative strategies. Thus, MI treatment must first avoid scar and infarct area progression. In addition, it should be able to induce the renewal of CMs and other cardiac cells in order to restore normal organ function. Concerning current treatments for MI such as bypass, balloon angioplasty, stents and pharmacological approaches (Toyoda *et al*., 2013), these are only focused on the palliative aspect, failing to address the fundamental issue of myocyte loss and replacement that underlies incipient cardiomyopathy. Therefore, although current strategies have helped to decrease the mortality rate over recent decades, MI unfortunately still constitutes a major clinical problem that every year causes the death of too many people.

Encouraged by the relevance of MI, a large number of scientists have focused their efforts on developing new therapies for treating this pathology, paying special attention to the regenerative requirements of the heart. Thus, in recent years, there has been an increasing number of data regarding potential new treatments, as will be discussed in the following section.

1.2. New treatments for myocardial infarction

Recent findings in the field of cardiac regeneration have changed previous assumptions and have demonstrated that mammalian hearts, including humans, have the ability to trigger cardiomyogenesis (Bergmann *et al*., 2015; Heusch, 2011), opening new therapeutic doors in the treatment of MI. However, it is important to note that the heart's capacity to induce proliferation of contractile cells is very low and is severely reduced over time (Zacchigna *et al*., 2014), making it insufficient to rescue cardiac function after a MI. Enhancing CM proliferation and recovery in the infarcted area constitutes a promising approach and one of the most important strategies in new therapies for adequate post-ischemic repair (Pascual-Gil et al., 2015a). Along similar lines, angiogenesis (Formiga *et al*., 2012) and recruitment of stem cells (Grimaldi *et al*., 2013; Matar and Chong, 2014) are crucial points that may help to address total heart regeneration.

To date, a number of preclinical and clinical studies have been carried out around the world to try to find the best way to regenerate the infarcted heart. Several strategies have been followed using different therapeutic agents, from siRNA to stem cells including growth factors (GFs) and inflammatory mediators (Awada *et al*., 2016; Feyen *et al*., 2016; Meng and Hoang, 2012; Monaghan *et al*., 2012). Among them, cell and protein therapies are the ones that have reached most success so far (Pascual-Gil et al., 2015a). It is important to note that the latest research trend strongly suggests that controlling the inflammatory response of the heart tissue after a MI may be the critical step to modulate in order to achieve full organ regeneration (Frangogiannis, 2014; Lavine *et al*., 2014; Uygur and Lee, 2016). Nevertheless, although this strategy may be the most promising one, it is still a relatively fresh field of research, and further investigations are needed before obtaining conclusive results.

1.2.1. Cell therapy

Cell therapy is based on the administration of living cells into a damaged organ or tissue to reverse or prevent a disease or condition. In the heart, stem cells are known to improve tissue repair through regeneration of vessels and cardiac muscle cells (Grimaldi *et al*., 2013). A number of different cell sources have already been tested in preclinical and/or clinical studies so far, including bone-marrow-derived mesenchymal stem cells (BM-MSCs), adipose-derived stem cells (ADSCs), induced pluripotent stem cells (iPSs), cardiac progenitor cells (CPCs), endothelial progenitor cells (EPCs) and induced CMs, among others (reviewed in (Pascual-Gil et al., 2015a)). Importantly, benefits derived from stem cells seem to be mainly due to a paracrine effect rather than differentiation towards cardiac lineages (Gnecchi *et al*., 2008). Consequently, GFs and exosomes released by stem cells are the major factors responsible for the therapeutic effects observed (Singla, 2016; Smits *et al*., 2005). Controversial results have been published in this field. Current findings vary from studies where injection of stem cells was related to improvements in cardiac function and angiogenesis, reduction

of fibrosis and generally positive remodeling of the heart (reviewed in (Sanganalmath and Bolli, 2013)) to other reports proving no relation between stem cell administration and recovery of cardiac function or differences with respect to conventional pharmacological treatments (Hirsch *et al*., 2011; Menasché *et al*., 2008; Traverse *et al*., 2011; Vu *et al*., 2012). Concerning the clinical application of stem cells, a limited level of success was obtained when cell therapy was translated into the clinical arena (revised in Emmert et al., 2014; Pascual-Gil et al., 2015a).

1.2.2. Protein therapy

This strategy consists of administering proteins, GFs or cytokines with specific therapeutic actions that can modulate determined biological processes and therefore, control the development of a disease or malignant event. Protein therapy in the heart has been mainly focused on promoting proangiogenic effects, since *de novo* formation of microvessels has the potential to salvage ischemic myocardium at early stages after MI (Cochain *et al*., 2013). To date, several GFs have been studied, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), neuregulin (NRG), hepatocyte growth factor (HGF), and stromal cell-derived factor-1 (SDF-1), among others (Jay and Lee, 2013). Similar to cell therapy, a wide variety of studies have been published examining protein therapy and its applications to treat MI, showing both favorable and unsuccessful results. In preclinical studies, injection of GFs promoted myocardial repair through reducing infarct size, enhancing angiogenesis and cardiac function and recruiting endogenous stem cells into the infarcted area (Awada *et al*., 2014; Tang *et al*., 2011). On the other hand, other researchers could not confirm such promising outcomes, and reported no improvements in cardiac function or infarct size (Engelmann *et al*., 2010; Ott *et al*., 2010). In addition, some authors associated protein administration with a higher risk of suffering adverse cardiac events (Kovacic *et al*., 2008). Remarkably, protein therapy was observed to be ineffective when this strategy was transferred to clinical trials (reviewed in (Jay and Lee, 2013; Pascual-Gil et al., 2015a). However, in general, together with cell therapy, protein therapy is one of the most promising new approaches to treat MI. Although there are still areas for improvement, a great effort is being put into making this strategy a clinical reality in the near future.

1.3. Drug delivery systems

As mentioned above, the results available so far offer contradictory findings regarding the efficacy of cell and protein therapies for MI. Nowadays, it is widely known that the lack of success when using these therapies is due to the harsh microenvironment of the ischemic tissue and the intrinsic characteristics of the therapeutic agents. Regarding cell therapy, poor cell engraftment, fast dissemination from the cardiac tissue, inadequate cell sources and difficulties in the establishment of the optimal timing for cell administration are responsible for the inefficacy of this therapy (Hastings

et al., 2014; Schulman and Hare, 2012; Sheng *et al*., 2013) (Fig. 1). On the other hand, proteins are labile molecules with half-lives of a few hours in the extracellular environment. This degradable nature means that proteins are eliminated rapidly after administration in any biological tissue, which results in low efficacy for this treatment (Hastings et al., 2014; Jain et al., 2013) (Fig. 1). Increasing the quantity of cells or proteins is not the solution, as important side effects could set in when large amounts of therapeutic agents are administered (Tayalia and Mooney, 2009). Therefore, it is of utmost importance to develop vehicles able to enhance cell and protein bioavailability, which act as suitable microenvironments for stem cell growth, survival and differentiation once they are administered (Naderi *et al*., 2011), and which overcome hurdles related to protein instability (Awada *et al*., 2016; Jay and Lee, 2013).

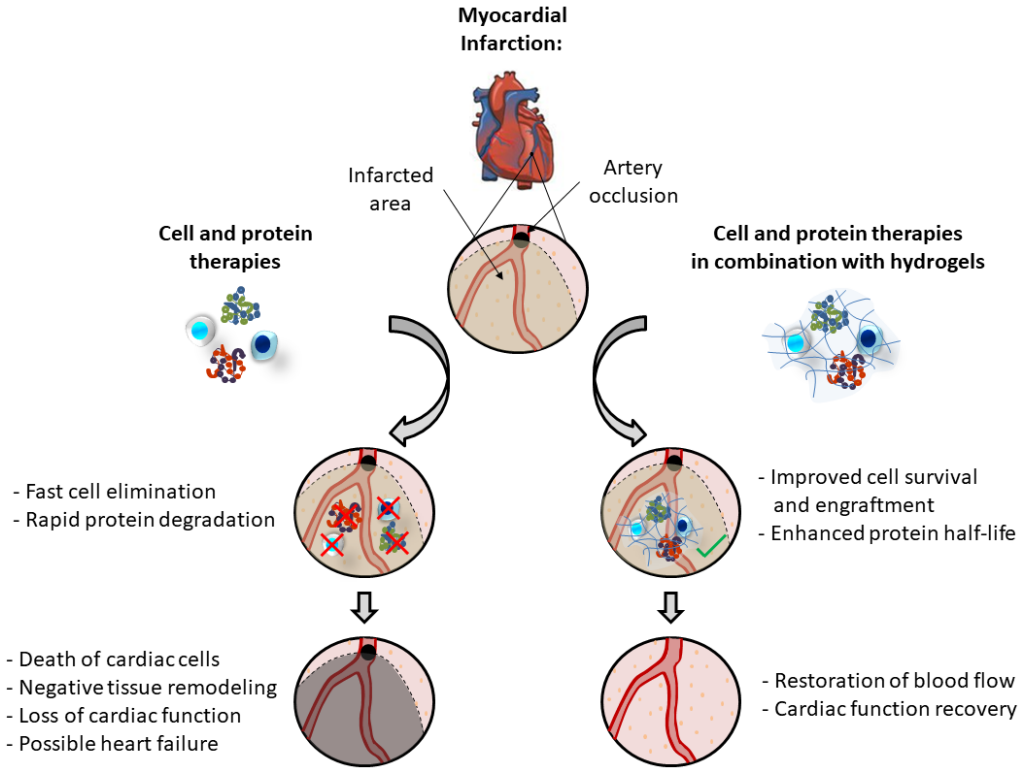

Figure 1. Limitations of cell and protein therapies as new treatments for myocardial infarction and benefits observed when combining such strategies with hydrogels.

In this regard, drug delivery systems (DDSs) made of biocompatible and biodegradable materials have emerged as the perfect carriers for cells and proteins. Nowadays, there is a wide variety of biomaterials available on the market, with different origins and physico-chemical properties, which provide exciting characteristics for each DDS (Cui *et al*., 2016; Rane and Christman, 2011; Venugopal *et al*., 2012). Several DDSs have already been tested in cardiac applications, including nano- and micro-particles (Chang et al., 2013; Formiga et al., 2014, 2013, 2010; Garbayo et al., 2016; Pascual-Gil et al., 2015b), nanofibers (Lin et al., 2012; Simón-Yarza et al., 2015), hydrogels and

liposomes (reviewed elsewhere (Crommelin and Florence, 2013; Spadaccio et al., 2009)). Importantly, all DDSs, regardless of their nature and specific features, share several common characteristics: 1) ability to encapsulate proteins and to protect them against *in vivo* degradation, 2) ability to serve as frames to support cell growth and survival, 3) possibility of being administered by more or less non-invasive techniques and 4) utility as controlled release reservoirs to locally deliver bioactive molecules (Pascual-Gil et al., 2015a). Among them, hydrogels represent one of the most interesting approaches. They are well defined cross-linked matrices that favor cell engraftment, work appropriately as protein reservoirs and can be designed to be liquid under storage conditions and gel only after being injected in the tissue, thus allowing minimally invasive administration (Johnson and Christman, 2013) (Fig. 1). In fact, as will be seen in the following sections, there are several ongoing clinical trials where hydrogels are being tested to treat heart failure, suggesting a promising future for this type of DDSs (McCune *et al*., 2016; Reis *et al*., 2014). Of special interest is the combination of hydrogels with stem cells and cytokines. This combinational therapy has been proven to produce synergistic effects, which improves the efficacy of the treatment when compared to the administration of each factor on its own (Sepantafar *et al*., 2016).

In this review we provide a comprehensive overview of the current state-of-the-art of hydrogels for potential application in cardiac tissue engineering. Recent advances in this field will be discussed, and preclinical and clinical efficacy studies published during the last 5 years using hydrogels will be analyzed. Finally, an outlook on future directions using cardiac hydrogels will be provided.

2. Hydrogels

Hydrogels are 3D cross-linked networks of polymers able to absorb a large amount of fluids, generally water, which becomes their principal component (Gauvin *et al*., 2012). The polymers used to prepare hydrogels are normally hydrophilic, which creates a cross-linked matrix that swells but does not dissolve in water (Zhu and Marchant, 2011). Generally speaking, the high fluid content makes them appropriate for cell seeding and encapsulation. In addition, they are also highly recommended for tissue implantation and other biomedical applications, due to their biocompatibility and excellent diffusion properties (Fujimoto *et al*., 2009). Importantly, the characteristics of the hydrogels depend on the biomaterials used for their manufacture since these will determine important factors such as conductivity, cross-linking, density, degradation and interaction with therapeutic agents (including the release and protection of drugs). In addition, the manufacturing process can also be determining in the final characteristics of the hydrogel, since it can enable precise control of surface topography, size, porosity, mechanical strength and chemical environment (Lakshmanan *et al*., 2013). This ability to control the microscale structure and features of hydrogels allows them to exactly mimic the natural extracellular matrix (ECM), which is the prime objective of DDS fabrication: to develop an analogue of the native ECM to serve as a temporary supportive scaffold.

The design and requirements of DDSs have evolved since the days they were first introduced. They have moved from devices designed to avoid the immune response of the host tissue (Lakshmanan *et al*., 2013), to bioactive, biocompatible and biodegradable DDSs with long-term objectives that include an inert host immune response, long-lasting mechanical and structural support and interaction with the endogenous tissue (Brown and Badylak, 2013). Moreover, current DDSs are able to deliver therapeutic agents to repair and regenerate the diseased tissue without necessitating retrieval of the implant materials. Hydrogels perfectly fit with the current requirements of DDSs, and their development has taken full advantage of advances in biomaterials science.

2.1. Hydrogel composition

Nowadays, hydrogels can be made from biomaterials that can be classified as either natural or synthetic, each one offering interesting properties. Both types of hydrogels are discussed below, as are hybrid hydrogels.

2.1.1. Natural-based hydrogels

Natural biomaterials are known for possessing high bioactivity, biocompatibility and biodegradability (Sepantafar *et al*., 2016). The best advantage that natural biomaterials offer over other materials is that they have intrinsic peptide sequences easily identifiable by cell-surface receptors. Accordingly, cell-biomaterial interactions are more predominant, resulting in favorable cell proliferation and differentiation (Sepantafar *et al*., 2016). On the other hand, natural biomaterials have poor mechanical properties and limited availability (Lakshmanan *et al*., 2013; Zhu and Marchant, 2011). Importantly, they have also proven to be immunogenic in some cases (Ige *et al*., 2012; Zhu and Marchant, 2011). The selection of the optimal natural biomaterial for each case is mandatory in order to obtain the best outcomes and avoid undesired secondary effects. Examples of natural biomaterials used to prepare hydrogels are proteins such as collagen, fibrin, gelatin, keratin, silk fibroin, sericin (Mano *et al*., 2014; Toh and Loh, 2014) and genetically engineered proteins like calmodulin and elastin (Zhu and Marchant, 2011). Other types of natural polymers widely used so far for hydrogel preparation are polysaccharides, including alginate, hyaluronic acid (HA), agarose, dextran, chitosan, cellulose and others that are not so common, such as pullulan, carrageenan and exudate gums (Kim et al., 2014; Mano et al., 2014; Toh and Loh, 2014). In particular, the natural biomaterials that have generated the most promising results for hydrogel production in cardiac tissue engineering are decellularized ECM constructs (biomimetic of the native biochemical and structural matrix composition) and alginate (presenting a structure similar to the ECM) (Landa *et al*., 2008; Sepantafar *et al*., 2016). Decellularization involves the physical, chemical, or enzymatic removal of the cellular content of an organ or tissue, to isolate the ECM (Singelyn and Christman, 2010). Decellularized materials offer the advantage of being cardiac-specific and potentially autologous

injectable therapies. On the other hand, alginate, a natural polysaccharide obtained from brown algae, has been extensively investigated in biomedical applications due to its attractive properties such as biocompatibility, low cost, non-toxicity, non-thrombogenicity and non-immunogenicity (Cheng et al., 2012; Ruvinov and Cohen, 2016). This natural biomaterial also presents some drawbacks, as it has slow and rather uncontrollable degradation (Ruvinov and Cohen, 2016; Silva et al., 2015). Following intracoronary injection, alginate is able to cross the leaky vasculature to the infarcted area. At that point, alginate undergoes gelation due to the local high concentration of calcium ions and forms a hydrogel (Ruvinov and Cohen, 2016). Notably, as discussed in Section 3, both decellularized ECM constructs and alginate biopolymer are among the few biomaterial-based hydrogels that are currently being evaluated in clinical trials for MI treatment.

2.1.2. Synthetic-based hydrogels

The first requirement of any biomaterial, regardless of its natural or synthetic origin, is biocompatibility. To date, thanks to the advances made in the field of biomaterials, several synthetic polymers are now known to be biocompatible and are approved by the Food and Drug Administration for certain applications within the human body (Place *et al*., 2009). Moreover, unlike natural biomaterials, synthetic biomaterials are easily obtained and exhibit high consistency in terms of their production, which allows total control over their composition and physical features, including conductivity, contractibility, density, porosity and hardness. This is translated into high levels of standardization and quality control during the manufacturing process (Jawad *et al*., 2008; Zhu and Marchant, 2011). Nevertheless, synthetic biomaterials lack recognition moieties or patterns for promoting desirable cell responses, which is considered to be their main drawback.

Synthetic materials can be broadly divided into non-biodegradable (maintaining physical and mechanical integrity over time) and biodegradable (ensuring that the biodegradation rate coincides with new tissue regeneration at the defect site). On the other hand, synthetic biomaterials can also be separated into non-bioactive and bioactive polymers (synthetic biomaterials that have bioactive elements attached to favor cell-material interactions) (Zhu and Marchant, 2011). Frequently, synthetic biomaterials comprise monomers that are present in the host metabolic pathway like lactic acid or glycolic acid (Barrett and Yousaf, 2009) or polymers made through industrial procedures such as poly(esters) and poly(urethanes) (Nair and Laurencin, 2006). The most common polymers used in the development of hydrogels so far are polycaprolactone (PCL), poly ethylene-glycol (PEG), poly-lactic acid, poly-glycolic acid and their derivate poly lactic-co-glycolide (PLGA) (Asti and Gioglio, 2014; Lin and Anseth, 2009). Of these, PEG stands out due to its versatility and excellent biocompatibility. Notably, PEG has also been combined with other biodegradable natural or synthetic polymers giving rise to a wide range of hydrogels with different applications in biomedicine (Klouda, 2015). These and other synthetic materials such as poly(N-isopropylacrylamide)

(PNIPAAm) (Ren *et al*., 2012) have already been used for cardiac applications, as will be reviewed in Section 3.

2.1.3. Hybrid hydrogels

One of the prerequisites for further progress in the design of DDSs is the creation of new carriers with precisely defined structures and properties that match the physiological characteristics of the tissue where such systems will be implanted, as well as being bio-recognized and not rejected by the organism (Kopeček and Yang, 2012; Kopeček, 2003). Given the variety of properties necessary for optimizing material activity in the biological environment, multicomponent hybrid hydrogels have attracted significant research interest. In this sense, hybrid polymeric hydrogels combining natural and synthetic polymers have drawn significant and continued attention for their full potential to mimic the ECM in both functional and biological functions (Lau and Kiick, 2015). Therefore, synthetic polymers have been blended with natural biomaterials for improving cellular adhesion and tissue response, while maintaining good manufacture control of physical properties (Klouda, 2015; Place *et al*., 2009; Ravichandran *et al*., 2012). Ideally, these hybrid biomaterials would possess cell-recognizable binding sites as well as enough mechanical stiffness to withstand cardiac contraction and enable tight control over their physical and chemical properties. For instance, VEGF loaded hydrogels made of PEG linked to a fibrinogen backbone proved to have beneficial effects in cardiac recovery after MI (Rufaihah *et al*., 2013). Their specific application in MI will be discussed deeper later in this review. It is interesting that apart from their applications after heart attacks (Rufaihah and Seliktar, 2016), hybrid hydrogels are currently under investigation for a variety of medical applications including spinal cord injury regeneration (Assunção-Silva *et al*., 2015) or brain repair after stroke (Nih *et al*., 2016), demonstrating their great potential for application in regenerative medicine.

2.2. Conventional and emerging hydrogels for cardiac tissue engineering

Cardiac administration of conventional hydrogels has led to enhanced angiogenesis, improved cardiac function and reduced infarct size in comparison to free cell or protein administration (reviewed in Rufaihah and Seliktar, 2016; Sepantafar *et al*., 2016). However, conventional hydrogels present some drawbacks including difficult manipulation of their swelling/shrinking kinetics, slow response rate and poor elasticity properties, which have hindered their practical applications so far (Xia *et al*., 2013; Zhang, 2005). Therefore, great efforts have been concentrated on the development of smart hydrogels, which are able to swap from liquid to gel state depending on external stimuli or initiators, as well as nanogels and microgels.

2.2.1. Smart hydrogels

Smart hydrogels are made of high-performance polymers that change their mechanical or physical properties in response to environmental stimuli (Samchenko *et al*., 2011). Biological structures are mostly formed by polymers characterized by being highly nonlinear, which means that small changes happen in response to a varying parameter until a critical point is reached, and after the transition is completed, there is no significant further response of the system (Galaev and Mattiasson, 1999). This nonlinear response of polymers is what makes them so unique and effective. In fact, smart hydrogels respond with a significant change in structure and properties when they are placed under certain conditions such as pH changes or temperature. Once that change occurs, there is no further change, meaning a predictable all-or-nothing response occurs, with complete uniformity throughout the polymer. In other words, smart hydrogels can mimic the natural feedback of body behavior, offering relevant improvements in drug delivery (Soppimath *et al*., 2002) or injectability (Kopeček and Yang, 2012).

Concerning drug release, the swelling or shrinking of smart hydrogels modifies the diffusion of the drug throughout the DDS, since it depends on the physical state. During the liquid phase, encapsulated therapeutic agents would be released faster than in the gel phase. Thus, the state of smart hydrogels can be controlled to only release the drug of interest under certain circumstances. This strategy was used in the 1990s to control the delivery of insulin (Kataoka *et al*., 1998), and more recently has been applied to other molecules such as plasmid DNA (Gustafson *et al*., 2010) or VEGF (Ehrbar *et al*., 2008). In particular, several smart hydrogels have been administered in the heart with therapeutic goals, either alone (Bastings *et al*., 2014) or loaded with GFs like VEGF (Zhu *et al*., 2015). Regarding *in vivo* administration, smart hydrogels make it possible to improve biocompatibility, reduce toxicity of exogenous cytotoxicity derived from some initiators (Bian *et al*., 2016) and provide convenience to the clinical operations later on (Reis *et al*., 2014). Both drug release and injectability improvements have been translated into direct benefits to tissue engineering technology (Nair, 2016; Samchenko *et al*., 2011). In addition, smart hydrogels have found a place acting as biological actuators and sensors helping, for instance, to regulate homeostasis in the body, as well as working as self-healing devices (Ebara *et al*., 2014; Lim *et al*., 2014).

The classification of environment-sensitive hydrogels is based on the external stimuli that cause hydrogel gelation/fusion (Samchenko *et al*., 2011; Ullah *et al*., 2015). Briefly, according to the nature of the changes in the environment, smart hydrogels can be classified as:

1. *Chemically responsive hydrogels*. In this case, the stimulus that generate the gel are changes in pH and glucose or oxidant concentrations. One of the most famous smart hydrogels is the pH sensitive hydrogel. These gels are composed of polyelectrolytes, which are polymers with a large number of ionic pendant groups, which either accept or donate protons in

response to changes in environmental pH (Kim *et al*., 2014). pH sensitive hydrogels are most frequently used to develop controlled release systems for oral administration (Samchenko *et al*., 2011). Nevertheless, important drawbacks of synthetic pH sensitive hydrogels are their non-biodegradability and the fact that pH changes cannot easily penetrate through materials (Ebara *et al*., 2014). This means that their biomedical applications are reduced (Samchenko *et al*., 2011). In any case, pH sensitive hydrogels have been used for MI treatment, providing promising results (Garbern *et al*., 2011; Koudstaal *et al*., 2014). Glucose and oxidation dependent hydrogels are based on the reduction of the local pH of the system when glucose is converted to gluconic acid by glucose oxidase in the presence of oxygen, which increases the swelling of cationic hydrogels and releases the charge (Ullah *et al*., 2015). No study using glucose and oxidation-responsive hydrogels in the heart is available at present.

2. *Biochemically responsive hydrogels*. This kind of smart hydrogel reacts when it interacts with antigens, enzymes or other types of ligands. Hydrogels in this category are normally degraded when they are placed in contact with a biological molecule that triggers a reaction of degradation of the matrix (Yang *et al*., 2002). In the absence of, for example, a free antigen, the structure of the hydrogel remains intact due to intra-chain antigen-antibody binding in the polymer network (Ullah *et al*., 2015). However, since any organism is a complex closed compartment of self-controlled molecules, it is difficult to guarantee that biochemical responsive hydrogels would be in the desired liquid or gel state and only swap to the other one when needed. Therefore, biochemically responsive hydrogels have not been used in MI treatments yet.
3. *Physically responsive hydrogels*. Factors like temperature, light, pressure, electricity or magnetic field are responsible for the transition between liquid and gel states of physically responsive hydrogels. In general, physical stimuli can be modified easier than other stimuli, making physically responsible hydrogels the preferred option for biomedical applications (Ebara *et al*., 2014). In particular, thermo-sensitive hydrogels are the most widely studied polymer systems (Kim *et al*., 2014). They are characterized by the presence of hydrophobic groups, for instance PNIPAAm or poly(propylene oxide), and exhibit phase separation properties in aqueous solution when the temperature is increased above a lower critical solution temperature, forming the gel. As pointed out previously, temperature is an easy parameter to control in research, and so is light. For this reason both temperature and light responsive hydrogels are the most common smart hydrogels used in cardiac and other applications (Ebara *et al*., 2014). Section 3 of this work will review the in *vivo* studies published so far using physically responsive hydrogels. In the case of light, the stimulus, which is generally UV or visible light, can be imposed instantly and delivered in specific amounts with high accuracy, allowing total control of the physical properties of the hydrogel. The concept that hydrogels may undergo pressure-induced volume phase transition is

explained by the fact that hydrogels that are collapsed at low pressure would expand at higher pressure (Lee *et al*., 1990). Hydrogels sensitive to electricity are usually made of polyelectrolytes, as are pH-sensitive hydrogels, and undergo shrinking or swelling in the presence of an applied electric field (Ebara *et al*., 2014). Electric field as an external stimulator has certain advantages in cardiac applications, for instance the presence of devices that can precisely control current value or electric impulse duration (Samchenko *et al*., 2011). Magnetic hydrogels are based on chemically crosslinked gelatin hydrogels and Fe_3O_4 nanoparticles, and although they can modulate the release of encapsulated factors when an electric field is applied (Liu *et al*., 2006), they have not been proved to have cardiac utility yet.

2.2.2. Nanogels and microgels

As pointed out in the previous sections, hydrogels have emerged as versatile and viable platforms for sustained protein release, targeted drug delivery, and tissue engineering due to their excellent biocompatibility and designable microstructure (Jiang et al., 2014). However, today, scientists and engineers are taking control of atoms and molecules individually, manipulating them and putting them to use with an extraordinary degree of precision. The nano- and microtechnology revolution has allowed the development of new and more specific DDSs. Translated into the field of hydrogels, nano- and microgels have been synthesized during the last decade, constituting the latest research trend in drug delivery (Soni and Yadav, 2016).

Nanogels are hydrogels confined to nanoscopic dimensions with the particularity of being made of polymer nanoparticles swollen in water. Such nanoparticles create three-dimensional networks, formed by chemical and/or physical cross-linking of polymer chains (Sasaki and Akiyoshi, 2010). Microgels have the same characteristics, but they fit in the micrometer scale instead of the nanometer one (Li and Ngai, 2013) (Fig. 2). Although nano- and microgels and conventional hydrogels share some characteristics, like high water content, biocompatibility, and desirable mechanical properties, the first two offer unique advantages over other DDSs (Gonçalves *et al*., 2010; Oh *et al*., 2008). For instance, owing to the reduced scale of the particles they are formed by, nanogels present a large surface area. This offers more possibilities for multivalent bioconjugation of the nanogels, as well as a higher load capacity of therapeutic agents (Oh *et al*., 2008). Thanks to this nanostructure, nanogels are able to respond faster to environmental changes and nowadays smart nanogels are thought to improve nanomedicine working not only as drug carriers but also as imaging and theranostic agents (Molina *et al*., 2015; Sivaram *et al*., 2015). Equally important is the ability of nanogels and microgels to reach body areas and compartments which are not easily accessed by hydrogels (Soni and Yadav, 2016). Thus, for example, nanogels are ideal candidates for intracellular delivery and are more stable for prolonged circulation in the blood stream (Oh *et al*., 2008). On the other hand, microgels cannot

be intravenously administered, being more oriented towards local sustained drug release and 3D cell culture. Finally, the nanogel particle size and surface properties can be manipulated to avoid rapid clearance by phagocytic cells, allowing both passive and active drug targeting (Gonçalves *et al*., 2010). Nevertheless, nanogels offer a fast drug release profile and it is more difficult to control the release of therapeutic agents than in microgels.

Concerning nano and microgel preparation, the methods of fabrication are simple, and importantly, do not involve the use of mechanical energy or organic solvents. In fact, drug loading occurs spontaneously in this type of hydrogel, meaning that the therapeutic agent is not exposed to any vigorous condition during preparation (Vinogradov *et al*., 2004). Two of the approaches most commonly used for preparation of nanogels and microgels are physical self-assembly of interactive polymers and chemical cross-linking of preformed polymers (Arnfast *et al*., 2014; Hui Zhang *et al*., 2016). Whereas physically synthesized nanogels offer a platform to encapsulate various types of bioactive compounds, particularly hydrophobic drugs and biomacromolecules, nanogels prepared by chemical cross-link have a wider application and greater flexibility (Hui Zhang *et al*., 2016). Interestingly, the hydrophilic nature of cross-linked nanogels is nowadays overcome by suitable engineering of the polymer structure, which allows high encapsulation of poorly soluble drugs (Soni and Yadav, 2014).

All this together makes nano and microgels the most promising approach within the field of hydrogels. In fact, nanogels have already been used for delivering a wide range of biomolecules, including genes, oligonucleotides and vaccines (Gonçalves *et al*., 2010). Moreover, they are ideally designed for targeted intracellular delivery of proteins. On the other hand, microgels have been shown to be useful for cell encapsulation and for the local controlled release of proteins. Thus, nano- and microgels combined with the ability of *in situ* gelation have demonstrated great potential as functional extracellular matrices for tissue engineering and as injectable systems for long-term sustained cell and protein release (Jiang et al., 2014). However, nano- and microgels still constitute a new field of research, and few preclinical studies in the arena of cardiac repair have been published so far (Cittadini et al., 2011; Mayfield et al., 2014; McGarvey et al., 2015).

2.3. Hydrogel delivery routes and time of delivery

The administration of a medicine is a common but important clinical procedure. In this regard, hydrogels should ideally be applied using minimally invasive approaches able to avoid open surgery, which facilitates their translation into clinical practice. At present, there are several strategies used for hydrogel administration. The most common ones include epicardial deposition and intramyocardial and intracoronary injections. They allow the best localized release of therapeutic agents at the site of injury (Nelson *et al*., 2011). Additionally, the latter two allow the use of cardiac catheters avoiding invasive surgical procedures.

Conventional hydrogels formed as patches can only be placed into the heart via epicardial deposition, needing an aggressive "open-chest" surgery procedure (Johnson and Christman, 2013; Nguyen *et al*., 2015). On the other hand, the development of injectable hydrogels based on stimuli-sensitive systems has allowed the use of catheter technology. Such hydrogels are administered in liquid state and become gel only when they have reached the myocardium, without blocking any vessel (Leor *et al*., 2009). In this regard, the intracoronary catheter administration of hydrogels has been possible thanks to stimuli-sensitive hydrogels. Liquid systems take advantage of the enhanced permeability and retention effects that occurs after myocardial injury (Galaup *et al*., 2012). Thus, the leaky vasculature allows the transport of materials to enter the infarcted zone (Nguyen *et al*., 2015). Alternatively, the intramyocardial injection using percutaneous-based catheter approaches has been shown to be accurate and reliable in many studies using cells and biomaterials (Amado et al., 2005; Garbayo et al., 2016; Singelyn et al., 2009; Wang and Christman, 2016). It is noteworthy that percutaneous catheters can be used in combination with 3D mapping systems such as NOGA to provide better results.

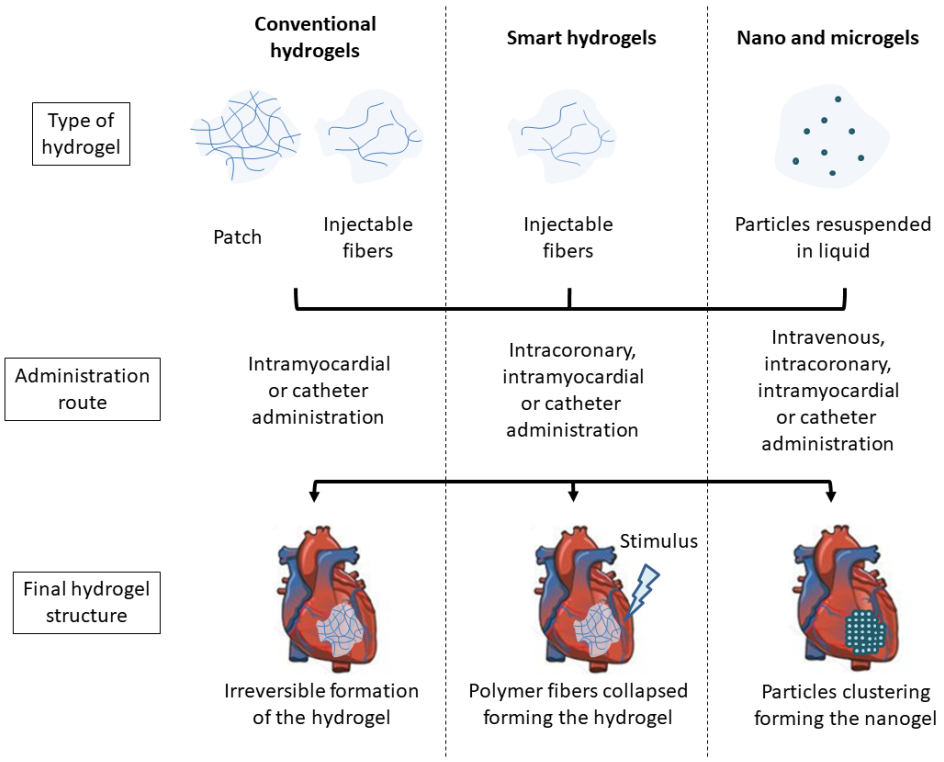

Figure 2. Types of hydrogels used for cardiac repair, administration routes that can be used to deliver each hydrogel into the heart and how hydrogels reach their final structure in the cardiac tissue.

Some hydrogels, such as nanogels, can also be administered by intravenous injection. Interestingly, novel nanogels can be designed for intravenous delivery by using targeted and non-aggregated nanoparticles (Nguyen *et al*., 2015). Intravenous delivery is minimally invasive but can show low

treatment retention Therefore, a great effort is currently being made to improve the biodistribution of the injected nanogels (Clares *et al*., 2012).

Apart from the route of administration, the timing of delivery is a key step that must be taken into consideration for obtaining the best outcomes. LV remodeling is a complex and time dependent process that can be divided into three phases: necrotic (starts after MI), fibrotic (from 3 days after MI to 2 weeks after MI approximately) and chronic (from 2 weeks after MI on out). Delivery of the hydrogel immediately after infarct induction resulted in no observable improvements (Kadner *et al*., 2012), and is unlikely to be clinically acceptable, due to an increased risk of ventricular rupture (Johnson and Christman, 2013; Ungerleider and Christman, 2014). Interestingly, if hydrogels are injected during the fibrotic phase, they may provide improved outcomes such as significant increase in scar thickness and fractional shortening (Kadner *et al*., 2012; Yoshizumi *et al*., 2016). However, other authors have suggested that the earlier the hydrogel is delivered, the better the prevention of negative ventricular remodeling and long-term deterioration of cardiac function (Blackburn *et al*., 2015). In any case, hydrogel degradation seems to play a crucial role in hydrogel success, confirming the importance of the biomaterials used for their preparation.

3. Recent advances in the use of hydrogels as therapeutic tools for cardiac repair

As outlined previously, hydrogels have attracted considerable interest regarding their use in drug delivery, owing to the benefits they offer to cell and protein therapies (Fig. 3). In this regard, many papers have been published involving the use of hydrogels alone or combined with cells and proteins in the context of cardiac repair. In this section, we revise the *in vivo* studies on hydrogels for cardiac regeneration after a heart attack performed during the last 5 years.

3.1. Hydrogels

A wide variety of biomaterials have been explored by many researchers as bulking agents in order to provide mechanical support to the weaken myocardium and prevent LV remodeling after a MI (Tous *et al*., 2011). Furthermore, biomaterials increase the thickness of the heart wall, which reduces wall stress by the law of Laplace. This law stipulates that wall stress is proportional to pressure and radius, and inversely proportional to the wall thickness (Plotkin *et al*., 2014; Tous *et al*., 2011). Previous approaches to combat mechanical changes relied on the surgical application of ventricular restraints on the heart surface to impede infarct expansion and ventricular dilatation, and to force the myocardium to maintain its structure (Silva *et al*., 2015). However, this procedure has never been implemented in routine clinical practice due to its invasive characteristics and the risk that it entails (Silva *et al*., 2015). Instead, hydrogels have gained great popularity thanks to their tunable mechanical properties and the possibility of delivery by non-invasive methods. The mechanical properties of hydrogels are intrinsically important and determine their efficacy as bulking agents. In

this sense, biomaterials must be chosen carefully in order to give the hydrogel the optimum conductivity, elastomeric, stress relaxation, indentation and Young's modulus properties, to match the requirements of heart muscle.

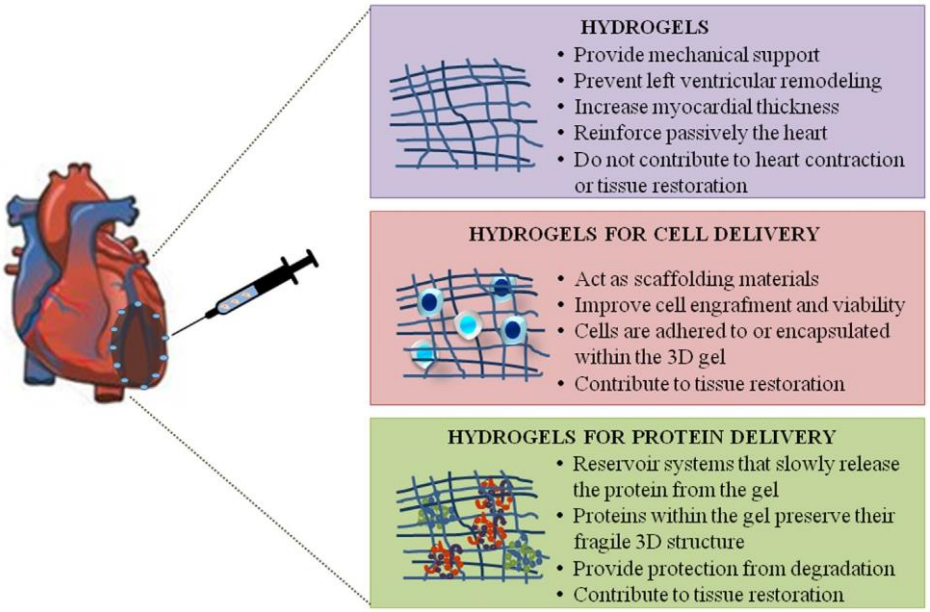

Figure 3. Strategies currently being investigated for MI treatment using hydrogels. Injectable hydrogels can be used as acellular scaffolds or as vehicles for cell and protein delivery.

3.1.1. Natural-based hydrogels

One of the most promising natural biomaterials explored for cardiac tissue engineering is alginate. Two different alginate hydrogels are among the few biomaterial-based scaffolds that have reached clinical trials. The safety and feasibility of one of these, IK-5001 (Bellerophon BCM LLC, previously referred to as BL-1040 from BioLineRx), was assessed in a first-in-man single-arm open-label clinical trial enrolling 27 patients with moderate-to-large ST-segment elevation MI, after successful revascularization (ClinicalTrials.gov Identifier NCT00557531). Within 7 days post-MI, IK-5001, a solution of 1% sodium alginate plus 0.3% calcium gluconate was administered into the infarct-related coronary artery using a catheter via percutaneous radial artery access. Coronary angiography demonstrated that the injection did not impair coronary flow and myocardial perfusion. In addition, cardiac markers were not altered, indicating no further myocardial damage. After 6 months follow-up, favorable tolerability could be confirmed, as well as absence of adverse events, arrhythmias, blood test abnormalities or death. Concomitantly, LV indices and left ventricular ejection fraction (LVEF) were preserved (Frey *et al*., 2014). Encouraged by the positive results of the preclinical and pilot studies, a pivotal trial was subsequently launched, the PRESERVATION-1. This ongoing placebo-controlled, multicenter, randomized, double-bind clinical trial was designed to evaluate the safety and effectiveness of IK-5001 for the prevention of LV remodeling and congestive heart failure

after acute MI (ClinicalTrials.gov Identifier NCT01226563). In this case, 303 patients who had successful percutaneous coronary intervention with stent placement after ST-segment elevation MI were enrolled. After 2-5 days of the surgical intervention, IK-5001 was injected via catheter proximal to the stent of the infarct-related artery. Although the complete outcomes remain unpublished, the results presented so far were disappointing. After 6 months, injectable hydrogel did not prevent LV remodeling compared to saline, nor did it reduce the occurrence of heart failure. Furthermore, other indices including New York Heart Association (NYHA) classification and functional capacity showed no differences associated with the alginate solution injection. The study is expected to finish in 2020 (Zeymer *et al*., 2015).

A different alginate hydrogel referred to as Algisyl-LVR™ (LoneStar Heart, Inc.) was also tested in a first-in-man safety and feasibility trial enrolling 11 patients with dilated cardiomyopathy undergoing open-heart surgery (ClinicalTrials.gov Identifier NCT00847964). Algisyl-LVR™ consists of two components that are mixed immediately before use: an aqueous solution of sodium alginate with 4.6% mannitol and water insoluble calcium alginate particles suspended in 4.6% mannitol solution. During the open-heart procedure, the alginate solution was delivered via 10-15 intramyocardial injections. Echocardiographic findings pointed out continual improvements in mean LVEF, left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and left ventricular end-systolic volume (LVESV) over time during the 24 months of the study. Magnetic resonance imaging was also performed in 4 patients. At 3 months, 2 patients had improvements in LVEF and LV volumes while 2 patients had worsening or no change in LVEF and modest improvements in LV volumes (Lee *et al*., 2015). These changes were accompanied by wall thickness improvement and myofiber stress decrease (L. C. Lee *et al*., 2013). Based on these promising outcomes, a randomized, controlled study was initiated in 78 patients with advanced heart failure receiving standard medical therapy to evaluate Algysil-LVR™ as a method of LV augmentation (ClinicalTrials.gov Identifier NCT01311791). Although currently still ongoing, at 12 months results showed an improvement in peak VO_2, six-minute walk test distance, NYHA assessment, Kansas City Cardiomyopathy Questionnaire and patient global assessment for patients in the Algysil group compared with the control group. Echocardiographic findings revealed favorable but not significant trends towards LVEF, LVEDD, LVESD and LV mass improvement in the Algysil group compared to the control (Mann *et al*., 2015).

The group of Christman *et al.* has great experience in the use of injectable decellularized myocardial matrix hydrogels for cardiac tissue engineering. This biomaterial has been validated in multiple animal models and is currently being tested in clinical trials. The main advantage of these hydrogels resides in their ability to mimic the native myocardial ECM. Thus, they provide biochemical cues to endogenous cells promoting cellular infiltration and differentiation. In addition, ECM-based hydrogels form a nanofibrous hydrogel at body temperature allowing injection through a catheter

(Singelyn *et al*., 2009; Wang and Christman, 2016). Beneficial effects were observed in a sub-acute rat MI model (Singelyn *et al*., 2012; Wassenaar *et al*., 2016). In particular, Singelyn *et al.* found larger areas of myocardium within the infarct region, and a higher density of proliferative cells and cardiac function preservation associated with decellularized matrix therapy (Singelyn *et al*., 2012). Wassenaar *et al*. also described reduced CM apoptosis, enhanced neovascularization and diminished cardiac hypertrophy and fibrosis (Wassenaar *et al*., 2016). Furthermore, an ECM-based hydrogel was for the first time successfully delivered via catheter and NOGA mapping in a porcine model by Seif-Naraghi *et al*. (Seif-Naraghi *et al*., 2013b). As a result, the myocardial matrix produced an improvement in cardiac function, LV volumes, infarct fibrosis, global wall motion scores and enhanced cardiac muscle (Seif-Naraghi *et al*., 2013b). In another study, porcine and human myocardium sources for developing ECM-based hydrogels were evaluated and compared. Despite some differences, the matrices were found to be similar. A human-derived matrix could avoid immunogenic challenges and issues related to xenogeneic disease transfer. However, other concerns related to its more complex processing, changes in ECM composition due to aging and patient-to-patient variability suggest that nowadays human matrices are not a viable option for clinical translation (Johnson *et al*., 2014). Based on the promising data, a phase I clinical trial using the porcine-derived matrix, VentriGel (Ventrix, Inc), has been recently initiated (ClinicalTrials.gov Identifier NCT02305602). This open label study will investigate the effects of percutaneous delivery of VentriGel in patients who have suffered a large ST elevation MI and have evidence of LV remodeling. Currently, the study is enrolling patients and it is estimated that it will be completed in mid-2017.

Chitosan hydrogels have also been tested in cardiac applications. Many of the intrinsic properties of thermosensitive chitosan-based hydrogels such as biodegradability, cytocompatibility and mechanical properties are determined by the degree of deacetylation of this biomaterial, converting chitosan into an easily tunable material (Rodríguez-Vázquez *et al*., 2015). Notably, chitosan hydrogels are liquid at room temperature but transit to gel consistency at 37ºC, which favors their injectability (Henning et al., 2015). In cardiac repair, a chitosan hydrogel injected in a rat MI model successfully reduced LVEDD and increased LVEF after 2 weeks. Furthermore, results showed reduced infarct size, thickened LV wall and increased arteriole density after 4 weeks (Henning *et al*., 2015). Modified hydrogels have also been developed by grafting different molecules to chitosan in order to achieve a more complex regenerative response in the tissue. For instance, the RoY synthetic peptide was introduced to a chitosan hydrogel to stimulate angiogenesis under hypoxia in a rat MI model. This peptide binds specifically to a glucose-regulated protein receptor that is expressed on vascular endothelial cells under hypoxia. In this case, an increase in vessel density accompanied by a reduction in fibrosis and infarct size, increase of ventricular wall thickness and improvement in cardiac function could be observed after 28 days (Shu *et al*., 2015).

HA has become very popular in many bioengineering fields due to its role in several wound-healing processes such as cellular signaling, angiogenesis, inflammation modulation and matrix organization (Abdalla *et al*., 2013; Tous *et al*., 2011). Regarding cardiac therapy, HA-based hydrogels have also been widely explored in recent years. In one example, HA hydrogel injection in the peri-infarcted area of rats substantially improved cardiac function, reduced scar area and collagen deposition and stimulated the formation of novel vasculature compared to control. Although regenerative effects could be appreciated from the third week post-implantation, differences between both groups were more pronounced after 4 weeks (Abdalla *et al*., 2013). Recently, Dorsey *et al*. used magnetic resonance imaging and finite element modeling to assess the impact of a HA injectable hydrogel on infarct tissue in a porcine MI model over a period of 12 weeks. The hydrogel injection led to a trend of reduced LV volumes and increased LVEF at all time points (1, 4, 8 and 12 weeks post-injection) compared to control. However, significant differences between both groups were only found at 1 week and 8 weeks for LV volumes and LVEF, respectively. By contrast, while LVEF of control animals was significantly worse than baseline at all-time points, the hydrogel group reached values similar to baseline after 8 weeks and was maintained at 12 weeks. It should be noted that these results presented great variability which explained controversial outcomes. Increased infarct thickness compared to control was also found at 4 weeks and persisted over the studied period. Concomitantly, finite element model simulations demonstrated that hydrogel therapy increased infarct stiffness for 12 weeks (Dorsey *et al*., 2015). Other interesting findings were described by Yoon *et al*. who proved that the injection time and composition of hydrogel influence final outcomes. Specifically, after testing different molecular weight hydrogels (50, 130 and 170 kDa), these authors found that the 50 kDa HA hydrogel showed the best regeneration and functional recovery. Moreover, the regenerative activity found after the administration of a HA hydrogel in a rat sub-acute MI model was significantly reduced in the chronic model. This underlies the importance of early administration of hydrogel therapy after a MI event as the majority of the ventricular expansion occurs within the first week, as stated elsewhere (Dorsey *et al*., 2015; Yoon *et al*., 2014). In another approach, Tous *et al*. observed reduced LV dilation together with increased thickness of the myocardial wall and enhanced presence of vessels in a sheep MI model when PLGA microspheres were introduced in the matrix of a HA hydrogel as a consequence of an enhancement of tissue bulking (Tous *et al*., 2012).

Less common biomaterials have been also tested during the last few years in cardiac regenerative medicine with promising results. For instance, keratin had never been applied to the heart even though its reparative potential in other tissues is well-documented. In this sense, keratin possesses more than 30 residual cytokines and GFs involved in tissue morphogenesis that could also be beneficial for cardiac repair. Remarkably, a keratin hydrogel has been reported to experience slow degradation, promote angiogenesis, attenuate adverse remodeling, preserve cardiac function and increase the amount of reparative factors such as bone morphogenetic protein 4 and transforming GF

beta 1 in a rat MI model (Shen *et al*., 2011). Another novel scaffold was developed by Song *et al*. who injected sericin, a major constituent of silk produced by silkworms, in the infarcted myocardium of mice where it underwent gelation. Silk sericin hydrogel was shown to reduce scar formation and infarct size, increase wall thickness and neovascularization and inhibit the inflammatory response and apoptosis (Song *et al*., 2016). The encouraging outcomes of these studies have opened the way for exploration of novel biomaterials suitable for cardiac repair.

Composite hydrogels based on the combination of different materials have also been explored in this field. For example, improved cardiac function was detected after 3 weeks of the implantation of a bio-adhesive and biodegradable epicardial patch of HA and lysed blood. The advantage of combining HA with blood resides in the possibility of blood to provide GFs and to be obtained in an autologous fashion. Furthermore, both components contain cell adhesion motifs that could activate endogenous signaling pathways (Chang *et al*., 2012). In another approach, Deng *et al*. explored the benefits of delivering an alginate-chitosan hydrogel to the myocardium of infarcted rats compared to the delivery of only chitosan or alginate hydrogels. The addition of chitosan to alginate helps to overcome some alginate-derived concerns such as limited stability and cell-matrix interactions. The composite hydrogel was found to enhance angiogenesis, reduce inflammation, prevent cell apoptosis, stimulate CM cell cycle re-entry and induce endogenous repair *in vivo*. However, important parameters to evaluate cardiac regeneration such as LV remodeling and cardiac function experienced further improvements following the injection of the alginate-only hydrogel rather than the composite hydrogel. The authors gave various explanations for this fact, such as the shorter gelation time, larger elasticity modulus and slower degradation rate of alginate (Deng *et al*., 2015). Recently, other preclinical studies have been carried out to test the efficacy in a rat MI model of a novel gelatinized capillary-alginate hydrogel (Capgel) (Della Rocca *et al*., 2016) and a chitosan-collagen hydrogel conjugated with a prosurvival angiopoietin-1-derived peptide (Reis *et al*., 2015). In the first case, Della Roca *et al*. found that the injection of the composite hydrogel could improve systolic function over time as assessed by left ventricular fractional shortening (LVFS) (Della Rocca *et al*., 2016). Similarly, Reis *et al*. demonstrated that the chitosan-collagen hydrogel incorporating the prosurvival peptide could improve LVEF, LVFS and decrease the systolic dimension and volumes but failed to reduce the diastolic parameters compared to controls. Interestingly, the incorporation of the pro-survival signal did not decrease the number of apoptotic cells compared to the non-modified hydrogel, but was able to produce a significantly higher survival of CMs in the infarcted area (Reis *et al*., 2015).

3.1.2. Synthetic-based hydrogels

The advantages of synthetic biomaterials have also been exploited for cardiac repair, although not as extensively as those of natural biomaterials. In one study, the effectiveness of two thermoresponsive

PNIPAAm hydrogels with different biodegradation times was assessed in a rat MI model (Ren *et al*., 2012). Hydrogels were prepared by blending PNIPAAm monomer with dextran-poly(ε-caprolactone-2-hydroxyethyl methacrylate) in a 19:1 ratio. Following *in vivo* administration, the time required for complete biodegradation of Gel A was 85 days while Gel B, prepared with twice the amount of dextran, was degraded in 28 days. A pretty complete regenerative response was obtained after 3 months with both gels. In fact, improved contractility, collagen deposition, neovascularization, LV dimension, wall thickness, LVFS, systolic function and infarct size were observed. Of note, contractility and systolic function showed significantly larger improvements in the presence of Gel B compared to Gel A, highlighting the importance of matching the rate of biomaterial degradation and tissue regeneration (Ren *et al*., 2012).

Likewise, biodegradable hydrogels based on PEG polymer and sebacic-acid-diacrylate have been used to make cardiac hydrogels. In this regard, Vilaeti *et al*. compared the beneficial effects obtained with a biodegradable ventricular restraint scaffold to those obtained with epicardial hydrogel application in a rat MI model (Vilaeti *et al*., 2013). A more prominent adverse fibroblastic reaction was observed after scaffold than after hydrogel implantation. LVEF, wall tension index and posterior wall thickness were improved with both approaches compared to control but LVEDD was shorter and sphericity was more attenuated after scaffold implantation compared to hydrogel. In spite of that, the authors pointed to the positive findings found with the latter approach and advocated performing further investigations due to its ease of implantation (Vilaeti *et al*., 2013).

Nowadays, self-assembly peptides are gaining attention in the development of synthetic hydrogels. They are normally formed by a sequence of 8-16 amino acids alternating hydrophilic and hydrophobic residues that form a stable nanofiber hydrogel that mimics the ECM upon exposure to physiological salt concentration of pH (French *et al*., 2016). In particular, two self-assembly peptides, known as RADA16-I and RADA16-II, are of special interest for the delivery of therapeutics in cardiac regeneration. For instance, Boopathy *et al*. functionalized a RADA16-II peptide with a peptide mimicking the Notch1-ligand Jagged1. When tested in infarcted rats, cardiac function was significantly improved and restored up to normal levels. In addition, the incorporation of the Notch1-ligand resulted in decrease fibrosis, increase endothelial vessel are and Ki67 expression compared to controls (Boopathy *et al*., 2015).

3.1.3. Hybrid hydrogels

In an attempt to further optimize hydrogel properties and maximize their beneficial effects, natural and synthetic polymers have been blended to give rise to hydrogels that combine advantages of both types of materials. One example of this was provided by Plotkin *et al*. In this approach, four bioactive hybrid hydrogels, two tetronic-fibrinogen- and two PEG-fibrinogen-based, with different mechanical

properties, were injected in a rat MI model to evaluate the effect of matrix stiffness on cardiac function preservation. Fibrinogen and its main derivate fibrin have been widely used in cardiac tissue engineering. Although fibrin contains multiple cell signaling domains, cell-adhesion motifs and protease degradation sites, control over its physical properties remains problematic. Therefore, conjugation with synthetic materials would help to address this concern (Plotkin *et al*., 2014). In this case, all four hydrogels proved to increase wall thickness, arterial density and viable cardiac tissue in the peri-infarct zone. These findings were accompanied by an improvement in heart function associated with the prevention of the remodeling process. Regarding the purpose of the study to elucidate the influence of matrix stiffness, it should be pointed out that hydrogels with the highest modulus exhibited the best rescue of heart function and highest neovascularization (Plotkin *et al*., 2014).

More recently, a chitosan hydrogel was conjugated with polypyrrole biomaterial to create a conductive scaffold capable of electrically connecting healthy myocardium and viable CMs in the infarct area. As a result, cardiac conduction and function significantly improved after 8 weeks in a rat MI model compared to non-modified hydrogel and saline. Mean LVESD and LVEDD were also improved in both chitosan hydrogels compared to saline (Mihic *et al*., 2015).

3.1.4. Limitations

It is undeniable that important advances have been achieved in the past few years in cardiac regenerative therapy with the administration of injectable hydrogels to reduce the detrimental impact of a MI in the heart. Nevertheless, this approach constitutes a passive strategy where the thickening of the wall is able to support cardiac function and prevent remodeling but do not contribute actively to either the contraction of the heart or tissue restoration (Kloner *et al*., 2015). Alternatively, some authors have hypothesized that passive reinforcement is insufficient to prevent LV remodeling and improve cardiac function. Instead, bioactivity or degradation properties of biomaterials are responsible of the beneficial effects observed (Rane *et al*., 2011). In fact, bioactive components of hydrogels such as cell binding motifs may stimulate endogenous cell recruitment and angiogenesis which results in positive outcomes (Rane *et al*., 2011). Overall, the combination of hydrogels with other therapeutic agents such as cells or GFs would further boost the reparative mechanisms of the cardiac tissue to achieve a more complete response. In addition, a very few preclinical reports have been published related to micro- and nanogels with most of the results being still from *in vitro* studies. Therefore their potential to restore the ischemic heart remain to be fully elucidated.

3.2. Hydrogels combined with cells

The combination of hydrogels with cells has been a widely explored strategy to overcome the issues that restrict the efficacy of cell therapy. Hydrogels help cells to avoid the harmful inflammatory

environment and mechanical washout from the beating myocardium, facilitate their long-term survival and support cellular functions (Gaffey *et al*., 2015). The lack of agreement about hydrogel's composition and characteristics and the cell source that best meets the demanding needs of the infarcted myocardium has led to the development and preclinical testing of different biomaterials and cells in recent years (Table 1). The ideal hydrogel should behave as a supportive physical, chemical and biological scaffold for cells. Therefore, it must to be designed using biocompatible, bioactive and consistent materials that possess intrinsic peptide sequences easily recognizable by cell-surface receptors (Sepantafar *et al*., 2016). Furthermore, it is essential for the matrix to allow the continuous diffusion of biomolecules between the ECM and the encapsulated cells (Seliktar, 2012). Regarding the manufacturing process, it is crucial that hydrogels undergo the gelation process under mild conditions without harming the cells. After polymerization, matrix should be susceptible to be remodeled by endogenous or injectable cells, favoring natural-occurring regeneration. Simultaneously, hydrogels highly vulnerable to proteolysis and degradation are not suitable for cell development (Sepantafar *et al*., 2016). Here, we summarize the most representative strategies combining hydrogels and cells that have been applied to MI treatment.

3.2.1. Natural-based hydrogels

Various studies have proposed the use of chitosan hydrogels to promote stem cell survival and engraftment in the ischemic myocardial microenvironment. As stated before [see section 1.2], regulating the post-MI environment and inflammatory response could be crucial to initiate the regenerative process and avoid damage to the delivered stem cells. For instance Liu *et al*. reported that a temperature-responsive chitosan hydrogel could be effective to attenuate the harsh microenvironment after a MI and therefore, enhance retention, engraftment and survival of ADSCs in the rat ischemic tissue. Investigation of the mechanisms underlying the contribution of chitosan hydrogels showed that they could rescue impaired cellular adhesion by removing reactive oxygen species (ROS), hostile factors responsible for graft cell death and CM injury. In addition, ROS scavenging by chitosan hydrogels increased myocardial homing of c-kit^{+} cells. Importantly, although significant improvements in cell engraftment were achieved, hydrogel encapsulation did not prevent the dramatic decrease of transplanted cells that occurred within the first week and that resulted in a quite low cell survival after 4 weeks. Despite the massive cell loss, several improvements were observed associated with therapy with ADSCs and chitosan hydrogel compared to either alone, including improved cardiac function, suppressed apoptosis, reduced infarct size, preserved wall thickness and enhanced neovascularization. Histological analysis revealed that ADSCs differentiated efficiently to vascular cells and occasionally also to immature CMs (Liu *et al*., 2012). In later work, Wang *et al*. used the same chitosan hydrogel to encapsulate brown ADSCs and obtained similar results. Infarcted rats treated with brown ADSCs/chitosan showed improved cardiac function,

Table 1. Preclinical efficacy studies in the last 5 years using cells and hydrogels combined with cells for treating myocardial infarction.

Biomaterial	Cell source	Animal model	Time of administration post-MI	Delivery method	End-point	Results compared to control	Reference
Natural							
Alginate	MSCs (9-15x10^6)	Pig	4 weeks	Intramyocardial injection	2 weeks	Improved impulse conduction and local conduction velocity. No improvement in fibrosis.	Panda *et al*., 2014
Chitosan	ADSCs (4x10^6)	Rat	Immediate	Intramyocardial injection	4 weeks	Decreased levels of ROS and homing of CSCs. Improved LVFS, LVEF and LVEDP. Reduced apoptosis, infarct size and enhanced neovascularization. Differentiation of ADSCs to vascular cells and CMs.	Liu *et al*., 2012
	BADSCs (4x10^6)	Rat	Immediate	Intramyocardial injection	4 weeks	Improved LVFS, LVEF, LVEDP. Decreased infarct size, fibrosis and enhanced neovascularization. Differentiation of BADSCs to vascular cells and CMs.	Wang *et al*., 2014
	EPCs (7x10^5)	Rat	Immediate	Intramyocardial injection	4 weeks	Increased migration and retention of EPCs. Improved LVEF, cardiac output, maximum pressure, dP/dt max and contractility. Decreased fibrosis and enhanced vascularization.	Gaffey *et al*., 2015
Hyaluronic acid	BM-MNCs (1x10^8)	Pig	10 min	Intramyocardial injection	8 weeks	Improved LVEF, LVEDP, LVEDV, contractility and wall thickness. Reduced scar size and fibrosis. Enhanced neovascularization.	C.-H. Chen *et al*., 2014
	CB-MNCs (1x10^8)	Pig	24 hours	Intramyocardial injection	8 weeks	Improved LVEF, LVEDP, LVEDV and wall thickness. Reduced scar area and fibrosis. Enhanced neovascularization. Differentiation of CB-MNCs to endothelial cells but not to smooth muscle cells.	Chang *et al*., 2016
Collagen	CLCs (5x10^5 or 5x10^6)	Rat	Immediate	Intramyocardial injection	7 weeks	Improved wall thickness (with 5x10^6 cells) and EDPVR. Reduced apoptosis (with 5x10^6 cells). No improvements in LV dimensions, contractile function and hemodynamic parameters. No increase in vascular density.	Qian *et al*., 2012
Gelatin	CMs (5x10^6)	Rat	1 week	Intramyocardial injection	3 weeks	Improved LVEF, LVFS and FAC. No improvements in LVEDD and LVESD nor infarct size. Enhanced vascularization and expression of VEGF, bFGF and HGF.	Nakajima *et al*., 2015
Fibrin	EPCs (1.9x10^6 or 4.5x10^6)	Rat	Immediate	Epicardial patch	4 weeks	Migration of EPCs to the infarcted myocardium. Increased angiogenesis and reduced infarct size and fibrosis. Improved cardiac output, LVEF, contractility, dP/dt max and max generated pressure.	Atluri *et al*., 2014
	ADSCs (2x10^6)	Rat	Immediate	Epicardial patch	6 weeks	Improved LVEF and LVFS. Reduced LVESD, LVEDD, LVESV and LVEDV. No increase in wall thickness. Enhanced expression of anti-inflammatory, anti-apoptotic, anti-fibrotic and angiogenic markers.	Sun *et al*., 2014
Celullose	BM-MSCs (3x10^6)	Rat	Immediate	Intramyocardial injection	8 weeks	Improved LVEF, LVFS and LVESD. No improvement in LVEDD.Reduced infarct size and fibrosis. Increased scar thickness.	Mathieu *et al*., 2012
Matrigel capsules	CPCs (1x10^5)	Mouse	1 week	Intramyocardial injection	3 weeks	Improved LVEF and reduced infarct size.	Mayfield *et al*., 2014
Synthetic							
PEG + alginate capsules	MSCs (1x10^6)	Rat	Immediate	Epicardial patch	4 weeks	Improved LVESD, LVFS and LVEF. No improvements in diastolic dimensions. Reduced scar area and increased microvessel density.	Levit *et al*., 2013
PEG-PCL	iPS derived CMs (2x10^6 to 4x10^6)	Rat	30 min	Intramyocardial injection	2 weeks	Improved LVFS, reduced LV enlargement, smaller LV chamber, thicker LV free wall and less fibrosis.	Wang *et al*., 2015
α-CD/PEG-PCL-PEG	BM-MSCs (2x10^7)	Rabbit	1 week	Intramyocardial injection	4 weeks	Increased LVEF, velocity in systole and early diastole and anterior wall thickness. Reduced LVEDD and infarct size. Enhanced neovascularization.	J. Chen *et al*., 2014
PEG-Fumarate	ESCs (1x10^6)	Rat	1 week	Intramyocardial injection	4 weeks	Improved LVFS, LVESD and LVEDD. Reduced infarct size and fibrotic area. Enhanced neovascularization. Differentiation of ESCs to CMs and vascular cells.	Wang *et al*., 2012
NapFF-NO	ADSCs (2x10^5)	Mouse	Immediate	Intramyocardial injection	4 weeks	Improved LVEF and LVFS. Reduced LVEDD and LVESD. Decreased collagen deposition and cardiac hypertrophy. Increased secretion of VEGF by ADSCs. Enhanced angiogenesis.	Yao *et al*., 2015
PNIPAAm + SWCNTs	BADSCs (2x10^6)	Rat	Immediate	Intramyocardial injection	4 weeks	Improved LVEF and LVFS. Reduced infarct area and increased wall thickness. Differentiation of BADSCs to CMs.	Li *et al*., 2014
RADA16-II	CPCs (1x10^6)	Rat	Immediate	Intramyocardial injection	3 weeks	Improved LVEF, LVESD, stroke work, stroke volume and cardiac output. No improvements in LVEDD. Reduced fibrosis.	Boopathy *et al*., 2014
Hybrid							
PEG/Fibrinogen	CMs (3x10^6)	Rat	1 week	Intramyocardial injection	4 weeks	Smaller and thicker scar and increased wall thickness. Increased LVFS.	Habib *et al*., 2011
	iPS (5x10^5)	Mouse	Immediate	Intramyocardial injection	4 weeks	Increased capillary density and angiogenesis and decreased in fibrotic and apoptotic indexes. Improved LVFS. Functional integration of transplanted cells and host myocardium.	Bearzi *et al*., 2014
HA/Gelatin/PEG	CDCs (1.5x10^5)	Mouse	Immediate	Intramyocardial injection	3 weeks	Improved LVEF. Reduced infarct area and increased wall thickness. Enhanced angiogenesis and disminished apoptosis.	Cheng *et al*., 2012

Table 1 (continued)

Biomaterial	Cell source	Animal model	Time of administration post-MI	Delivery method	End-point	Results compared to control	Reference
poly(NIPAAm-*co*-AAc-*co*-HEMAPCL) /Collagen	BM-MSCs ($1x10^6$)	Mouse	10 min	Intramyocardial injection	4 weeks	Reduced interstitial fibrosis, increased capillary density and improved LVEF and LVFS.	Xia *et al*., 2015
OAC-PEG-OAC/Collagen	BM-MSCs ($3x10^6$)	Rat	30 min	Intramyocardial injection	4 weeks	Improved LVEF. Reduced infarct size, increased wall thickness and arteriole density.	Xu et al., 2014
PCL-NIPAA-HEMA-DBA/Collagen	CDCs ($3.5x10^5$)	Rat	Immediate	Intramyocardial injection	4 weeks	Improved LVEF and reduced infarct size.	Matsushita *et al*., 2016

ROS: reactive oxygen species. *LVFS*: left ventricular fractional shortening. *LVEF*: left ventricular ejection fraction. *LVEDP*: left ventricular end-diastolic pressure. *LVEDV*: left ventricular end-diastolic volume. *EDPVR*: end-diastolic pressure volume relationship. *FAC*: fractional area change. *LVEDD*: left ventricular end-diastolic diameter. *LVESD*: left ventricular end-systolic diameter. *LVESV*: left ventricular end-systolic volume. *LVEDV*: left ventricular end-diastolic volume.

decreased infarct size and fibrosis and neovascularization. Importantly, neovascularization was partially attributed to the differentiation of transplanted cells to vascular cells. In comparison with ADSCs, more brown ADSCs differentiated to CMs (Wang *et al*., 2014).

HA-based hydrogels have also been used to retain stem cells after their injection in the heart. For instance, enhanced cellular retention and migration to the damaged tissue was observed when EPCs were encapsulated in a shear-thinning HA hydrogel and tested in a rat MI model. Shear-thinning hydrogels are pre-formed before injection but with the application of a shear force they are able to deform and flow through a syringe and then reassemble again at the injection site. After 4 weeks, according to the potential of EPCs to promote vasculogenesis and the higher engraftment of cells achieved by hydrogel encapsulation, a greater number of blood vessels were observed following combinational treatment with HA hydrogel and EPCs compared to direct cell injection. Regarding cardiac remodeling and function, important improvements were observed. Administration of either hydrogel or cells alone produced a reduction of fibrosis and scar fraction accompanied by an increase in LVEF, cardiac output and contractility. Interestingly, better outcomes were observed after combinational therapy (Gaffey *et al*., 2015).

Apart from rodent MI models, positive results were obtained encapsulating stem cells in HA hydrogels in a clinically relevant animal model by Chen *et al*. In this case, autologous bone marrow mononuclear cells (BM-MNCs) were encapsulated in a HA hydrogel and intramyocardially injected 10 minutes after MI. One month later, LVEF and systolic and diastolic interventricular septum thicknesses were significantly improved and those improvements were maintained after 2 months. In fact, LVEF experienced a progressive increase over the duration of the study in animals that received hydrogel/BM-MNCs while this parameter decreased in control groups. Moreover, encapsulation of BM-MNCs in a HA hydrogel successfully augmented the number of grafted cells, improved systolic and diastolic function, increased left ventricular end-diastolic pressure (LVEDP) and left ventricular end-diastolic volume (LVEDV), reduced scar size and fibrotic area and induced the formation of capillaries and arterioles (C.-H. Chen *et al*., 2014). More recently, Chang *et al*. reported that the use of autologous BM-MNCs could not be the best source for cell therapy owing to

the decrease in the regenerative capability of these cells as a result of aging or chronic diseases. Instead, authors suggested human umbilical cord blood MNCs (CB-MNCs) as a better alternative for cardiac repair. Therefore, these cells were encapsulated in a HA hydrogel and injected in a pig MI model 24 hours after the induction of ischemia. Animals were previously immunosuppressed to avoid immunological issues that could compromise therapy success. After 2 months, the group that received the combinatorial treatment showed the highest LVEF as well as improved systolic and diastolic function compared to controls. In addition, the injection of CB-MNCs alone or in combination with HA hydrogel significantly decreased scar area and fibrosis, and promoted angiogenesis in the infarcted region. Assessment of the fate of transplanted CB-MNCs indicated that these cells differentiated to endothelial cells but not smooth muscle cells (Chang *et al*., 2016).

The lack of elucidation about the best scaffold for cardiac repair led Qian *et al*. to develop a comparative study about the efficacy of transplanting CM-like cells (CLCs) in different scaffolds: a collagen-based epicardial patch, a low-dose injectable collagen hydrogel (LDH) with the same number of cells as the patch or a high-dose injectable collagen hydrogel (HDH) with 10 times more cells (Qian *et al*., 2012). To this end, CLCs were obtained from human MSCs after being subjected to a differentiation procedure. Controversial results were obtained in a rat MI model. The patch provided a better cellular retention compared to the injectable hydrogel which resulted in a partially improved cardiac function. In fact, similar cell retention was obtained in the cell patch group and the HDH, where 10-fold more cells were injected. Wall thickness was significantly increased in the cell-seeded patch and HDH groups compared with LDH and control groups while only the cell-seeded patch and acellular patch groups were able to decrease wall stress. Apoptosis was reduced in all the groups containing CLCs but not in acellular groups. By contrast, no improvements were found in any group related to LV dimensions and vascular density (Qian *et al*., 2012). More recently, in another report it was suggested that collagen administration may induce the aggregation of platelets leading to a coronary microembolization, whereas the use of gelatin, a biomaterial made of collagen, does not trigger this thrombogenic effect (Nakajima *et al*., 2015). Hence, in this case rat CMs were transplanted with a gelatin hydrogel in the myocardium of rats one week after induced MI, resulting in an enhanced cellular engraftment compared to direct cell administration. After 3 weeks, LVEF, LVFS and fractional area change (FAC) were significantly increased in the cells/hydrogel group while neither hydrogel nor CMs alone improved cardiac function. By contrast, similar LV dilatation was detected in all the groups. The same functional study was performed on infarcted rats injected with hydrogels seeded with five-fold less cells. In this case, cellular therapy failed to recover cardiac function, suggesting that therapeutic effects depend on the cell number. Moreover, a smaller infarct size and higher angiogenesis were observed in the cells/hydrogel group compared to controls (Nakajima *et al*., 2015).

Fibrin hydrogels have also been devised for improving stem cell-based therapies in the context of MI. In a recent study, Atluri *et al*. reported a robust vasculogenic response to therapy with a fibrin hydrogel encapsulating EPCs in a rat MI model. In this study, hydrogels were sutured to the epicardial surface. After 1 week, a high rate of EPCs had migrated from the hydrogel to the ischemic tissue. In fact, there was a greater number of transplanted cells within the myocardium in the EPCs/hydrogel group compared with the free EPCs group. Consistent with this, increased vasculogenesis was observed in the EPCs/hydrogel group after 4 weeks, which seemed to have contributed to scar size reduction and cardiac function improvements. An interesting finding was that not only EPCs contributed to vasculogenesis but also the fibrin patch itself, as it was evidenced in the patch only group, thereby maximizing the vasculogenic response. Furthermore, the reparative efficacy of patches with different fibrin concentrations and EPCs densities was compared. Best results were found after treatment with the highest fibrin concentration and cellular density (Atluri *et al*., 2014).

Microgels have also been explored to enhance engraftment of transplanted cells as is the case with CPCs encapsulated in matrigel-based microcapsules. Although encapsulation did not fully prevent cell loss (only 10% of CPCs remained in the tissue after 3 weeks), injection of the CPCs/capsules in the ischemic myocardium of mice resulted in better cardiac function and a lower degree of fibrosis compared with the non-encapsulated CPCs (Mayfield *et al*., 2014).

3.2.2. Synthetic-based hydrogels

Although synthetic hydrogels lack biological functions and may not be as suitable for cell encapsulation as their naturally-derived counterparts, positive results could be also detected after transplantation of stem cells in several of these hydrogels. In particular, PEG hydrogels are especially useful as scaffolds for promoting stem cell survival. Interestingly, hydrogels based solely on PEG do not break down easily and cannot support cell culture. However, PEG hydrogels can be easily modified to incorporate chemical and physical cues able to control cellular functions. In this regard, autologous CMs differentiated from human iPSs were seeded in a temperature-sensitive hydrogel composed of PEG and PCL. In order to increase the binding of the hydrogel to the infarcted area, a collagen-binding peptide was also immobilized in the matrix. When tested in a rat MI model, all groups presented ventricular dilation and decreased LVFS after 2 weeks. Nonetheless, the encapsulated iPS group showed significantly less worsening of these parameters. Furthermore, only animals that received iPSs/hydrogel had thicker LV free wall and less fibrosis compared to the control group, suggesting that neither cells nor material alone produced any benefits. These positive findings could be attributed to the enhanced survival of transplanted cells in this group compared to direct cell injection as evidenced by the presence of iPSs in the myocardium at 2 weeks only following delivery in combination with the polymer (Wang *et al*., 2015). A similar strategy was

developed by Chen *et al*. with the aim of enhancing the retention of BM-MSCs in the ischemic myocardium and hence, boost cardiac repair. In this case, poly(ethylene glycol)-d-polycaprolactone-(dodecanedioic acid)-polycaprolactone-poly(ethylene glycol) polymer was blended with α-cyclodextrin to form a self-assembly synthetic hydrogel. BM-MSCs were seeded and the efficacy of the system was tested in a rabbit MI model. The administration of the hydrogel alone gave rise to the same results as the control group. Conversely, positive outcomes were found in the free BM-MSCs and BM-MSCs/hydrogel groups including improved LV dilation, wall thickness, LVEF and infarct size, with further improvements in the latter group. Besides, encapsulated BM-MSCs produced greater neovascularization compared with all other groups (J. Chen *et al*., 2014).

Since low cell retention and survival in the targeted tissue is considered the main factor limiting cell therapy success, Levit *et al*. designed a more sophisticated approach in order to further improve this aspect (Levit *et al*., 2013). First, MSCs were encapsulated in alginate capsules, and then capsules were seeded in a PEG hydrogel patch. The system exerts a dual function on transplanted cells: alginate capsules act as a mechanical barrier preventing washout of cells from the myocardium and concomitantly, the PEG hydrogel patch secures the capsules to the ischemic heart. Both capsules and hydrogel were designed to allow free diffusion of cytokines and molecules. 4 weeks after implantation in a rat MI model, cardiac function, infarct area and microvessel density were improved only after treatment with encapsulated cells plus hydrogel. In this regard, the administration of non-encapsulated MSCs seeded in the PEG hydrogel, hydrogel seeded with empty capsules or hydrogel alone did not produce any improvements compared to control (Levit *et al*., 2013).

Beyond PEG-based hydrogels, interesting results have been obtained by the administration of cells encapsulated in other synthetic matrices. In particular, ADSCs were suspended in a hydrogel synthesized by the combination of naphthalene with a short peptide, FFGGG, and β-galactose caged nitric oxide (NO) which can release NO in response to β-galactosidase (NapFF-NO). The efficacy of the system to trigger a regenerative response was tested in a mouse MI model. First, it was confirmed that the encapsulation of cells in the hydrogel allowed an enhanced cell retention that was further increased in the NO releasing group. Besides, the incorporation of caged NO in the hydrogel stimulated the release of VEGF from transplanted ADSCs resulting in a higher density of blood vessels and reduced cardiac hypertrophy compared to co-transplantation of cells in a hydrogel lacking NO molecule. However, the enhanced angiogenesis in the ADSCs-NapFF-NO group was not fully translated to cardiac function and collagen content, since the administration of encapsulated cells improved functional parameters, regardless of the presence or absence of NO (Yao *et al*., 2015).

In another approach, brown adipose tissue derived stem cells (BADSCs) were seeded into a PNIPAAm hydrogel modified with single-wall carbon nanotubes (SWCNTs) to improve scaffold bioactivity, especially adhesion of the matrix to transplanted cells. After 4 weeks of administration

in a rat MI model, a further recovery of cardiac function and inhibition of cardiac remodeling could be detected in the BADSCs/hydrogel group compared to treatment with either component alone, but which only produced small improvements. Remarkably, BADSCs were able to differentiate to CMs in the MI environment, which could be one of the reasons for the regeneration response observed (Li *et al*., 2014).

Self-assembly peptides have also been used to deliver cells to the heart. For instance, Boopathy *et al*. seeded CPCs in a RADA16-II hydrogel and administered the system in a rat MI model. A mimetic peptide of ligand Jagged1 had previously been tethered to the matrix to activate the Notch signaling pathway, fundamental in cardiac development and differentiation of cardiac stem cells, in transplanted cells. As a result, cardiac functional parameters and fibrosis were significantly improved compared to the non-modified hydrogel group (Boopathy *et al*., 2014).

3.2.3. Hybrid hydrogels

The combination of natural and synthetic materials has also brought interesting benefits in the field of biomaterial-based cellular therapy. In particular, some studies have blended PEG with natural polymers to formulate hydrogels suitable for cell transplantation (Bearzi *et al*., 2014; Habib *et al*., 2011). In a first approach, Habib *et al*. PEGylated fibrinogen to synthesize a photopolymerizable and biodegradable hydrogel matrix where neonatal rat ventricular CMs were seeded. The permanence of the hydrogel in the myocardium was augmented from less than one month in the absence of additional PEG-diacrylate (PEG-DA) crosslinker to more than one month by the incorporation of 2% additional PEG-DA. Owing to the treatment with combined CMs and hydrogel, rat hearts experienced an increase in LVFS and functional outcomes after 4 weeks. Smaller and thicker scar and higher wall thickness were also detected in the CMs-hydrogel group compared to controls. Next, the authors also assessed the feasibility of transplanting human embryonic stem cell derived CMs. This resulted in favorable cardiac remodeling with a relatively thickened infarcted area, a non-dilated LV chamber and increased LVFS (Habib *et al*., 2011). In a related approach, Bearzi *et al*. used a PEGylated fibrinogen hydrogel to deliver iPSs derived from neonatal mouse CMs to the ischemic myocardium of mice. To further improve vascularization and cellular engraftment, iPSs were engineered to overexpress placental growth factor or matrix metalloproteinase 9. Animals that received hydrogel seeded with iPSs, regardless of whether cells were engineered or not, showed increased capillary density and LVFS and reduced fibrotic and apoptotic indexes. However, the combination of both type of engineered iPSs within the hydrogel resulted in further improvements. Importantly, transplanted iPSs were found to integrate functionally with the host myocardium (Bearzi *et al*., 2014).

Apart from PEG-containing hydrogels, PNIPAAm is another biomaterial that has been explored for the formulation of hybrid hydrogels and subsequent cell encapsulation. For example, Xia *et al*.

copolymerized PNIPAAm with acrylic acid and 2-hydroxyethyl methacrylate-poly(ε-caprolactone) in a ratio of 88:9.6:2.4 to synthesize poly(NIPAAm-co-AAc-co-HEMAPCL). Then, type I collagen was conjugated with the polymer to enhance biocompatibility. This process led to the formation of a thermosensitive smart material that was used for BM-MSCs encapsulation and tested in a mouse MI model. After 4 weeks, favorable improvements in cardiac function were observed associated with therapy with either hydrogel or cells alone, although the combination of both produced better outcomes. Similar patterns were observed for capillary density and interstitial fibrosis (Xia *et al*., 2015). Likewise, cardiac function and remodeling were significantly improved in infarcted rats following administration of a hybrid hydrogel based on thiolated collagen and multiple acrylate-containing oligo(acryloyl carbonate)-b-poly(ethylene glycol)-oligo(acryloyl carbonate) copolymers and seeded with BM-MSCs. Interestingly, although BM-MSCs improved LVEF, only combined therapy with cells and hydrogel could restore cardiac function up to normal levels, possibly as a consequence of enhanced engraftment, which also resulted in decreased infarct size and increased wall thickness and arteriole density (Xu *et al*., 2014).

3.2.4. Limitations

Unfortunately, the development of strategies based on cell delivery presents some limitations that should be considered. First, cellular therapies require dedicated centers able to afford the high-cost resources needed to isolate, culture and handle stem cell products. Secondly, the use of autologous sources needs a time span to collect and culture cells from the patients, which is not always available in patients suffering a MI event (Koudstaal *et al*., 2014). Moreover, the regenerative potential of autologous cells could be restricted due to aging and chronic diseases. By contrast, allogeneic sources from healthy patients would provide a better cardiac repair but entail possible immunological problems (Dhingra *et al*., 2013). One critical aspect that requires further investigation is to understand, from a mechanistic point of view, stem cell behavior in response to hydrogels. Furthermore, some hydrogels are formed very fast on the implantation area forming a consistent structure that does not allow the correct oxygen and nutrient exchange for stem cell survival.

3.3. Hydrogels for protein delivery

Sustained release of therapeutic agents takes on special importance in the heart since this organ is very vascularized and blood flow constantly removes delivered proteins from the tissue (French *et al*., 2016). In this sense, encapsulation of bioactive molecules in hydrogels allows local sustained release over time, ensuring a prolonged therapeutic effect. Additionally, hydrogels enable control over the rate of diffusion of biomolecules from the matrix to the ECM by altering their chemical and physical properties. They also provide a platform that protects agents from rapid *in situ* degradation due to proteinases. Regarding the loading of proteins into hydrogels, this can be achieved via

different strategies including physical entrapment, surface adsorption and affinity binding to specific ligands of the matrix. Overall, since hydrogels appeared on the scene as a suitable scaffold to encapsulate and deliver proteins, several therapeutic strategies have now been pre-clinically tested (Table 2). Generally speaking, hydrogels intended to deliver proteins should be designed with adequate pore size and porosity to ensure the retention and prolonged sustained release of encapsulated molecules. Moreover, smart hydrogels made of intelligent materials have become increasingly important in the hydrogel protein delivery field because of their ability to release therapeutic payloads in response to, for instance, disease-related stimuli.

3.3.1. Natural-based hydrogels

Given the biocompatibility of HA, there is great interest in the development of HA-based hydrogels for controlled protein delivery to the infarcted myocardium. For instance, positive results were obtained when NRG was encapsulated in a HA hydrogel and the system was tested in infarcted mice. After 6 days, NRG could be detected in the myocardium of NRG/hydrogel-treated animals while this was not possible when NRG was injected in saline. As a result, significantly enhanced CM mitotic activity and cardioprotection were observed. Echocardiographic assessments 2 weeks post-treatment showed reduced LV dilation and improved LVEF in the NRG/hydrogel group compared with controls (Cohen *et al*., 2014). Other therapeutic agents have also been successfully released in a sustained manner in the infarcted myocardium thanks to encapsulation into a HA hydrogel, as is the case with SDF-1 and engineered SDF-1 analogue (ESA) (MacArthur *et al*., 2013; Song *et al*., 2014). SDF-1 and ESA are key factors that induce recruitment of bone marrow stem cells to areas of ischemia (Song *et al*., 2014). In an acute rat MI model, a reduction was observed in LV areas and infarct fraction accompanied by an increase in capillary density following ESA/hydrogel therapy. Furthermore, this was translated into a better cardiac function with significant improvements in LVEDD, LVEF, cardiac output and contractility. Of note, the hydrogel group alone produced some benefits, therefore it is likely that positive outcomes resulted from the synergistic effect of the HA hydrogel itself and ESA (MacArthur *et al*., 2013). Further evidence about the promising restorative effects of SDF-1 encapsulated in a HA hydrogel was provided by Song *et al.* (Song *et al*., 2014). It is noteworthy that in this case, treatment was assessed in a chronic MI rat model where regeneration raises a more challenging issue. N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) angiogenic peptides were also immobilized in the hydrogel matrix to boost endogenous response. Compared to non-treated infarcted animals, dual therapy with SDF-1 and Ac-SDKP resulted in the inhibition of cardiac remodeling, an increased number of arterioles and capillaries and functional recovery up to normal levels after 4 weeks. SDF-1 proved to be effective in the recruitment of stem cells since these cells were observed in the groups containing the mentioned GF but not in other groups (Song *et al*., 2014).

Table 2. Preclinical efficacy studies of the last 5 years using hydrogels combined with proteins for treating myocardial infarction

Biomaterial	Protein	Animal model	Time of administration post-MI	Delivery method	End-point	Results compared to control	Reference
Natural							
Alginate	GH (5 μg)	Rat	10 min	Intramyocardial injection	3 weeks	Improved LVEDD, LVESD, ventricular sphericity, wall tension index and infarct thickness. Enhanced angiogenesis. No improvements in LVEF, fibrosis and infarct area.	Daskalopoulos *et al.*, 2015
Alginate + PLGA microspheres	TAT-HSP27 (375 ng)	Rat	Immediate	Intramyocardial injection	1 week	Improved LVEF, LVESV and maximum pressure development. Reduced fibrosis. Suppressed apoptosis.	Lee *et al.*, 2013
Alginate + gelatin nanoparticles	IGF-1 (100 ng) + 6-Bromo-indirubin-3-oxime (17,8 ng)	Rat	1 week	Intramyocardial injection	6 weeks	Improved LVEF, LVFS, LVEDD and LVESD. Enhanced proliferation of CMs and revascularization.	Fang *et al.*, 2015
Chitosan	$VEGF_{165}$ (5 μg) + bFGF (5 μg)	Dog	Immediate	Epicardial INJEX needle-free injection system	6 weeks	No improvement in infarct size and fibrotic area. Open channels with luminal endothelization. Increased density of small vessels.	Zhou *et al.*, 2013
	ESA (25 μg)	Rat	Immediate	Intramyocardial injection	4 weeks	Increased LVEDD, LVEF, cardiac output and contractility. Reduced LV areas, infarct fraction and increased capillary density.	MacArthur *et al.*, 2013
	SDF-1 (500 μg)	Rat	4 weeks	Intramyocardial injection	4 weeks	Decreased infarct size and greater wall thickness. Increased angiogenesis. Improved stroke work, LVEF, cardiac output and arterioles elastance.	Song *et al.*, 2014
Hyaluronic acid	rTIMP-3 (20 μg)	Pig	Immediate	Intramyocardial injection	2 weeks	Improved LVEF, decreased LVEDD and LVEDV. Attenuated MI expansion. Reduced proinflammatory cytokines and increased myofibrobast proliferation.	Eckhouse *et al.*, 2014
	rTIMP-3 (20 μg)	Pig	Immediate	Intramyocardial injection	4 weeks	Reduced LV dilation and increased wall thickness. Improved LVEF and PCWP.	Purcell *et al.*, 2014
	NRG (2.5 μg)	Mouse	Immediate	Intramyocardial injection	2 weeks	Increased CM proliferation and cardioprotection Improved LVEF, decreased LV area and increased border zone thickness	Cohen *et al.*, 2014
Extracellular matrix	bFGF (10 μg)	Rat	1 week	Intramyocardial injection	5 days	Increased functional arteriole density	Seif-Naraghi *et al.*, 2013
	HGF fragment (10 μg)	Rat	1 week	Intramyocardial injection	4 weeks	Improved FAC, prevented LV remodeling and increased arteriole density.	Sonnenberg *et al.*, 2015
Gelatin microspheres	VEGF + IGF-1 (dose not specified)	Rat	10 min	Intramyocardial injection	4 weeks	Improved systolic and dyastolic function. Decreased infarct size and increased CM diameter. Enhanced angiogenesis. Reduced inflammation and apoptosis.	Cittadini *et al.*, 2011
Collagen/ Chitosan	Thymosin β4 (1.5 μg)	Rat	Immediate	Intramyocardial injection	3 weeks	Increased wall thickness. Enhanced vascularization.	Chiu *et al.*, 2012
Synthetic							
PEG	HGF (1 μg) + VEGF (1 μg)	Rat	Immediate	Intramyocardial injection	3 weeks	Increased angiogenesis, progenitor cell migration, reduced fibrosis. Improved LVEDV, LVESV and cardiac function.	Salimath *et al.*, 2012
PEG microrods	MGF E domain (1 μg)	Mouse	10 min	Intramyocardial injection	10 weeks	Improved LVEF, LVESV, dP/dt max, dP/dt min and contractility.	Peña *et al.*, 2015
sP(EO-*stat*-PO)	Met-CCL5 (0.5 μg) + SDF-1 (3 μg)	Mouse	Immediate	Intramyocardial injection	4 weeks	Reduced infarct area. Prevented neutrophil infiltration. Improved LVEF and increased neovascularization but without improvements in LVEDD and LVESD.	Projahn *et al.*, 2014
UPy-PEG	HGF (200 ng) + IGF-1 (200 ng)	Pig	4 weeks	Intramyocardial catheter	4 weeks	Reduced infarct scar.	Bastings *et al.*, 2014
UPy-PEG	HGF (1 μg) + IGF-1 (1 μg)	Pig	4 weeks	Intramyocardial catheter	4 weeks	Reduced LVESD and hypertrophy. Improved LVEF and FAC. Formation of new CMs and increased eCSCs population at the border zone. Increased capillary density.	Koudstaal *et al.*, 2014
Poly(NIPAAm-*co*-HEMA-*co*-MAPLA)+ PLGA microparticles	bFGF (10 μg) + IGF-1 (400 ng)	Rat	2 weeks	Intramyocardial injection	16 weeks	Improved FAC. Reduced LV dilation and increased wall thickness. No improvements in fibrosis. Similar results regardless of the incorporation of GFs.	Nelson *et al.*, 2014
Hybrid							
Dex-PCL-HEMA/ PNIPAAm	HMGB1 (2.5 μg)	Rat	Immediate	Intramyocardial injection	4 weeks	Reduced LV diameter and collagen content. Improved LVEF. Increased angiogenesis and proliferation of cardiac stem cells.	He *et al.*, 2013
Dex-PCL-HEMA/ PNIPAAm	$VEGF_{165}$ (2 μg)	Rat	Immediate	Intramyocardial injection	4 weeks	Reduced LV diameter, infarct size and collagen content. Improved LVEF. Increased angiogenesis and inhibited apoptosis.	Zhu *et al.*, 2015
PEG-DA /Fibrinogen	VEGF-A (5 μg)	Rat	Immediate	Intramyocardial injection	4 weeks	Improved FAC, LVEF, cardiac output and contractility. Reduced LVEDD, LVESD, LVEDV, LVESV and infarct size. Enhanced angiogenesis.	Rufaihah *et al.*, 2013

Table 2 (continued)

Biomaterial	Protein	Animal model	Time of administration post-MI	Delivery method	End-point	Results compared to control	Reference
PVA/Dex	bFGF (100 µg)	Sheep	20-30 min	Epicardial patch	8 weeks	Increased wall thickness and attenuated LVESD. Enhanced vascular density. No improvements in functional parameters, perfusion and infarct size.	Fathi *et al*., 2013

LV: left ventricle. *LVEDD*: left ventricular end-diastolic diameter. *LVESD*: left ventricular end-systolic diameter. *LVEDV*: left ventricular end-diastolic volume. *LVEF*: left ventricular ejection fraction. *LVFS*: left ventricular fractional shortening. *PCWP*: pulmonary capillary wedge pressure. *FAC*: fractional area change.

The combination of HA hydrogels with bioactive molecules has also been evaluated in larger animal models, with positive outcomes (Eckhouse *et al*., 2014; Purcell *et al*., 2014). In a recent study by Purcell *et al*. recombinant tissue inhibitor of metalloproteinases-3 (rTIMP-3) was encapsulated in a novel hydrogel and injected into the infarcted myocardium of pigs. The inhibition of metalloproteinases (MMPs) by rTIMP-3 represents an important therapeutic target since MMPs are greatly upregulated in the heart after a MI, leading to the degradation of the ECM, which finally ends up in adverse LV remodeling. To locally adjust the release rate of rTIMP-3 from hydrogel to the tissue requirements, a MMP-degradable hydrogel was developed. At 2 weeks post-injection, very few remnants of hydrogel could be observed in the heart but LV remodeling had been reverted and LVEF improved. Furthermore, these results were confirmed after 4 weeks (Purcell *et al*., 2014).

The ability of decellularized ECM-derived hydrogels to enhance retention and delivery of heparin-binding GF through their glycosaminoglycan content has recently led to the individual encapsulation of bFGF and an HGF fragment (HGFf) into these hydrogels (Seif-Naraghi et al., 2013a; Sonnenberg et al., 2015). In a first approach, Seif-Naraghi *et al*. demonstrated in a subacute rat MI model that enhanced tissue retention of bFGF by an ECM hydrogel compared to controls produced a significant increase in functional arteriole density in the short-term (Seif-Naraghi *et al*., 2013a). In a similar study, sustained release of HGFf from ECM hydrogels resulted in increased arteriole density, decreased CM diameter and improved cardiac function after 4 weeks. On the other hand, all treatment groups including ECM hydrogel alone, free HGFf and ECM hydrogel/HGFf were capable of preventing LV remodeling and fibrosis without significant differences (Sonnenberg *et al*., 2015).

Fibrin hydrogels may also become a suitable delivery system for the controlled delivery of proteins. In a pioneering approach, Ye L. et al. investigated the use of a fibrin patch encapsulating insulin-like growth factor (IGF-1) loaded into gelatin MPs to improve cardiac function when administered in combination with human induced pluripotent stem cell-derived cardiovascular cells. Interestingly, in order to extend IGF-1 release, the protein was encapsulated in gelatin microspheres which were added to the fibrin patch created over the site of myocardial injection. Strikingly, the combination of cells and the fibrin patch improved heart function and metabolism in a large animal model of MI without inducing ventricular arrhythmias (Ye et al., 2014).

Some studies also support the use of gelatin microgels as a platform for protein delivery. Interestingly, a dual therapy was developed by Cittadini *et al*. who delivered VEGF and IGF-1 encapsulated in biodegradable gelatin hydrogel microspheres to the infarcted tissue of rats (Cittadini *et al*., 2011). After 4 weeks, IGF-1 prevented LV remodeling, improved LV systolic and diastolic function, increased CM diameter and reduced inflammation and apoptosis. On the other hand, the main function of VEGF was to induce angiogenesis. Therefore, better regeneration was achieved after treatment with both GFs than after treatment with each factor alone (Cittadini *et al*., 2011).

Less clear is the use of chitosan for protein delivery applications. In a recent study, controversial results were obtained by Zhou *et al*. after using a chitosan hydrogel to improve outcomes of transmyocardial revascularization (TMR) surgery (Zhou *et al*., 2013). In TMR, channels are created in the ischemic wall to enhance myocardial perfusion with endoventricular blood. However, it is common that thrombi and collagenous fibers occupy the inside of the channels, closing them in a short period of time. In this approach, it was demonstrated that filling the channels with a chitosan hydrogel loaded with $VEFG_{165}$ and bFGF is useful to maintain channel patency and increase small vessel density in a canine MI model. However, infarct size and fibrotic area did not improve in the chitosan/GFs group compared to saline (Zhou *et al*., 2013).

3.3.2. Synthetic-based hydrogels

PEG hydrogels are increasingly used for the delivery of bioactive molecules such as therapeutic proteins. For example, a synergistic reparative response was achieved by Salimath *et al.* by the dual delivery of VEGF and HGF to the ischemic tissue of rats into a bioactive PEG hydrogel. Since PEG is an inert material that lacks biological functions, it was functionalized by the incorporation of the Arg-Gly-Asp (RGD) cell adhesive motifs. Short-term echocardiographic assessment after 7 days did not show any cardiac improvement associated with the GF therapy. By contrast, at day 21 sustained release of VEGF increased the number of blood vessels, an effect that was significantly enhanced by co-encapsulation with HGF. Furthermore, hydrogels loaded with both GFs induced a decrease in fibrosis, stimulated the migration of c-kit^+ progenitor cells and improved cardiac function while single factor therapy failed to induce regeneration (Salimath *et al*., 2012). These data suggest that hydrogel-based therapies combining more than one bioactive agent could be a promising approach to achieving an enhanced endogenous response.

Apart from conventional PEG hydrogels, smart hydrogels have also been tested for sustained delivery of proteins, as with a pH-switchable hydrogel made of 2-ureido-4-pyrimidone (UPy) and PEG (Bastings *et al*., 2014; Koudstaal *et al*., 2014). This system was combined with IGF-1 and HGF aiming to stimulate the *in situ* activation of endogenous CPCs in a chronic pig MI model. In this sense, dual GF/hydrogel therapy produced an increase in the number of CPCs in the infarct border

zone, induced the formation of new CMs and enhanced capillary density. Furthermore, this was accompanied by improvements in LVESV, LVEF, FAC and hypertrophy. However, controversial results were obtained when these cardiac parameters were compared with those of the free GF administration group. LVEF, FAC and hypertrophy were similar in both groups (Koudstaal *et al*., 2014).

Continuing with smart hydrogels, a thermosensitive synthetic hydrogel composed of dextran grafted with hydrophobic poly(e-caprolactone)-2-hydroxyethyl methacrylate (PCL-HEMA) in combination with PNIPAAm was also developed by the group of Jiang *et al*. (He *et al*., 2013; Zhu *et al*., 2015). In the first approach, high-mobility group box 1 (HMGB1), a cytokine that attenuates cardiac remodeling after MI, was incorporated into the hydrogel and tested in a rat MI model. Combination of Dex-PCL-HEMA/PNIPAAm hydrogel with HMGB1 resulted in the enhanced proliferation of cardiac progenitors at 24 hours. Long-term assessment at 4 weeks, showed improved LV diameter and LVEF accompanied by a decrease in collagen content compared with all other groups. Notably, a similar significant increase in neovascularization was observed in both groups that received hydrogel therapy, with or without HMGB1 (He *et al*., 2013). More recently, the same hydrogel and *in vivo* model was used to evaluate the effects of the sustained delivery of $VEGF_{165}$ on cardiac repair. Improved LVEF, prevented LV dilation, decreased infarct size and collagen content, inhibited apoptosis and increased angiogenesis were observed after 4 weeks in the hydrogel/$VEGF_{165}$ group compared with either alone (Zhu *et al*., 2015). It is noteworthy that in these studies the Dex-PCL-HEMA/PNIPAAm hydrogel demonstrated efficacy in improving cardiac function and preventing LV remodeling by itself although significantly better results were obtained after combination with HGMB1 or $VEGF_{165}$.

Interesting outcomes in protein delivery have also been observed with poly(ethylene oxide-*stat*-propylene oxide) hydrogels. In this regard, Met-CCL5, a chemokine that inhibits neutrophil infiltration, and SDF-1 were encapsulated in a star-shaped poly(ethylene oxide-*stat*-propylene oxide) hydrogel (Projahn *et al*., 2014). In this approach, different mechanisms of synthesis led to the formation of hydrogels with different release profiles. Met-CCL5 was encapsulated in a fast degradable hydrogel to avoid neutrophil infiltration at early stages post-MI, while SDF-1 was encapsulated in a slow degradable hydrogel to provide a sustained signal for stem cell recruitment. Four weeks after administration in infarcted mice, cardiac function was significantly improved after treatment with both hydrogels. Neutrophil infiltration was reduced when Met-CCL5 hydrogel was delivered alone or in combination with SDF-1 hydrogel. On the other hand, infarct area and neovascularization were improved when SDF-1 hydrogel was administered alone or in the combinational therapy (Projahn *et al*., 2014).

Finally, regarding self-assembling peptides, RADA 16-I peptide was combined with heparin-binding domain sequences for improving the sustained delivery of VEGF in the cardiac tissue (Guo et al., 2012). When such systems were tested in a MI rat model, the authors reported that the addition of heparin-binding domain sequences resulted in improved cardiac function and angiogenesis compared to animals treated with VEGF encapsulated in RADA 16-I naked systems.

3.3.3. Hybrid hydrogels

A few semi-synthetic materials have also been examined as protein carriers for cardiac repair, although it is still a relatively unexplored field. In one approach, a hybrid hydrogel was developed by the covalent bound of PEG to a fibrinogen backbone (Rufaihah *et al*., 2013). In addition, acrylate groups were added to PEG to enable polymerization following exposure to UV light. The angiogenic factor VEGF-A was loaded into the hydrogel and the system was injected into a rat MI model. After 4 weeks, improvements in cardiac function and remodeling as well as angiogenesis were observed associated with hydrogel only and hydrogel/VEGF-A treatments, suggesting that the mechanical support provided by the matrix is able to stabilize and strengthen the infarct. As expected, the incorporation of VEGF-A resulted in a higher arteriole density that led to enhanced positive results (Rufaihah *et al*., 2013).

Limited positive results were obtained following implantation of another hybrid material composed of poly(vinyl alcohol) (PVA) blended with dextran and carrying bFGF in an ovine MI model (Fathi *et al*., 2013). In this case, the hydrogel was sutured to the epicardial surface as a patch 20-30 min after MI induction. Echocardiographic assessment, magnetic resonance imaging and histological studies were performed after 2 months. As a result, the bFGF patch increased wall thickness which resulted in attenuated LVESD. However, LVEDD did not improve, and infarct size was similar in the patch/bFGF group and the non-treated MI group. Concomitantly, since bFGF is an angiogenic factor, the formation of new blood vessels was evaluated. Although vascular density, especially capillary density, was significantly increased after treatment with bFGF/hydrogel compared to hydrogel only, this was not accompanied by either improved cardiac perfusion or function. As a possible explanation, the authors suggested that newly formed vessels may not be large enough in caliber and not sufficiently well-organized to translate the induced angiogenesis into cardiac improvements (Fathi *et al*., 2013).

3.3.4. Limitations

First of all, growth factor denaturation related to the gelation chemistry of the hydrogel can be found. Moreover, one of main disadvantages associated with the delivery of proteins encapsulated in hydrogels is the limited potential to develop a multifactorial response. Another limitation that should

be considered is that hydrogels constitute porous matrices that release the encapsulated molecules in a rapid fashion. Therefore, methods to achieve a more sustained release should be investigated to guarantee long-term beneficial effects. Furthermore, an effective protein therapy requires optimization of the concentration of bioactive molecule that should be delivered in order to obtain a sufficient endogenous response but avoid undesirable side-effects. Cardiac tissue regeneration is a complex process dependent on the activation of numerous signaling pathways and biological processes in a sequential manner. In this regard, biomaterial-based protein therapy delivers one or two types of therapeutic agents to the damaged myocardium. By contrast, cell therapy enables a more multifaceted approach owing to the ability of cells to secrete a great variety of GFs, to interact with the ECM and in some cases, to differentiate into cardiac lineages. Thus, a more complete regenerative response could be achieved combining stem cells, therapeutic proteins and hydrogels in the same strategy. Following this hypothesis, our group is currently working on an approach based on administering HA hydrogels encapsulating ADSCs and NRG loaded into PLGA microparticles.

4. Conclusions and future perspectives

This review summarizes the state of the art in cardiac tissue engineering using hydrogels. These DDSs offer great promise in addressing the existing challenges associated with regenerative therapies for MI. Important advances have been made in the development of new biomaterials that have allowed the preparation of more sophisticated hydrogels over recent years. We expect that these novel approaches will lead to wider use of hydrogels in cardiac tissue engineering. However, as this review has illustrated, despite the significant advances made in the use of hydrogels for cell and protein delivery, several issues are yet to be solved. We cannot forget that only a few strategies have reached clinical practice, which means that these findings should be considered preliminary in nature. Although some hydrogel systems have shown efficacy in preclinical studies, differences between animal-human biology and issues regarding timing and technology of deployment may be responsible for clinical failure. Closely related to the previous point, one of the issues still pending before clinical translation is the need to scale up the manufacture of hydrogel-based approaches to industrial levels. Moreover, further investigation of hydrogel integration within the host myocardium would help to avoid possible adverse events such as arrhythmias. Finally, going a step forward, we envisage that a more complete regenerative response at the site of injury could be achieved combining stem cells, therapeutic proteins and hydrogels in the same strategy.

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HYPOTHESIS AND OBJECTIVES

The most effective clinical approach following a cardiac ischemic event is reperfusion. However, reperfusion is a double-edged sword, since it also induces severe tissue damage. In addition, surviving patients have to deal with a deteriorating heart and many develop heart failure. Cardioprotective drugs and stem cells have been tested in preclinical and clinical studies aiming to protect or repair the heart. Unfortunately, both have failed to prove consistent benefits, mainly due to delivery issues associated with poor cell survival and retention in the heart as well as short half-life and side effects of administered drugs.

The initial hypothesis of this thesis is that drug delivery systems could bring substantial improvements to the clinical management of the infarcted heart by providing enhanced delivery of therapeutic agents.

On that basis, the main objective of this doctoral thesis was to design, develop and evaluate tissue engineering strategies for addressing heart damage after a myocardial infarction from two different therapeutic approaches: stimulation of cardiac repair and cardioprotection from reperfusion injury.

To that end, the following partial objectives were set:

1. To design, prepare and evaluate in a murine myocardial infarction model a strategy for improving cell delivery to the ischemic heart by combination of stem cells with biomimetic microparticles.

2. To optimize the isolation of extracellular vesicles from cardiac progenitor cells for their application as therapeutic agents for cardiac repair and subsequent combination with a novel injectable hydrogel for a potential improved delivery.

3. To evaluate the biodistribution and cardioprotective effect of squalene-adenosine nanoparticles and multidrug squalene-adenosine/vitamin E nanoparticles in a rat ischemia/reperfusion myocardial infarction model.

CHAPTER 1

Transplantation of adipose-derived stem cells combined with neuregulin-microparticles promotes efficient cardiac repair in a rat myocardial infarction model

Long-term engraftment of human cardiomyocytes combined with biodegradable microparticles induces heart repair

TRANSPLANTATION OF ADIPOSE-DERIVED STEM CELLS COMBINED WITH NEUREGULIN-MICROPARTICLES PROMOTES EFFICIENT CARDIAC REPAIR IN A RAT MYOCARDIAL INFARCTION MODEL

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ABSTRACT

Tissue engineering is a promising strategy to promote heart regeneration after a myocardial infarction (MI). In this study, we investigated the reparative potential of a system that combines adipose-derived stem cells (ADSCs) with microparticles (MPs) loaded with neuregulin (NRG), named ADSC-NRG-MPs, on a rat MI model. First, cells were attached to the surface of MPs encapsulating NRG and coated with a 1:1 mixture of collagen and poly-D-lysine. One week after *in vivo* administration, the system favored the shift of macrophage expression from a pro-inflammatory to a regenerative phenotype. At long-term, the adhesion of ADSCs to MPs resulted in an increased cell engraftment, with cells being detectable in the tissue up to three months. In consonance, better tissue repair was observed in the animals treated with cells attached to MPs, which presented thicker left ventricles than the animals treated with ADSCs alone. Moreover, the presence of NRG in the system promoted a more complete regeneration, reducing the infarct size and stimulating cardiomyocyte proliferation. Regarding vasculogenesis, the presence of ADSCs and NRG-MPs alone stimulated vessel formation when compared to the control group, but the combination of both induced the largest vasculogenic effect, promoting the formation of both arterioles and capillaries. Importantly, only when ADSCs were administered adhered to MPs, they were incorporated into newly formed vessels. Collectively, these findings demonstrate that the combination of ADSCs, MPs and NRG favored a synergy for inducing a greater and more complete improvement in heart regeneration and provide strong evidence to move forward with preclinical studies with this strategy.

Keywords: Tissue engineering, cardiac repair, myocardial infarction, microparticles, stem cells, growth factor.

1. Introduction

Tissue engineering (TE) is a promising strategy for the regeneration of damaged tissues. The combination of stem cells and growth factors (GFs) with a biomaterial scaffold [1] has been demonstrated to protect GFs from rapid degradation [2] and to provide three-dimensional support that favors cell engraftment and survival [3]. Moreover, the combination of GFs and stem cells increases the possibility of activation of different pathways to promote tissue repair [4,5]. All this considered, TE seems to be a promising therapy in heart damage [6,7]. Within cardiovascular diseases, myocardial infarction (MI) is the most frequent, causing millions of deaths per year worldwide according to the World Health Organization [8]. After a MI, the heart is dramatically damaged and to date, there is no treatment available to repair or reduce the massive loss of cardiomyocytes. Accumulative research evidence indicates beneficial effects of adipose-derived stem cells (ADSCs) for treating MI in both animal models and humans, even though low cell survival and engraftment have been observed [9–11]. ADSCs are easily isolated from adipose tissue, grow fast in culture media and share common properties with bone marrow stem cells in terms of multipotency and immunoregulatory properties [12,13]. ADSCs implantation participates in the repair of the damaged cardiac muscle by inducing angiogenesis, mainly due to a paracrine effect in the infarcted area [14,15]. These adult stem cells are able to secrete various GFs, such as vascular endothelial growth factor and hepatocyte growth factor, among others [16,17]. As the secretion of GFs by cells is regulated by tissue microenvironment signals, the concentration of secreted GFs are in the physiological range and can be adapted according to the requirements of the different stages of heart healing [18]. Although some studies have already reported the efficacy of ADSCs in cardiac regeneration [9,10], the possibility of combining them with GFs different from those secreted by those cells, such as neuregulin-1 (NRG), may induce a better regenerative response. NRG is a GF that plays a crucial role in the adult cardiovascular system [19] by inducing sarcomere membrane organization and integrity, cell survival, cardiomyocyte proliferation and angiogenesis [20–22]. Our group has recently shown that microparticles (MPs) allow controlled delivery of therapeutic proteins like NRG in the MI region, accompanied by a significant improvement in cardiac function in both rat and pig models of MI [23–25]. We have also demonstrated that NRG-MPs combined with ADSCs, known as ADSC-NRG-MPs, is totally biocompatible with infarcted rat hearts [26]. Based on these results, in the present study we sought to enhance ADSCs survival in the injured myocardium of a rat MI model and likewise to improve tissue repair by the adhesion of the cells to NRG-MPs. The potential reparative activity of ADSCs and NRG-MPs, alone or in combination, was first investigated. We then determined cell survival and cardiac differentiation. Finally, the interactions between MPs and ADSCs with the macrophages of the innate immune system were examined to determine whether a shift to regenerative macrophages was induced. Collectively, the results obtained indicate that the use of NRG-MPs combined with ADSCs led to increased cell

engraftment, thus improving treatment efficacy and providing a rationale for the future application of this technique in clinical studies.

2. Material and methods

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (Mw: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; Mw: 400), human serum albumin (HSA), bovine serum albumin, dimethylsulfoxide, carboxymethyl-cellulose, mannitol, polysorbate 80, sodium azide, sigmacote (SL2) and monoclonal anti-actin α-smooth muscle-Cy3 antibody (C6198) were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane was obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (Mw: 125,000) was obtained from Polysciences, Inc. (Warrington, PA, USA). Collagen I of rat tail 3 mg/mL, Minimum Essential Medium Alpha (α-MEM) Medium, 0.05% Trypsin-EDTA, Heat inactivated Fetal Bovine Serum (FBS) and Phosphate Buffered Saline pH 7.2 (PBS) were provided by Gibco-Invitrogen (Carlsbad, CA, USA). ADSCs were obtained from inguinal adipose tissue of male Sprague-Dawley transgenic rats. H9c2 cells were obtained from ATCC. Poly-D-Lysine (PDL) 1 mg/mL was obtained from Merck-Millipore (Darmstadt, Germany) and recombinant human Neuregulin-1b-iso (NRG) from EuroBioSciences (Friesoythe, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish peroxidase conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-GFP antibody (ab290), chicken polyclonal anti-GFP (ab13970), mouse monoclonal anti-cardiac troponin-T antibody [1C11] (ab8295) and rabbit monoclonal [Y59] anti-CCR7 (ab32527) were supplied by Abcam (Cambridge, UK). Alexa Fluor 488 goat anti-rabbit (A11008), Alexa Fluor 594 goat anti-mouse (A11032), Alexa Fluor 594 goat anti-rabbit (A11012), Alexa Fluor 488 goat anti-chicken (A11039) and DAPI nucleic acid stain (D1306) were supplied by Molecular Probes-Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-CD163 antibody (MCA342R) was provided by Bio-Rad AbD Serotec (Raleigh, NC, USA). Goat serum (X0907) was provided by Dako (Barcelona, Spain). Donkey anti-rabbit FITC (711-096-152) was provided by Jackson ImmunoResearch (West Grove, PA, USA). Rabbit anti-caveolin-1 antibody (#3238) was provided by Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 647 mouse anti-human Ki-67 (558615) was provided by BD Pharmingen (San Jose, CA, USA).

2.2. Preparation of NRG-releasing particles

NRG-releasing PLGA particles were prepared by multiple emulsion solvent evaporation method using total recirculation one-machine system (TROMS) as previously described [26]. Briefly, the organic phase (O) composed of 100 mg of PLGA dissolved in 4 mL of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 200 µg of NRG, 5 mg of HSA, and 5 µL of PEG 400 dissolved in 200 µL of PBS. Next, the inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 mL of a 0.5% w/v PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion (W_1/O/W_2), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. MPs were washed three times with ultrapure water by consecutive centrifugations at 4ºC (20,000 g, 10 min) and lyophilized for 48 h without cryoprotective agents (Virtis Genesis 12 EL, Gardiner, NY, USA). Unloaded MPs were formulated without adding NRG.

2.3. Characterization of NRG particles

Particle size and size distribution were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). Particles were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers. Particle surface charge was determined by zeta potential measurement using ZetaPlus®, based on the analysis of complete electrophoretic mobility distributions (Brookhaven Instruments Corp., NY, USA). The morphology of MPs was analyzed by scanning electron microscope (SEM, Philips XL 30 ESEM-FEG). Briefly, 0.1 mg of MPs were resuspended in 50 µL of deionized water and sputtered with a thin metallic layer before analyzed. Encapsulation efficiency was studied by western blot assay, as described elsewhere [26]. The bioactivity of MPs-released proteins was evaluated *in vitro* by determining H9c2 proliferative capacity following GF treatment by MTS assay as previously described [26].

2.4. Particle surface modification

In order to favor cell attachment to the particle surface, MPs were overlaid with 0.1 or 0.5 µg/cm^2 of collagen type I or PDL alone, or 0.5 µg/cm^2 of a 1:1 mixture of both. Particle coating was performed in 15 mL sigmacote falcon tubes. MPs were re-dispersed in acidified PBS (pH 5.7) and the mixture was sonicated until the particles were completely dispersed in the solution. Then, coating molecules were added to the falcon tube and mixed with the particles under rotation at room temperature for 60 min. Coated particles were washed with distilled sterile water and lyophilized for long term storage

without cryoprotectant. Zeta potential and morphology of coated MPs were examined as described in section 2.3.

2.5. Isolation and culture of ADSCs

ADSCs were obtained by *in vitro* culture of the stromal vascular fraction isolated from inguinal adipose tissue of 5 male Sprague–Dawley transgenic rats that expressed the green fluorescent protein (GFP) as previously described [9]. A homogeneous population of ADSCs was cultured in α-MEM medium supplemented with 10% FBS, 1 ng/mL bFGF and 1% penicillin/streptomycin. Cells were sub-cultured when 80% confluence was reached. The percentage of ADSCs that expressed GFP was assessed by flow cytometry.

2.6. Adhesion of ADSCs to the particles

For ADSCs adhesion, 1 mg of coated MPs was re-dispersed with complete α-MEM medium, and was then ultrasounded and quickly vortexed prior to the addition of $5x10^5$ cells. The mixture was then gently flushed and plated in Costar® Ultra Low Cluster Flat Bottom Sterile Polystyrene Plate. Plates were incubated at 37 ºC. The evolution of the adhesion of the cells to the particles was observed by bright field microscopy within different time points (0, 10, 30, 60 and 90 min).

2.7. *In vivo* studies using chronic myocardial infarction model

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC.

Female Sprague-Dawley rats (Harlan-IBERICA, Spain) of 8 weeks of age underwent permanent occlusion of the left anterior descending coronary artery, as previously described [23]. A total of 35 animals with left ventricular ejection fraction (LVEF) between 40-50% at day 2 post-MI were included in the study. LVEF was obtained by echocardiographic measurements with Vevo 770 ultrasound (Visualsonics, Toronto, Canada). One week post-MI, rats were randomized to receive: 1) $5x10^5$ ADSCs (n=6), 2) 1 mg of NRG-MPs (1294.06 ng NRG, n=4), 3) $5x10^5$ ADSCs adhered to 1 mg of non-loaded MPs (ADSC-MPs; n=7), 4) $5x10^5$ ADSCs adhered to 1 mg of loaded NRG-MPs (ADSC-NRG-MPs; 1294.06 ng NRG; n=10) or 5) control medium (n=8). Treatments were dispersed in 80 µL of injection medium and injected into 2 regions surrounding the border of the infarct implanted with a 23-gauge needle as previously described [26]. At one week and three months post-injection the animals were sacrificed to perform the histological studies.

2.8. Morphometric and histological studies

After sacrifice, the hearts were perfused-fixed in 4% paraformaldehyde at 4ºC and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4ºC, embedded in paraffin and 5-µm-sections were cut. All histology sections were stained and analyzed in a blinded manner. Hematoxylin-eosin (HE) staining was performed to visualize tissue structure and to locate the tract of the injection and the treatments.

Cell survival was quantified one week and three months post-treatment after staining with anti-GFP antibody. Moreover, at one week, GFP-ADSCs co-localization with the MPs was studied by evaluating contiguous slices with immunofluorescence against GFP and HE stain, respectively. Proliferation of the ADSCs and surrounding cardiomyocytes was quantified by immunofluorescence against GFP, troponin T (cTnT) and Ki-67 and subsequent observation using a LSM 800 confocal microscope (Zeiss, Madrid, Spain) in the animals sacrificed one week after treatment. At this term, the differential immunological response to the treatments was evaluated by immunofluorescence against $CCR7^+$ (M1) and $CD163^+$ (M2) macrophages and expressed as the ratio M2:M1. Besides, ADSCs differentiation to capillaries, arterioles and cardiomyocytes was studied in the animals that had received treatment for three months by immunofluorescence against GFP and caveolin-1, smooth muscle actin (α-SMA) or cTnT, respectively. Furthermore, heart tissue remodeling and revascularization were investigated three months post-treatment. Infarct size and left ventricular (LV) wall thickness were measured in images made with a 2.5× objective of Sirius Red-stained sections viewed with a Zeiss Axio Imager M1 microscope (Carl Zeiss AG, Oberkochen, Germany) and captured using an Axio Cam ICc3 video camera and Axiovision software (4.6.3.0 version). Infarct size was measured as the percentage of collagen area (red) vs. total tissue area. To appraise the revascularization effect induced by the different treatments, immunofluorescence was performed against α-SMA for the vessels and caveolin-1 for the capillaries.

2.9. Statistical analysis

Statistical analysis was performed with Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). Differences among treatment groups were assessed by ANOVA with Tukey post hoc correction, when the values measured were normally distributed. Normality was tested with the Shapiro–Wilk and Kolmogorov–Smirnov normality tests. Statistical significance was determined by P values < 0.05.

3. Results

3.1. Characterization of NRG particles

Particles prepared by TROMS had a mean particle size of 20 ± 5 µm and a Z potential of -19.92 ± 2.49 mV. Encapsulation efficiency was 65 ± 2 %, which corresponded to a final loading of 1294.06 ng of NRG per mg of polymer. The bioactivity of the NRG released from the MPs was assessed by induction of H9c2 cell proliferation, and we observed that NRG remained bioactive after the encapsulation process, as in previous studies [26].

3.2. Particle surface modification

Two different molecules, PDL and collagen type I, were used to favor cellular adhesion to the MPs. Different concentrations were analyzed: 0.1 and 0.5 µg/cm^2 of PDL and collagen alone, or 0.5 µg/cm^2 of a 1:1 mixture of both. Zeta potential was measured to analyze changes on surface charge and therefore, to estimate the adherence of the cells to the MPs (Fig. 1A). Particles coated with PDL showed a positive charge with an increased magnitude corresponding to an increase in concentration (0.1 µg/cm^2 resulted in 5.92 ± 1.67 mV and 0.5 µg/cm^2 was 30.87 ± 0.72 mV). Collagen also increased zeta potential values with respect to the uncoated MPs, but particle surface charge remained negative and the Z values were not concentration-dependent (0.1 µg/cm^2 was -4.19 ± 1.58 mV and 0.5 µg/cm^2 was -5.56 ± 3.58 mV). The 0.5 µg/cm^2 collagen:PDL coating produced a zeta potential close to neutrality (1.16 ± 1.52 mV). SEM images showed that the incorporation of biomimetic substances in the particles induced a change in surface appearance, becoming foamier with respect to the uncoated particles, as it can be seen in Fig. 1B. In addition, no changes in the structure of the MPs were observed and the biomimetic substances were homogenously distributed through the surface of all the particles.

3.3. Isolation and culture of ADSCs

ADSCs were obtained from transgenic rats that expressed GFP in order to track ADSCs once injected in the tissue. The cytometry analysis showed that 80.09 ± 9.82 % of the ADSCs expressed GFP. A representative cytometry analysis can be seen in Fig. 1C.

3.4. Adhesion of ADSCs to the particles

Adhesion of 5x10^5 ADSCs to MPs with different coatings and at different time points was examined. Complete cell adhesion to the particles was only observed after 60 min in the MPs coated with 0.5 µg/cm^2 of the 1:1 collagen:PDL mixture (Fig. 1D), while the MPs coated either with PDL or collagen

alone, independently of the concentration, required longer times for the total adhesion of the cells, and in some cases more than 90 min. Therefore, MPs coated with 0.5 µg/cm^2 of the 1:1 collagen:PDL mixture were selected for cellular adhesion in further studies.

A)

Coating	Uncoated	Collagen		PDL		Collagen:PDL
[µg/cm^2]	-	0.1	0.5	0.1	0.5	0.5
Z Potential (mV)	-19.92 ± 2.49	-4.19 ± 1.58	-5.56 ±3.58	5.92 ± 1.67	30.87 ± 0.72	1.16 ± 1.52

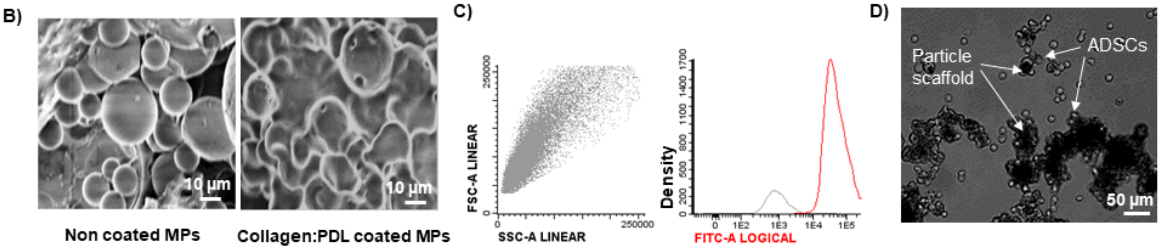

Figure 1. *In vitro* characterization of ADSC-NRG-MPs. A) Zeta potential values of the uncoated and coated MPs. Zeta potential results are presented as means ± SD. B) SEM images of the uncoated and coated MPs. C) Representative example of ADSC-GFP$^+$ expression by flow cytometry analysis. D) Bright field image of the complexes formed by 5x10^5 ADSCs and 1 mg of the 0.5 µg/cm^2 collagen:PDL 1:1 coated MPs after 60 min of attachment.

3.5. Morphometric and histological studies

3.5.1. Cell fate *in vivo*

Cell engraftment and survival was analyzed one week and three months after transplantation by immunofluorescence against GFP. One week post-treatment, ADSCs-GFP$^+$ cells were detected in the heart tissue of the ADSCs, ADSC-MPs and ADSC-NRG-MPs groups. The amount of cells detected at this time was similar in the three groups (± 5%) (Fig. 2A). Then, it was demonstrated that the GFP$^+$ cells co-localized with the MPs when compared with HE consecutive stains, indicating that ADSCs remained in the injection track (data not shown). Interestingly, three months after transplantation some ADSCs-GFP$^+$ could also be detected (< 1%), but only in the animal groups treated with the particle-scaffolds (ADSC-MPs and ADSC-NRG-MPs) (Fig. 2B). These observations suggest that MPs have substantially improved the long-term engraftment and survival of ADSCs.

We next investigated the differentiation of ADSCs to smooth muscle, endothelial and cardiac muscle cells three months after transplantation by immunofluorescence against GFP and α-SMA, caveolin-1 or cTnT, respectively. On the one hand, some of the ADSCs-GFP$^+$ were positively stained for α-SMA in the ADSC-MPs and ADSC-NRG-MPs groups indicating that these cells differentiated into smooth muscle cells. Moreover, some GFP$^+$/α-SMA$^+$ cells were incorporated into newly formed vessels (Fig. 3A). On the other hand, ADSCs-GFP$^+$ did not co-stain with caveolin-1 nor cTnT (Fig.

3B and 3C). These results suggest that transplanted ADSCs differentiate into the smooth muscle of blood vessels but not into endothelial cells nor cardiomyocytes.

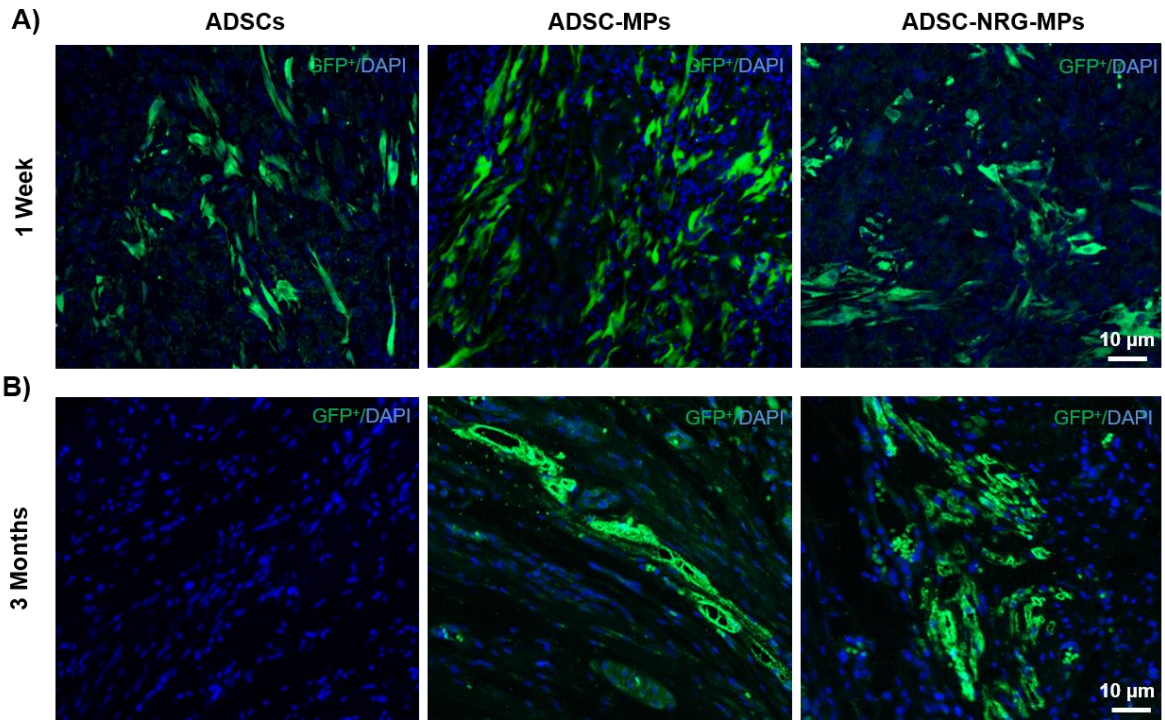

Figure 2. Analysis of ADSCs survival one week and three months post-administration into the hearts of rats with MI. Representative images of the ADSC-GFP$^+$ at short (one week) (A) and long (three months) (B) terms.

3.5.2. Cardiomyocyte and ADSC proliferation

The ability of NRG to induce proliferation of cardiomyocytes and ADSCs in the cardiac tissue was evaluated by the expression of Ki-67 marker in cTnT$^+$ and GFP$^+$ cells, respectively. Although we did not observe any proliferation of ADSCs, a significant increase in the number of Ki-67$^+$ cardiomyocytes was observed following treatment with ADSC-NRG-MPs (4.16 ± 0.55, P<0.001) compared with control (1.11 ± 0.16), ADSCs (1.00 ± 0.34) and ADSC-MPs (0.63 ± 0.27) groups at one week post-implantation (Fig. 4A and 4B). This result indicates that NRG released from MPs could be stimulating the mitotic activity of cardiomyocytes.

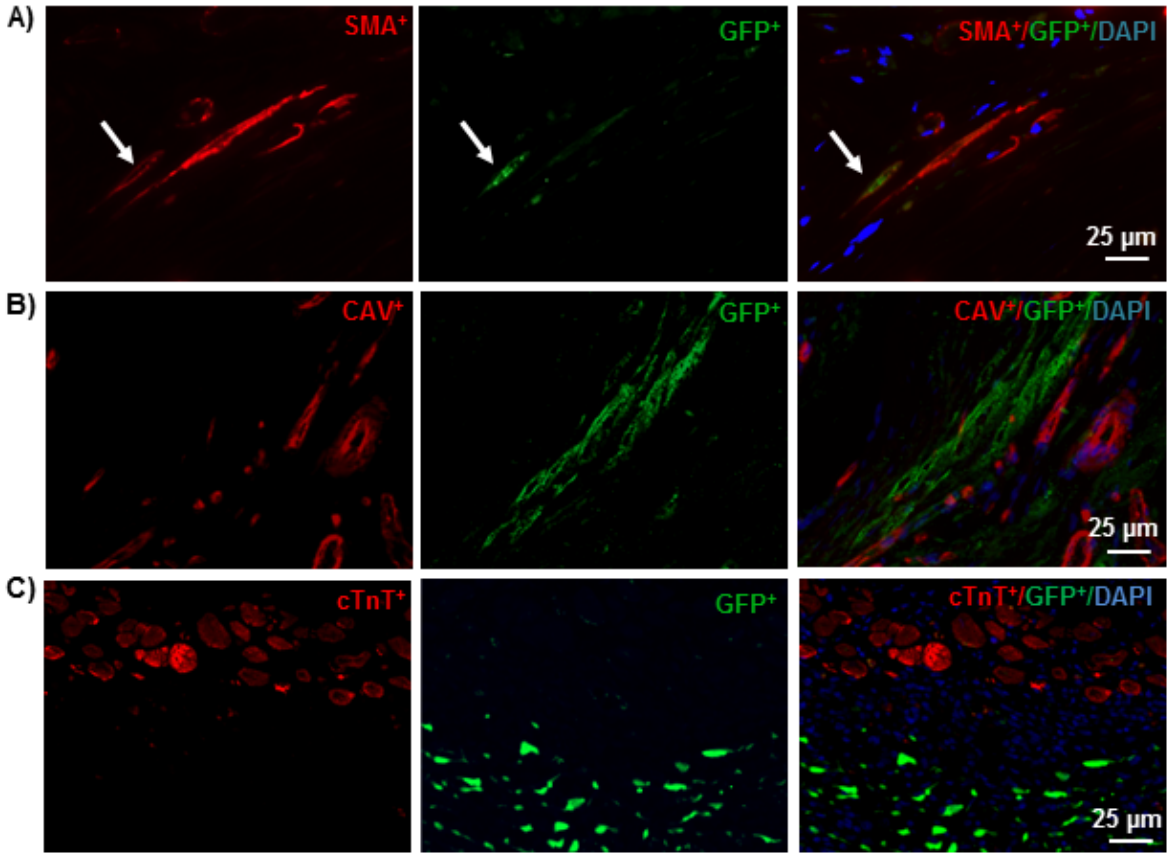

Figure 3. Analysis of ADSCs differentiation. A) Representative fluorescence image showing that some ADSCs-GFP$^+$ co-stained with α-SMA three months after treatment. B) Representative fluorescence image of ADSCs-GFP$^+$, which did not co-stain with caveolin-1 (CAV) three months after treatment. C) Representative fluorescence image of ADSCs-GFP$^+$, which did not co-stain with cTnT three months after treatment.

3.5.3. Response of macrophages to the implanted tissue engineering strategy

Macrophage phenotype was evaluated one week following treatment administration to assess the activation state of implant-associated macrophages. A statistically significant higher ratio of M2 (CD163$^+$):M1 (CCR7$^+$) macrophages was observed in the surrounding tissue of ADSCs (1.20 ± 0.06), ADSC-MPs (0.87 ± 0.04) and ADSC-NRG-MPs (1.00 ± 0.07) treated animals when compared with the control group (0.20 ± 0.02, P<0.001) one week after graft (Fig. 5A and 5B). This increased CD163 expression suggests a shift of macrophage phenotype towards an anti-inflammatory pro-healing phenotype.

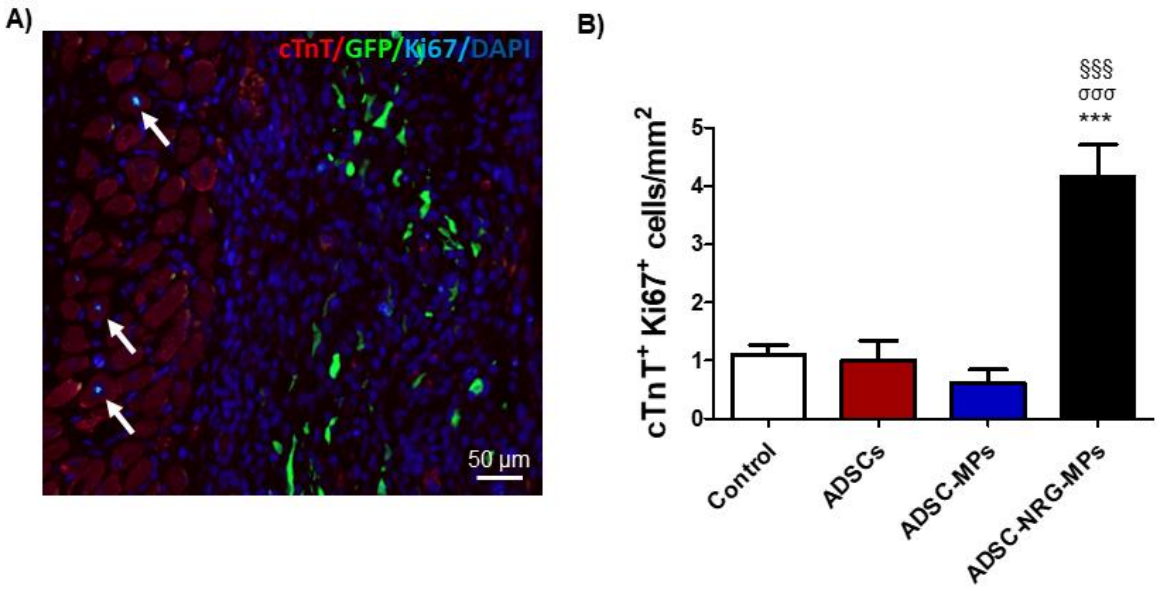

Figure 4. Quantification of proliferative cardiomyocytes at one week post-treatment. A) Representative immunofluorescent image showing proliferative cardiomyocytes (white arrows) in the ADSC-NRG-MPs group at one week. B) Quantitative analysis of proliferating cardiomyocytes showing that ADSC-NRG-MPs induced a significant higher cardiomyocyte proliferation. Data are means ± SEM (***P<0.001 vs. control; $^{\sigma\sigma\sigma}P$<0.001 vs. ADSCs and $^{\S\S\S}P$<0.001 vs. ADSC-MPs).

3.5.4. Cardiac tissue remodeling

Having shown that MPs were able to improve cell engraftment, we sought to analyze whether the strategy was able to induce positive tissue remodeling. For this, infarct size, LV wall thickness and vasculogenesis were studied. Three months following transplantation, the highest reduction in infarct size was observed in the ADSC-NRG-MPs group (6.84 ± 0.57%, P<0.01 vs. control) followed by the NRG-MPs group (7.49 ± 0.57%, P<0.05 vs. control). However, there were no significant differences between the ADSCs (7.96 ± 0.73%), ADSC-MPs (8.13 ± 1.09%) and control (10.74 ± 0.92%) groups (Fig. 6A and 6B). LV wall thickness was significantly increased in the groups treated with ADSC-MPs (0.85 ± 0.03 mm, P<0.01 vs. ADSCs and P<0.001 vs. NRG-MPs and control) and ADSC-NRG-MPs (0.89 ± 0.02 mm, P<0.001 vs. control, NRG-MPs and ADSCs groups) when compared to control (0.66 ± 0.01 mm), NRG-MPs (0.71 ± 0.02 mm) and ADSCs (0.73 ± 0.02 mm) groups. Interestingly, the highest improvement was observed in the ADSC-NRG-MPs group (Fig. 6C). These results suggest that the greatest prevention in tissue remodeling is achieved by the combinational treatment of NRG-MPs and ADSCs (ADSC-NRG-MPs).

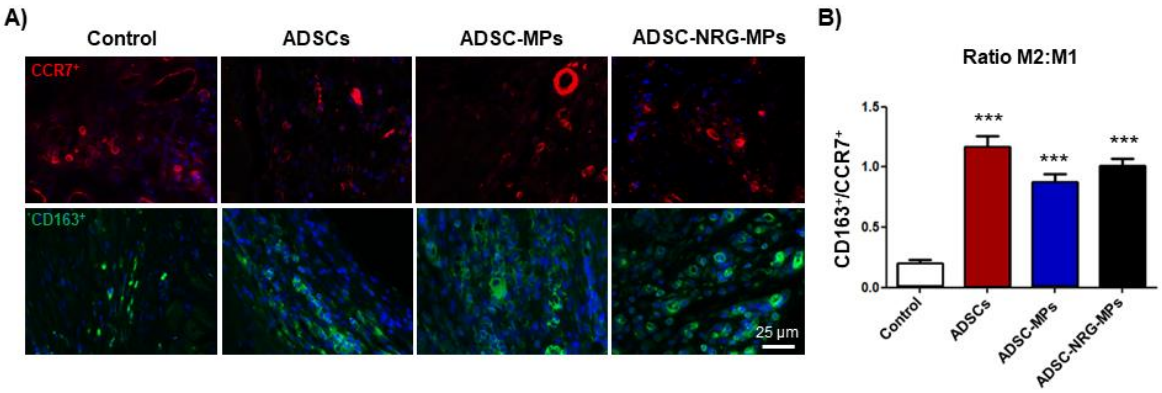

Figure 5. Response of macrophages to the implanted TE strategies. A) Representative immunofluorescence images of M1 ($CCR7^+$) and M2 ($CD163^+$) macrophages. B) Quantification of the ratio M2:M1 macrophages one week post-treatment showing that the presence of ADSCs produced a significant increase of M2 macrophages compared to control. Data are means ± SEM (***P<0.001 vs. control).

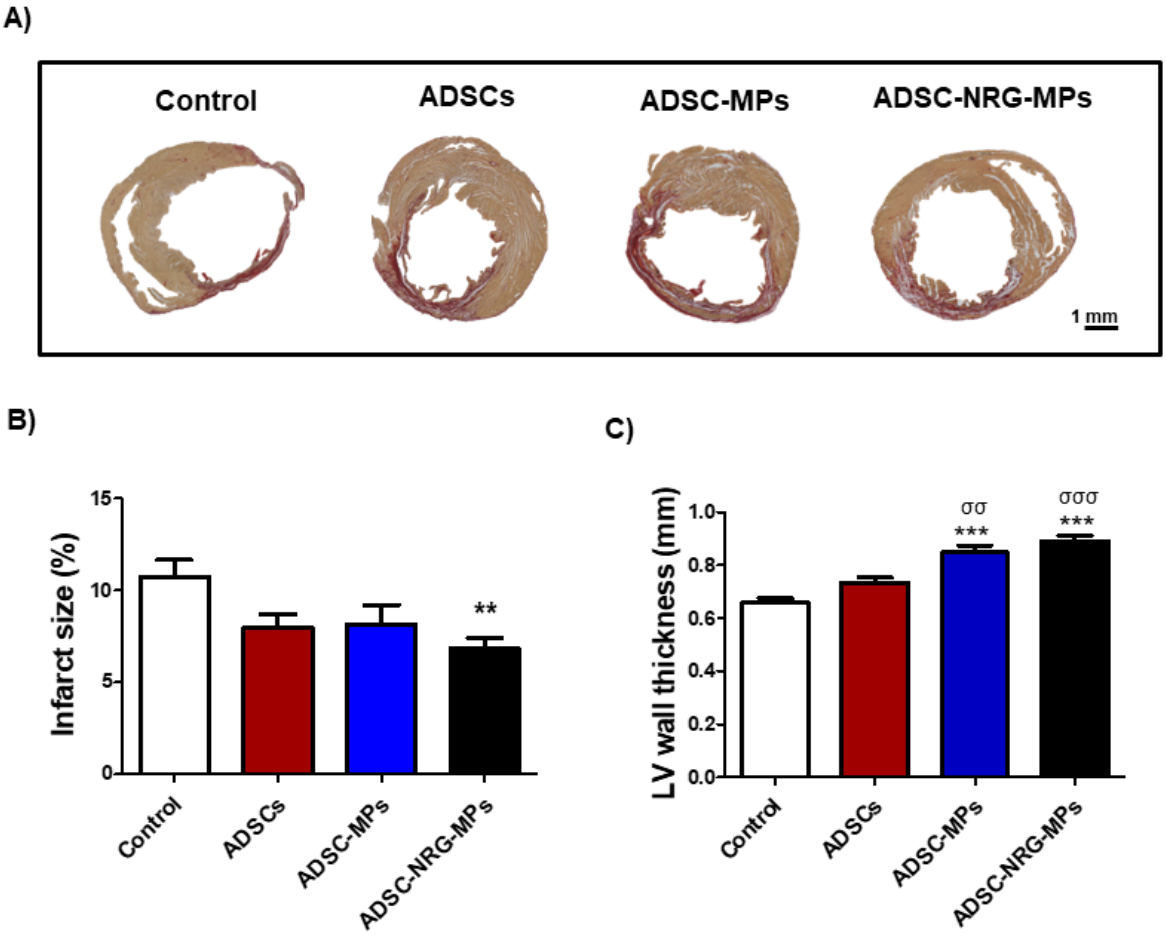

Figure 6. ADSC-NRG-MPs prevented LV remodeling. A) Representative images of infarcted hearts that received different treatments stained with Sirius red. B) Quantification of infarct size showing that the ADSC-NRG-MPs group presented a significantly reduced infarct size compared with control. C) Quantification of the LV wall thickness showing that both ADSC-MPs and ADSC-NRG-MPs treatments resulted in a significantly thicker LV wall compared with control and ADSCs. Data are means ± SEM (**P<0.01 and ***P<0.001 vs. control; $^{\sigma\sigma}P$<0.01 and $^{\sigma\sigma\sigma}P$<0.001 vs. ADSCs).

Finally, we evaluated the effect of the cardiac TE strategy on vasculogenesis. To do this, we quantified the number of arterioles (α-SMA$^+$ vessels) and capillaries (small caliber caveolin-1$^+$ vessels) on the infarcted and peri-infarcted myocardium. Regarding the density of α-SMA$^+$ vessels, a significantly greater number of arterioles was observed in all the treatment groups (NRG-MPs: 168.10 ± 11.31 μm^2, P<0.05; ADSCs: 217.20 ± 10.20 μm^2, P<0.001; ADSC-MPs: 225.37 ± 10.32 μm^2, P<0.001 and ADSC-NRG-MPs: 203.45 ± 7.87 μm^2, P<0.001) when compared to the control group (129.57 ± 6.68 μm^2) (Fig. 7A). Meanwhile, the highest number of capillaries were found in the animals treated with ADSC-NRG-MPs (738.62 ± 22.09 μm^2, P<0.01 vs. control). The ADSC-MPs group (738.34 ± 25.53 μm^2, P<0.5 vs. control) also showed a significant higher number of vessels than the control group (612.10 ± 27.95 μm^2). No statistical differences were found between these two groups. On the other hand, the NRG-MPs (559.60 ± 26.62 μm^2) and ADSCs (685.96 ± 32.46 μm^2) groups showed similar density of capillaries as the control (Fig. 7B). Altogether, these findings reveal that the largest effect on vasculogenesis is obtained after treatment with ADSC-NRG-MPs.

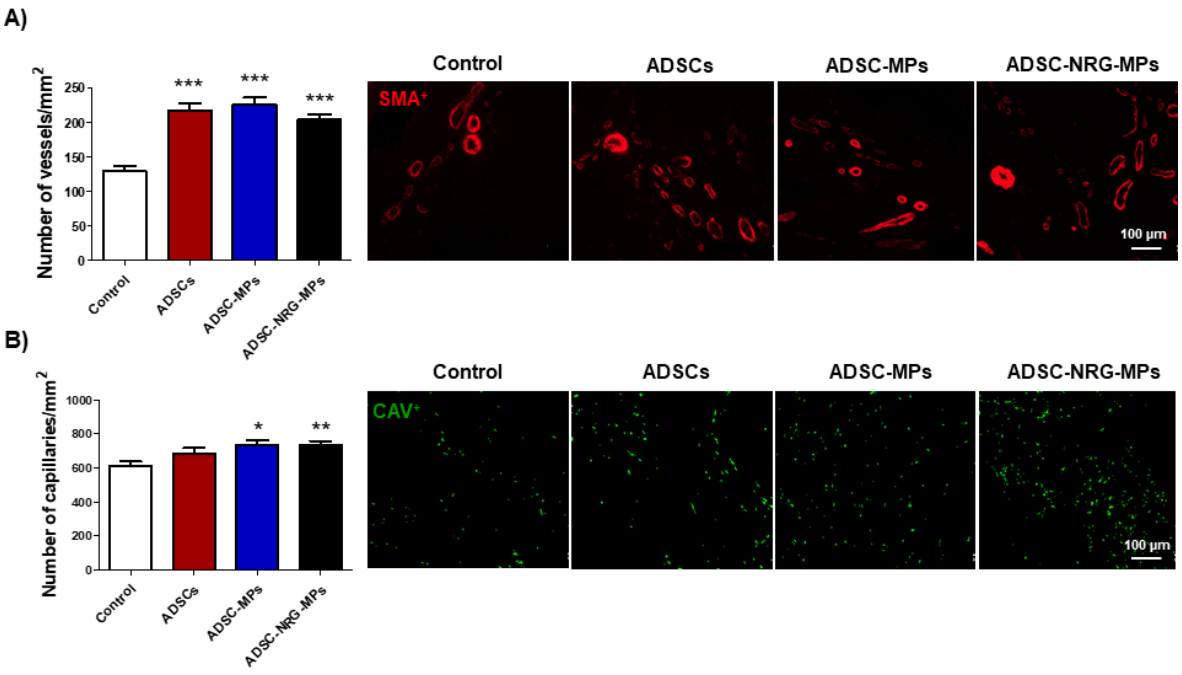

Figure 7. Transplanted cardiac TE strategies induced vasculogenesis. Quantification and representative images of: A) arterioles (α-SMA$^+$ vessel density, arteriogenesis) showing that all treatment groups induced the formation of vessels and B) small caliber vessel density (caveolin-1$^+$ (CAV$^+$), angiogenesis) showing that the administration of ADSCs adhered to MPs promoted neoangiogenesis in the infarcted and peri-infarcted myocardium three months after injection of the treatments. Data are means ± SEM (*P<0.05, **P<0.01, ***P<0.001 vs. control).

4. Discussion

Most of the strategies developed to date for myocardial regeneration have been focused on the combination of either proteins or cells with different biomaterial scaffolds. However, only a few studies have investigated the effect of the three compounds administered together [27,28]. Since the combination may enable the activation of different regenerative pathways, this strategy might favor a greater regeneration of the damaged tissue compared with each compound administered alone. We report here for the first time an approach that combines ADSCs, NRG and PLGA MPs, showing more efficient cardiac remodeling than when these elements are administered individually. Our data clearly demonstrate that the adhesion of ADSCs to NRG-MPs enhanced ADSCs survival once in the tissue. This might account for the higher cardiac remodeling detected in the rat MI model.

Previous studies on cardiac TE were performed using hydrogels or sheet scaffolds typically made of natural biomaterials such as alginate, collagen, chitosan and hyaluronic acid, which brought about improvements in cardiac performance [29–33]. However, these drug delivery systems may exhibit certain limitations [34]. On the one hand, sheet scaffolds require an invasive administration procedure, as the chest has to be opened to place the system directly on the heart, which restricts its application in humans. Conversely, MPs of a safe size to be intramyocardially administered can be easily delivered to target areas in the heart through minimally invasive approaches, using cardiac catheters [24], which would facilitate the transition to a future clinical application. At the same time, MPs allow a more prolonged sustained controlled release than hydrogels, where the porous nature of the latter facilitates the diffusion of molecules to the external medium. Interestingly, we employed MPs made of PLGA, a synthetic polymer that has received Food and Drug Administration (FDA) approval for clinical application [35] and that has been widely investigated for heart TE applications [36,37]. We have previously shown that MPs are good candidates as heart regenerative devices, having a mean particle size which is safe for intramyocardial administration, good cardiac retention [38], enough surface to favor cell adhesion [26] and providing a sustained release of the bioactive proteins for at least three months [25]. Although the use of MPs is more common for protein than for cell delivery, in the present study we move a step forward employing the MPs as cell carriers. Moreover, since MPs can only incorporate one or two GFs at the same time and it could remain difficult to release each factor with different kinetics [23,39,40], the combination of GFs and MPs with cells such as ADSCs, with a high paracrine activity, is a promising alternative.

One of the most interesting findings of this study is the demonstration that MPs are good carriers to improve cell retention in the tissue and to enable localized delivery of cells. One of the goals of the particles developed was to tackle the low proportion of grafted cells that survive in the infarcted heart after transplantation, which is the most important drawback of myocardial cell therapy [17]. The combination of stem cells with a polymeric device has previously shown to improve cell survival

rate. However, although a certain improvement in cell engraftment has been observed, to our knowledge the longest these cells were detected in the heart was one month [41–43]. Notably, in our study MPs were able to enhance cell survival up to three months. This could be explained by the three-dimensional support that MPs provide to the cells that avoids the cellular washout from the injection site [36,44,45]. Moreover, the synthesized MPs of 20 μm diameter remained in the tissue for up to three months post-implantation with no particle migration toward other organs [38], facilitating retention of therapeutic agents in the cardiac tissue.

Going a step further, we investigated whether ADSCs were able to proliferate or to promote cardiomyocyte proliferation after being injected in the cardiac tissue. As MI induces a high loss of cardiomyocytes, it is important to repopulate the infarcted area. Our results demonstrate that although ADSCs did not proliferate, a significantly higher number of proliferative cardiomyocytes were found in the ADSC-NRG-MPs group compared to other groups, indicating that a putative trophic effect could be induced by the NRG released from the MPs. In fact, similar results were previously reported by our group after the administration of NRG-MPs [23] and Kühn *et al.* [46] after the administration of free NRG, thus further supporting the use of this cardiovascular therapeutic protein for the treatment of MI. Furthermore, the enhanced mitotic activity of cardiomyocytes found in our study after the engraftment of ADSCs in combination with NRG-MPs may result in a more complete tissue repair since the highest reduction of infarct size was denoted in this group. Interestingly, the administration of NRG-releasing MPs alone also produced a significant reduction of infarct size compared with control group whereas free ADSCs and ADSC-MPs did not prevent infarct expansion. Here we also shown that injected ADSCs did express vascular cell markers three months after administration, as previously described by others [47,48]. Our results showed that certain ADSCs-GFP$^+$ co-stained with α-SMA, but not with cTnT nor caveolin-1 in both ADSC-MPs and ADSC-NRG-MPs treated groups, demonstrating that ADSCs contribute principally to blood vessel formation, providing blood flow to the tissue [9,49,50]. Accordingly, although the treatment with NRG-MPs and ADSCs alone resulted in a certain increase in the number of vessels, the group that received cells attached to the surface of MPs exhibited a further increase in vasculogenesis, inducing the formation of both arterioles and capillaries. Indeed, the majority of the studies with ADSCs that reported cardiac function improvement displayed neoangiogenesis in the ischemic tissue [9,51,52], which is also consistent with ADSCs ability to differentiate into smooth muscle cells and to secrete GFs such as VEGF [16]. Moreover, tissue revascularization may also be helpful for improving cell survival and proliferation [16,53].

On the other hand, the characteristics of the tissue-engineered implant determine the host response to the treatment by interacting positively or negatively with the immune system. In this way, biomaterials and therapeutic agents may modulate wound healing and may induce a shift in the local macrophage phenotype that may be associated with better tissue recovery [54–56]. The present study

also demonstrates that our cardiac tissue-engineered implant can modulate the immune response by inducing a shift in macrophage phenotype toward the M2 state in the short term. These findings, which are particularly important given the complexity of the *in vivo* environment, are consistent with previous studies that have shown that both biomaterials and ADSCs can prompt M1 macrophages, which correspond to classically-activated pro-inflammatory macrophages, towards M2 macrophages associated with regulatory and homeostatic functions [56–58]. This increase of M2 macrophage expression has been shown to induce positive LV remodeling of the infarcted myocardium in animals treated with both biomaterials [59] and cells [60]. In our study, the ADSCs, ADSC-MPs and ADSC-NRG-MPs groups induced a shift towards M2 macrophages in the short-term, which correlates with similar cell survival in these groups. However, in the long-term, although both groups with cells and MPs exhibited a thickened LV wall, only the ADSC-NRG-MPs group induced also a reduction in infarct size, indicating that the TE strategy globally produced a greater improvement. This correlates with the enhanced cell survival in the MPs groups and the positive trophic effect of NRG.

In summary, the findings of this study demonstrate that MPs were able to improve cell survival, and hence consistent higher efficacy of the treatment was observed, resulting in a better outlook.

5. Conclusion

This is the first report describing a method to deliver ADSCs to the infarcted myocardium based on the use of polymeric MPs loaded with NRG as a three-dimensional support for cells. The results show that cells could be found in the host tissue up to three months, while participating to neoangiogenesis, stimulating macrophage maturation to a pro-healing phenotype and improving the overall cardiac remodeling. Moreover, the combination with NRG stimulated cardiomyocyte proliferation and provided a more complete healing. The next step will be to study the efficacy of the system in large, clinically relevant animal models in order to confirm the potential of the system as a regenerative strategy. To this end, special attention should be paid to adjust the microparticle size, loading and release of the cytokines as well to demonstrate the functional effect of this therapeutic approach.

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LONG-TERM ENGRAFTMENT OF HUMAN CARDIOMYOCYTES COMBINED WITH BIODEGRADABLE MICROPARTICLES INDUCES HEART REPAIR

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ABSTRACT

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) are a promising cell source for cardiac repair after myocardial infarction (MI) as they offer several advantages, such as potential to remuscularize infarcted tissue, integration in the host myocardium and paracrine therapeutic effects. However, cell delivery issues have limited their potential application in clinical practice, showing poor survival and engraftment after transplantation. In this work, we hypothesized that the combination of hiPSC-CMs with microparticles (MPs) could enhance the long-term cell survival and retention in the heart and consequently improve cardiac repair. CMs were obtained by differentiation of hiPSC by small-molecule manipulation of the Wnt-pathway, and adhered to biomimetic poly(lactic-co-glycolic acid) MPs covered with collagen and poly-D-lysine. The potential of the system to support cell survival was analyzed *in vitro*, demonstrating a 1.70-fold and 1.99-fold increase in cell survival after 1 and 4 days, respectively. The efficacy of the system was tested in a mouse MI model. Interestingly, two months after administration, transplanted hiPSC-CMs could be detected in the peri-infarct area. These cells not only maintained the cardiac phenotype but also showed *in vivo* maturation and signs of electrical coupling. Importantly, cardiac function was significantly improved, which could be attributed to a paracrine effect of cells. These findings suggest that MPs represent an excellent platform for cell delivery in the field of cardiac repair, which could also be translated into an enhancement of the potential of cell-based therapies in other medical applications.

1. Introduction

Myocardial infarction (MI) continues to represent the leading cause of mortality and morbidity worldwide (Reed *et al.*, 2017). Despite the great medical advances in recent years, treatments are still palliative, with heart transplant constituting the only option providing a definite and reparative solution. However, it is severely limited by the availability of donors and major complications associated with such an invasive procedure (Tonsho *et al.*, 2014). After a MI, it is estimated that 50 g of the human heart muscle becomes dysfunctional due to the irreversible loss of around 1 billion native cardiac cells (Gepstein, 2002). The endogenous regenerative mechanisms of the heart are insufficient to replace the dead tissue, which remains chronically impaired. Therefore, a large number of patients that suffer from a cardiac ischemic event undergo frequent rehospitalizations, multimedication and progress towards end-stage heart failure (Bahit *et al.*, 2018).

Cardiac cell therapy arose at the turn of the century as a novel strategy aiming to counteract the massive loss of tissue by repopulating the ischemic area with functional cardiac lineage cells. Since then, a large number of cell sources have been explored in preclinical studies with several progressing to the clinical stage (Telukuntla *et al.*, 2013). As cardiomyocytes (CMs) are responsible for heart contraction, many efforts have been focused on the development of *in vitro* protocols to generate contractile cardiac muscle cells similar to native heart cells (Boheler *et al.*, 2002; Batalov and Feinberg, 2015). So far, embryonic stem cells have been the major source for obtaining CMs (Liu *et al.*, 2018). However, ethical, immunogenic and availability issues have limited their potential application (Nussbaum *et al.*, 2007). Recently, the generation of induced pluripotent stem cells (iPSC) from adult cells by Yamanaka et al. (Takahashi and Yamanaka, 2006) and the progresses in cell reprogramming techniques came to provide another attractive source of CMs. CMs differentiated from iPSC (iPSC-CMs) present potential advantages. These cells can be expanded to obtain a large number of cells, are non-immunogenic (autologous) and do not require the destruction of embryonic tissue (Lalit *et al.*, 2014; Cahill *et al.*, 2017).

Research conducted over the last few years on cell therapy has taught us several lessons. Originally, it was believed that transplanted stem cells develop into cardiac cell types. However, when the fate of engrafted cells was investigated, most of the studies reported that only a few cells, if any, actually gave rise to new cells (Nigro *et al.*, 2018). Instead, cells were found to stimulate cardiac functional recovery by the secretion of a wide variety of biologically active molecules that modulate nearby cells behaviour, which is known as the paracrine effect (Hodgkinson *et al.*, 2016). Moreover, efficacy results originated from these studies have been controversial due to the low percentage of local engraftment and survival of transplanted cells (Hou *et al.*, 2005; Terrovitis *et al.*, 2010).

In this framework, the association of stem cells and drug delivery systems is now taking cell therapy one step further. Biocompatible implantable/injectable hydrogels have been extensively explored to

deliver and retain cells in the target tissue, bringing significant improvements in cardiac function (Saludas *et al.*, 2017). However, these systems have also shown limitations, such as lack of long-term cell engraftment (Chow *et al.*, 2017), administration procedures based on open-chest surgeries (Atluri *et al.*, 2014) or need for a careful adjustment of the gelation and mechanical properties. An alternative less widely investigated approach to deliver cells could be the use of biodegradable and biocompatible microparticles (MPs) (Saludas *et al.*, 2018). MPs represent an excellent delivery platform, as they allow for minimally invasive administration procedures using catheter technology (Garbayo *et al.*, 2016) and are localizable to the injection site (Leong and Wang, 2015). Among the diverse cell-based applications, MPs may be used as tridimensional scaffolds, as microcapsules or as key elements to deliver molecules and control the architecture of cell aggregates (Ahrens *et al.*, 2017).

In this study, we developed an effective strategy for cardiac repair by the combination of human iPSC-CMs (hiPSC-CMs) and biomimetic MPs. First, cell-MP complexes were prepared and *in vitro* cell viability was analyzed. We then studied the potential of the strategy to enhance long-term hiPSC-CMs survival and engraftment in a mouse MI model, and its impact on cardiac function and adverse ventricular remodeling. Finally, the phenotype, electrical coupling and maturation of the engrafted cells were characterized. Altogether, the results obtained indicate that the combination of hiPSC-CMs with MPs is a promising approach to stimulate heart repair after a MI.

2. Materials and Methods

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (Mw: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG, Mw: 400 Da), human serum albumin (HSA), Sigmacote®, fibronectin from bovine plasma, poly-D-lysine (PDL), cadmium chloride, Fast Red, hydrochloric acid, rabbit anti-connexin-43 antibody (C6219) and rabbit anti-α-sarcomeric actinin (A7811) were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane, acetone and formaldehyde were obtained from Panreac Química S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (Mw: 125 kDa) was obtained from Polysciences, Inc. (Warrington, PA, USA). Collagen I rat protein and phosphate buffered saline pH 7.2 (PBS) were provided by Gibco-Invitrogen (Carlsbad, CA, USA). Live/Dead™ Viability/Cytotoxicity kit was obtained from Molecular Probes (Carlsbad, CA, USA). Mouse anti-human mitochondria (ab92824) and rabbit anti-dystrophin (ab15277) primary antibodies were purchased from Abcam (Cambrigde, UK). Alexa Fluor 488 goat anti-mouse (A10680) and Alexa Fluor 594 goat anti-rabbit (A11012) secondary antibodies were supplied by Invitrogen (Carlsbad, CA, USA). Anti-cardiac troponin T (MA5-12960), AlamarBlue™ Cell Viability Reagent,

the Fix & Perm kit, the Human Episomal iPSC Line, Essential 8™ Medium, RPMI basal medium, B27 and B27 minus insulin supplements, as well as other cell culture reagents were obtained from ThermoFisher (Carlsbad, CA, USA). PrimeScript RT Reagent kit was provided by TaKaRa (Paris, France). PowerUp™ SYBR™ Green Master Mix was obtained from AppliedBiosystems (Waltham, MA, USA). Small molecules CHIR99021 and C-59 were from Axon Medchem (Groningen, The Netherlands), while ROCK inhibitor Y27632 was purchased from Tocris (Bristol, UK) and Growth Factor Reduced Matrigel Matrix from BD Bioscience (Madrid, Spain).

2.2. Differentiation and isolation of CMs from hiPSCs

CMs were obtained by differentiation of hiPSCs as described in (Lian *et al.*, 2013). Briefly, cells were maintained in Essential 8 Medium on 1:180 Growth Factor Reduced Matrigel coated plates until subconfluence, when hiPSCs were routinely passaged at a 1:15 ratio. For differentiation, cells were plated on 12-well plates as above. When the culture reached 80-90% confluence, the medium was changed to RPMI supplemented with 1X B27 minus insulin (RPMI-minus insulin) plus 8 µM CHIR99021 for 24 hours. Then, the medium was replaced with RPMI-minus insulin for 48 hours, followed by 5 µM C-59 in RPMI-minus insulin for another 48 hours. Next, the medium was shifted again to RPMI-minus insulin for 48 hours, before changing to RPMI supplemented with 1X B27 (RPMI-B27). This medium was renewed every other day until the appearance of beating (usually 7-9 days after the start of the differentiation), when a metabolic selection of hiPSC-CMs by culturing for 72 hours in RPMI without glucose supplemented with 1X B27 and 4 mM lactate was applied. Cells were returned to conventional RPMI-B27 for 48 hours before another 72 hours of metabolic selection in the above mentioned medium. After that, cells were returned to RPMI-B27 for 48 hours and isolated. At this point hiPSC-CMs formed beating monolayers. For this, cells were washed 3 times with PBS plus 0.5 mM EDTA, and incubated for 7-15 minutes in warm TryplE. hiPSC-CMs were then mechanically dissociated with a micropipette tip and counted with trypan blue to exclude dead cells. The required amount of cells was suspended in RPMI-B27 plus 1 µM Y27632 and spun down at 1000 g for 10 minutes.

2.3. Characterization of hiPSC-CMs

Differentiation purity was assessed by fluorescence-activated cell sorting (FACS), while hiPSC-CMs identity was confirmed by functionality (beating), gene and protein expression. For FACS, cells were stained with anti-cardiac troponin T (1:100, MA5-12960) using the Fix & Perm kit and analyzed. RNA extraction was carried out with TRIreagent®, while RT was performed using TaKaRa PrimeScript RT Reagent Kit, following the manufacturer´s instructions, with a maximum of 500 ng of RNA per sample. RT-qPCR was performed with primers shown in Supplemental Table 1, using

Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix in a QuantStudio 5 (Thermo Fisher Scientific, Carlsbad, CA, USA). GAPDH was selected as a housekeeping gene and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method. For protein expression, cells were labelled with anti-α-sarcomeric actinin (1:200, A7811) and visualized with a Zeiss confocal microscope.

2.4. Preparation of MPs

PLGA particles were prepared by the multiple emulsion solvent evaporation method using Total Recirculation One-Machine System (TROMS) (Formiga *et al.*, 2013; Pascual-Gil *et al.*, 2015). Briefly, the organic phase (O) consisting in 50 mg of PLGA 503H dissolved in a mixture of 4 ml of acetone/dichloromethane (1:3) was injected into the inner aqueous phase (W_1) formed by 5 mg of HSA, 5 µl of PEG 400 and 200 µl of PBS pH 7.4. The W_1/O emulsion was allowed to recirculate through the system for 1 min and 30 sec. Then, this emulsion was added to the outer aqueous phase (W_2) consisting of 20 ml of PVA 0.5% and allowed to recirculate for 2 mins and 30 sec. Finally, the $W_1/O/W_2$ emulsion was stirred at RT for 3 hours to allow total solvent evaporation. MPs were washed three times with ultrapure water by consecutive centrifugation at 20,000 g, 4ºC for 5 mins and lyophylized for 48 hours (VirTis Genesis Freeze Dryer 12 EL, Gardiner, New York, USA). Lyophilized MPs were stored at 4ºC.

2.5. Characterization of MPs

Particle size, size distribution and zeta potential were determined after lyophilization. Particle size and size distribution were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). MPs were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter. Particle surface charge was determined by zeta potential measurement using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), based on the analysis of complete electrophoretic mobility distributions.

2.6. Particle surface modification

In order to facilitate the adhesion of hiPSC-CMs to MPs, particle surface was functionalized by coating with biomimetic molecules. Different mixtures of molecules and concentrations were tested to select the most adequate to favor the adhesion of hiPSC-CMs. The different coatings studied were: I) a mixture of 30 µg/ml of collagen type I and 100 µg/ml of PDL, II) a mixture of 60 µg/ml of collagen type I and 100 µg/ml of PDL and III) a mixture of 30 µg/ml of fibronectin and 100 µg/ml of PDL. In all cases, particle coating was performed in sigmacoted tubes. MPs were dispersed in acidified PBS (pH 5.7). Then, biomimetic molecules were added to the particles solution and the

mixtures were incubated at 37ºC for 1 hour under rotation. Coated particles were washed with distilled sterile water by consecutive centrifugations (25,000 g, 4ºC, 10 mins) and lyophilized for 48 hours. Zeta potential was measured to confirm that the particles had been successfully coated.

2.7. Adhesion of hiPSC-CMs to MPs

For the adhesion of cells, 0.150 mg of coated MPs were dispersed in RPMI-B27 medium prior to the addition of 150,000 hiPSC-CMs. The mixture was plated in a Costar® Ultra Low Cluster Flat Bottom Sterile Polystyrene Plate and incubated at 37ºC. The evolution of the adhesion of cells to MPs was assessed at 30 mins, 1, 1.5, 2 and 4 hours by bright field microscopy (Nikon TMS, Amsterdam, The Netherlands).

2.8. *In vitro* cell survival

The survival of cells cultured alone or adhered to MPs was evaluated by Live/Dead® and Alamar Blue assays, following manufacturer's instructions. For that, 150,000 cells alone or adhered to 0.150 mg of MPs were incubated in Costar® Ultra Low Cluster Flat Bottom Sterile Polystyrene Plate at 37ºC. After 1 and 4 days of incubation, samples were stained with Live/Dead staining kit for 30 min at 37ºC, and then examined using a LSM 800 confocal microscope (Zeiss, Madrid, Spain). The survival rate of cells was quantified by incubation with Alamar Blue kit at 37ºC. After 6h of incubation, absorbance was measured at 570 nm and 600 nm using a Power Wave XS Microplate Spectrophotometer. Survival was calculated following manufacturer's instructions. Four to eight replicates were used in each treatment and the test was performed in quadruplicate.

2.9. *In vivo* studies using a chronic MI model

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Navarra and performed according to the requirements of the EU legislation.

Permanent myocardial ischemia was induced in male BALB/C (Rag-2) mice aged 8-12 weeks. Briefly, animals were anesthetized with isoflurane and intubated for mechanical ventilation. Prior to surgery, animals received ketoprofen, fentanest and enrofloxacin. Mice underwent a left thoracotomy through the fourth intercostal space and the left anterior descending coronary artery was permanently occluded. After 15 min of artery occlusion, animals received one of these treatments: MPs coated with PDL and collagen (0.150 mg, n=9) or hiPSC-CM-MPs (150,000 hiPSC-CMs adhered to 0.150 mg of MPs coated with PDL and collagen, n=11). Treatments were dispersed in 12 µl of injection medium and injected intramyocardially into two areas using a 27G syringe. Finally, animals were closed, administered with buprex and allowed to recover.

2.9.1. Cardiac functional evaluation

Echocardiography was performed using a Vevo 770 ultrasound system (Visualsonics, Toronto, Canada) at 2 and 60 days following ligation of the left anterior descending coronary artery. Measurements were optimized for small animals and performed as previously described (Benavides-Vallve *et al.*, 2012). Left ventricular ejection fraction (LVEF), fractional area change (FAC), end-systolic volume (ESV) and end-diastolic volume (EDV) were studied. A total of 24 animals with a LVEF below 45% at day 2 post-MI were included in the study.

2.9.2. Morphometric and histological studies

Male mice were sacrificed at day 7 (n=3 for hiPSC-CM-MPs group) or at day 60 (n=9 for MPs group, n=8 for hiPSC-CM-MPs group) to perform the morphometric and histological studies. Briefly, mice were anesthetized, injected with 100 µl of 0.1 mM cadmium chloride for diastole cardiac arrest, and perfusion-fixed for 15 min with Zn-Formalin under physiological pressure. The hearts were excised, fixed o/n in Zn-Formalin at 4ºC, cut into 3 equally-sized blocks (apical, mid-ventricular, and basal), dehydrated in 70% ethanol (4°C, o/n), and embedded in paraffin. For histological analysis, 5 µm serial sections were prepared.

Infarct size and heart wall thickness were determined using Sirius Red stained sections. For the Sirius Red staining, sections were deparaffinized and immersed for 90 min in 0.1% Fast Red, which was diluted in a saturated solution of picric acid. They were then differentiated for 2 min in 0.01 N HCl, dehydrated and mounted in DPX. Infarct size was assessed by quantifying images from 12 serial heart sections, 50 µm apart. Images were analyzed with Image J 1.48v software and data were expressed as a percentage of the ischemic area *vs.* the total left ventricular area. For quantifying infarct wall thickness, 12 images from serial heart sections of the infarct zone were analyzed using Image J 1.48v software.

The survival and engraftment of transplanted cells was analyzed and quantified at 7 days and 60 days post-administration in the ischemic tissue. Due to the human origin of transplanted cells, these cells could be identified *in vivo* by immunostaining using a mouse anti-human mitochondria antibody (1:1000, ab92824). The number of engrafted cells was calculated by quantifying images from 15 serial heart sections 50 µm apart, and extrapolating this number to the whole length of the graft area. Data were expressed as a percentage of the number of engrafted cells vs. the number of injected cells. Electrical coupling of transplanted cells and preservation of the cardiac phenotype were studied using rabbit anti-connexin-43 (1:500, C6219) and rabbit anti-dystrophin antibodies (1:100, ab15277), respectively. In addition, Alexa Fluor 488 goat anti-mouse (1:200, A10680) and Alexa Fluor 594

goat anti-rabbit (1:200, A11012) secondary antibodies were used. Fluorescently stained tissue slides were observed with a camera attached to a Zeiss Axio Imager M1 fluorescence microscope.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA). Differences between both groups for each time point were analyzed by a t test for independent samples. Results are expressed as mean ± SEM. Statistical significance was determined by p values < 0.05.

3. Results

3.1. Cardiac differentiation of hiPSCs

Cardiac differentiation was performed by small-molecule manipulation of the Wnt-pathway, followed by enrichment through metabolic selection (Fig. 1A). This resulted in the final obtaining of beating monolayers of hiPSC-CMs (Supplementary Video 1), with all experiments showing a purity above 90% of cardiac troponin T-positive cells by FACS (Fig. 1B). RT-qPCR analysis (Fig. 1C) showed a robust downregulation of pluripotency-associated genes (NANOG, PU5F1 and SOX2), concomitant with an upregulation of a cardiac-specific gene expression profile (NKX2-5, GATA4, MEF2C, MYH6, MYH7, MYL2, MYL7 and HCN4). hiPSC-CMs were mononucleated, with well-defined sarcomeres, as depicted by α-sarcomeric actinin staining (Fig. 1Di, ii).

3.2. Microparticle characterization

MPs were manufactured using PLGA, a biocompatible and biodegradable FDA-approved polymer (Makadia and Siegel, 2011). PLGA MPs were prepared by the multiple emulsion solvent evaporation method using TROMS. A homogenous population of MPs with a mean particle size of 10.4 ± 0.8 µm was obtained. Particles presented a negative surface charge with a zeta potential of -20.4 ± 3.3 mV.

3.3. Particle surface modification

The surface of particles was functionalized with biomimetic molecules to facilitate the adhesion of hiPSC-CMs. Collagen type I and fibronectin are proteins of the cardiac extracellular matrix that bind cell adhesion molecules on the surface of cells, while PDL increases the surface charge of MPs to positive values. This switch of zeta potential may attract cells by non-specific interactions between the negatively charged cell membrane and the positively charged surface of MPs (Delcroix *et al.*,

2011; Garbayo *et al.*, 2011). Zeta potential was measured to confirm the successful coating. All coated particles presented positive zeta potential values, with those particles coated with a larger proportion of PDL presenting higher values. In particular, the coating of MPs with 30 µg/ml of collagen and 100 µg/ml of PDL increased zeta potential to +20.0 ± 2.2 mV, the coating with 60 µg/ml of collagen and 100 µg/ml of PDL to +16.3 mV and the coating with 30 µg/ml of fibronectin and 100 µg/ml of PDL to +25.0 mV.

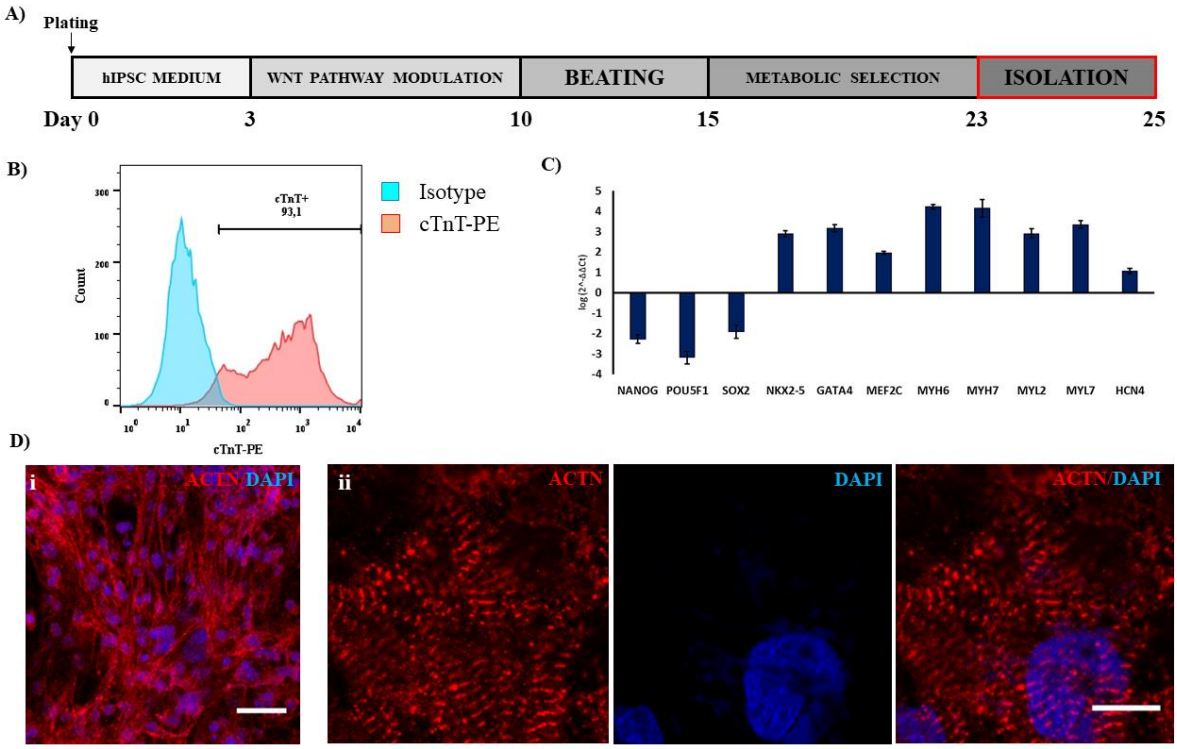

Figure 1. Cardiac differentiation of hiPSCs. A) Diagram of small molecule-based CM differentiation and metabolic selection. B) Representative FACS staining for cTnT, showing a purity of >90% of hiPSC-CMs. C) Gene expression profile of hiPSC-CMs, demonstrating proper downregulation of pluripotency genes (NANOG, POU5F1 and SOX2) and upregulation of cardiac transcription factors (NKX2-5, GATA4, MEF2C) and contractile proteins (MYH6, MYH7); co-expression of genes for CM subtypes is detected, namely ventricular (MYL2), atrial (MYL7) and pacemaker (HCN4). D) Immunofluorescence for α-sarcomeric actinin, confirming the high purity of cultures (i), with cells displaying evident sarcomeric striations (ii). Data is represented as mean ± SD. Scale bars: Di: 25 µm; Dii: 10 µm.

3.4. Adhesion of hiPSC-CMs to MPs

The evolution of the adhesion of hiPSC-CMs to MPs with different coatings was studied at 30 mins, 1, 1.5, 2 and 4 hours by bright field microscopy. On the one hand, when cells were incubated with MPs covered with 30 µg/ml of fibronectin and 100 µg/ml of PDL, only a small number of cells were adhered to particles after 1.5 hours of incubation (Fig. 2A). By contrast, the coating of particles with 30 or 60 µg/ml of collagen and 100 µg/ml of PDL induced a greater adhesion of hiPSC-CMs. After 1.5 hours of incubation, most of the cells were successfully adhered to the surface of particles (Fig.

2A). Incubation for longer times did not increase cell adhesion. Similar results were obtained in terms of particle surface charge and cell adhesion properties with both concentrations tested of collagen, which suggests that 30 µg/ml of collagen creates a large enough biomimetic surface for the adhesion of 150,000 hiPSC-CMs. From a practical point of view, the coating formed by the lowest concentration of collagen blended with PDL (i.e. 30 µg/ml of collagen and 100 µg/ml of PDL) was selected for further studies to obtain a less expensive protocol with potential for future clinical translation.

3.5. *In vitro* survival of cells

The potential of MPs to provide cells with a 3-dimensional microenvironment that enhances cell survival and facilitates cell biological functions was analyzed by the Live/Dead and Alamar Blue assays. As seen in the Live/Dead assay, after 1 day of incubation the number of viable cells was increased when cells were cultured associated with MPs compared to cells alone (Fig. 2B). This higher survival was maintained up to day 4. These results were quantified and confirmed with the Alamar Blue assay. The use of particles as a physical support for cells produced a significant 1.70-fold increase in cell survival over the culture of cells alone after 1 day ($p < 0.001$) and a 1.99-fold increase after 4 days ($p < 0.01$). These results demonstrate that the adhesion of cells to the surface of biomimetic MPs stimulates cell viability by providing cells with a 3-dimensional support.

3.6. Cardiac functional analysis

We then tested the efficacy of the complexes to repair the damaged myocardium in a mouse MI model. LVEF, FAC, ESV and EDV were analyzed by echocardiography at 2 and 60 days post-treatment administration. After 2 days, no differences could be found between MPs and hiPSC-CM-MPs groups for any of the functional parameters studied. Two months after treatment administration, LVEF was significantly enhanced ($p < 0.05$) in animals treated with hiPSC-CM-MPs (41.25 ± 2.57 %) compared to animals treated with MPs (33.21 ± 1.86 %) (Fig. 3A). Similar results were obtained when FAC was studied. This parameter was significantly increased ($p < 0.05$) in the hiPSC-CM-MPs group (30.31 ± 2.28 %) compared to the MPs group (23.26 ± 1.46 %) (Fig. 3B). Furthermore, functional analysis revealed that pathological hypertrophy of the left ventricle was prevented after 2 months with the combinatorial treatment, as reflected in the ESV and EDV parameters. In this sense, a significant reduction was found in the hiPSC-CM-MPs group compared to the MPs group in both ESV (MPs: 96.95 ± 8.21 µl, hiPSC-CM-MPs: 63.16 ± 3.09 µl, $p < 0.01$) and EDV (MPs: 142.70 ± 8.77 µl, hiPSC-CM-MPs: 111.80 ± 4.8 µl, $p < 0.05$) (Fig. 3C and D). These findings reveal that the combinatorial use of hiPSC-CMs and MPs as a therapeutic option is able to induce significant cardiac repair, as shown by the improvement in cardiac function.

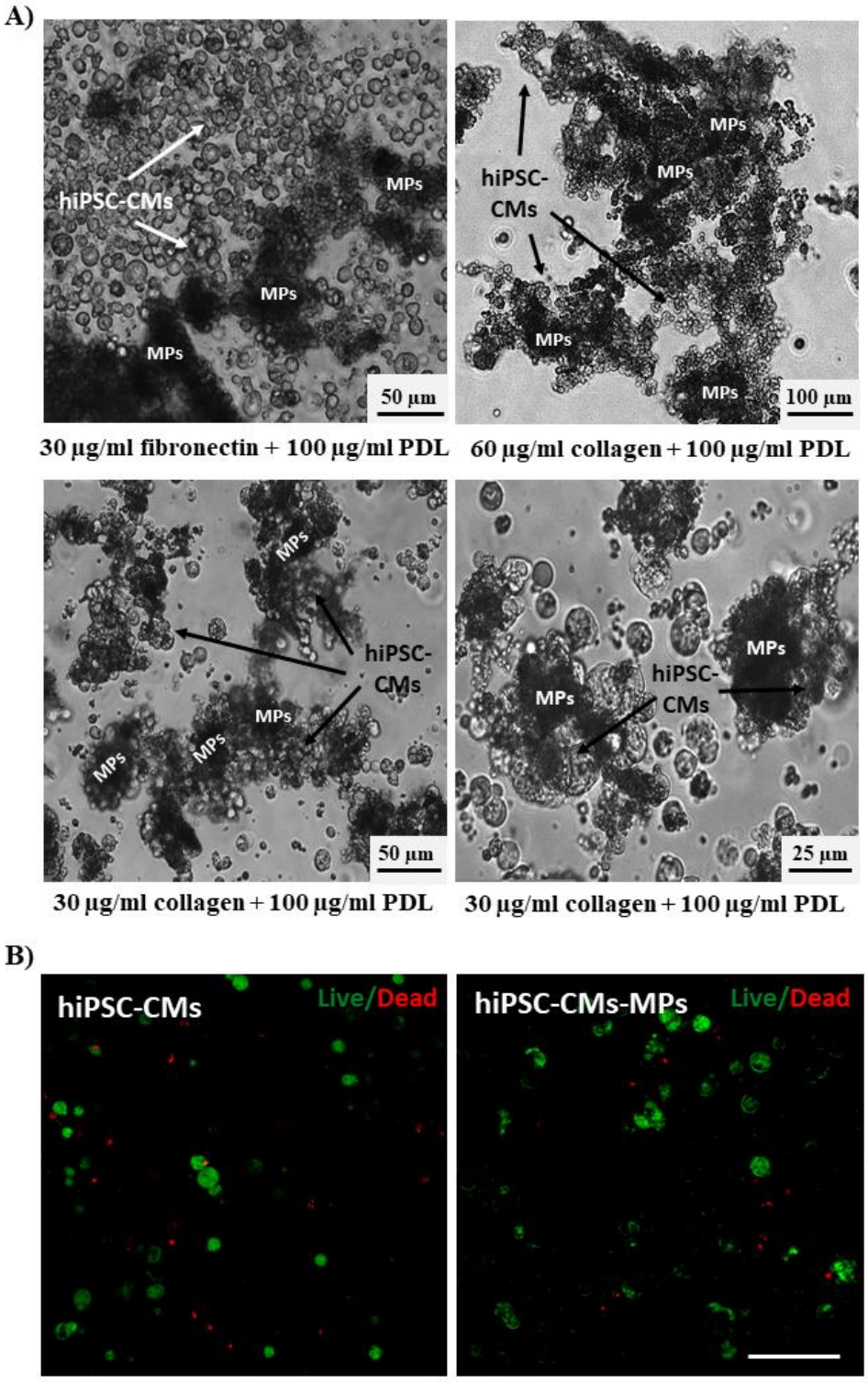

Figure 2. Adhesion of hiPSC-CMs to biomimetic MPs and *in vitro* cell viability. A) Bright field images of the complexes formed by 150,000 hiPSC-CMs and 0.150 mg of MPs covered with different mixtures of biomimetic molecules after 1.5 hours of incubation. MPs coated with 30 µg/ml of collagen type I and 100 µg/ml of PDL showed the largest adhesion of hiPSC-CMs and were selected for further studies. B) Representative confocal images of hiPSC-CMs cultured alone or in combination with biomimetic MPs for 24 hours. Calcein AM (green) stains for live cells and ethidium homodimer-1 (red) for dead cells. Scale bar: 50 µm.

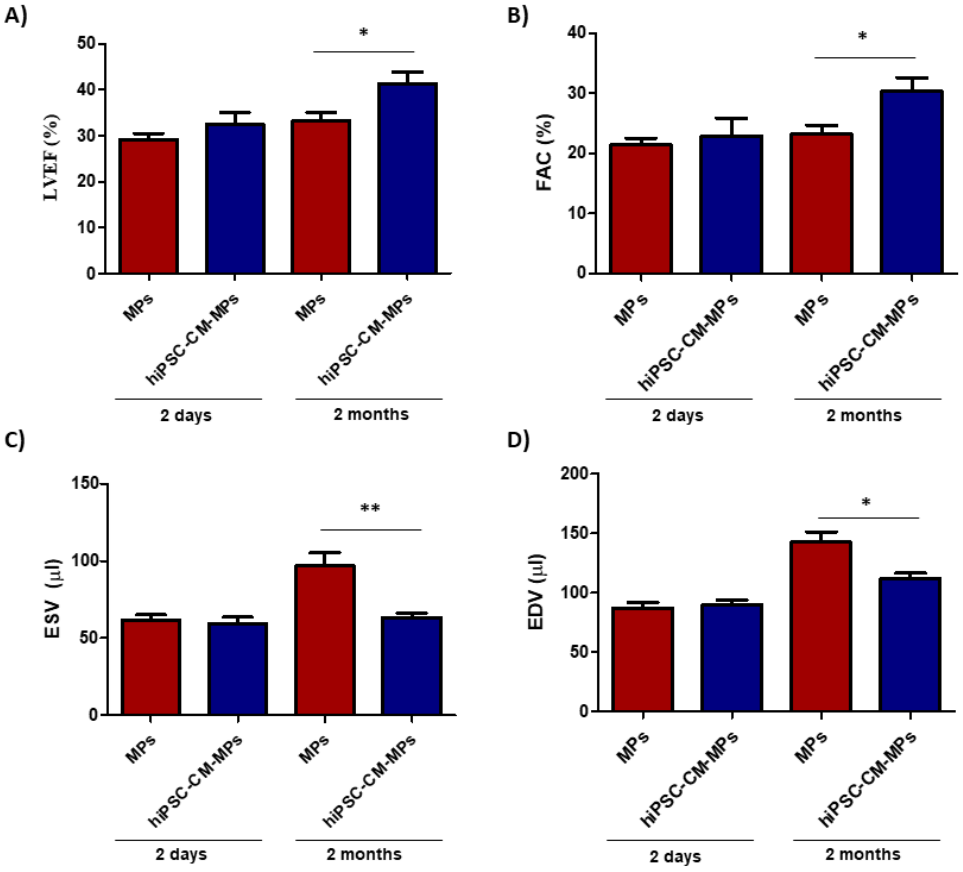

Figure 3. Effects of hiPSC-CM-MPs on cardiac function. LVEF (A), FAC (B), ESV (C) and EDV (D) measured by echocardiography at 2 days and at 2 months post-treatment in animals injected with MPs alone or with hiPSC-CMs adhered to biomimetic MPs (hiPSC-CM-MPs). Results show that combinatorial treatment of MPs and hiPSC-CMs improves cardiac function after 2 months compared to MPs. Data are expressed as mean ± SEM (t test for independent samples, * $p < 0.05$, ** $p < 0.01$).

3.7. Morphometric and histological studies

3.7.1. Left ventricular remodeling

Left ventricular wall thickness and infarct size were analyzed after 2 months of treatment administration. Left ventricular wall thickness was slightly increased in the animals treated with hiPSC-CM-MPs (499.0 ± 41.7 µm) compared to animals treated only with MPs (446.2 ± 58.4 µm) (Fig. 4A). Similarly, a tendency towards a reduction of infarct size was observed in the hiPSC-CM-MPs group (19.93 ± 2.91 %) compared to MPs group (25.09 ± 4.28 %) (Fig. 4B). These findings may suggest that MPs alone or in combination with cells induce a similar prevention of adverse ventricle remodeling.

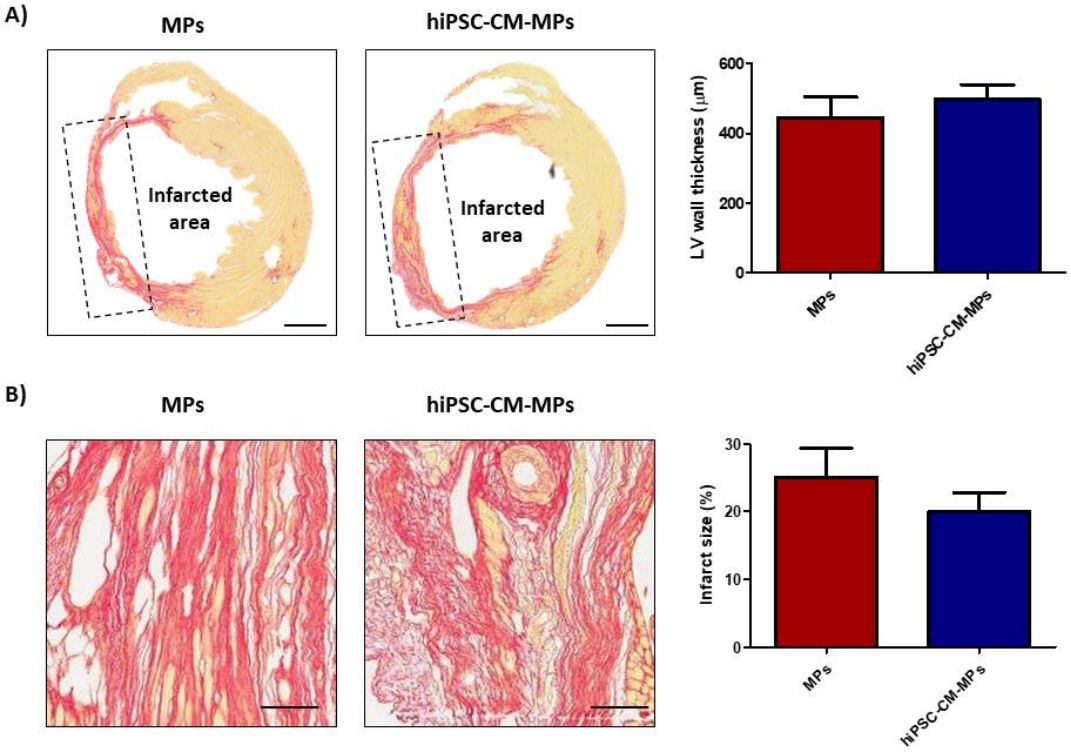

Figure 4. Effects of hiPSC-CM-MPs on ventricular remodeling. Representative images and quantification of left ventricular (LV) wall thickness (A) and infarct size (B) as measured by Sirius Red staining after 2 months of treatment administration. Both treatment groups presented similar wall thickness of the left ventricle, whereas administration of hiPSC-CM-MPs showed a tendency to reduce infarct size. Data are expressed as mean ± SEM. Scale bars: A) 1 mm, B) 100 μm.

3.7.2. Fate of transplanted cells

The engraftment and survival of transplanted hiPSC-CMs was analyzed at one week and two months post-administration in the ischemic tissue by immunofluorescence using an anti-human mitochondria antibody, that allows the specific detection of cells of human origin. One week after administration, transplanted cells could be detected at the graft site in the infarct and peri-infarct area. hiPSC-CMs were relatively small, presented a round-shape morphology and were arbitrarily distributed. Interestingly, when cell survival was analyzed after 2 months, engrafted cells could still be detected at the injection site surrounding the damaged area (<1%) (Fig. 5A). At this time, engrafted cells showed a certain maturation reflected in their larger and elongated morphology and the appearance of clusters of aligned hiPSC-CMs. Additionally, engrafted cells preserved their cardiac phenotype as reflected in the expression of dystrophin throughout the cytoplasm (Fig. 5B). Finally, we investigated whether engrafted cells formed gap junctions with other cells and therefore, if they showed signs of electrical coupling to native cardiac cells. For that, the expression of Cx-43 was analyzed. After 2 months of administration in the cardiac tissue, transplanted hiPSC-CMs showed expression of Cx-43 at the interface between transplanted hiPSC-CMs (Fig. 5C). Altogether, the results indicate that

the transplantation of cells in combination with MPs enhances cell survival and engraftment. In addition, engrafted hiPSC-CMs maintain a cardiac muscle phenotype and express cardiac Cx-43 gap-junction protein after 2 months of administration.

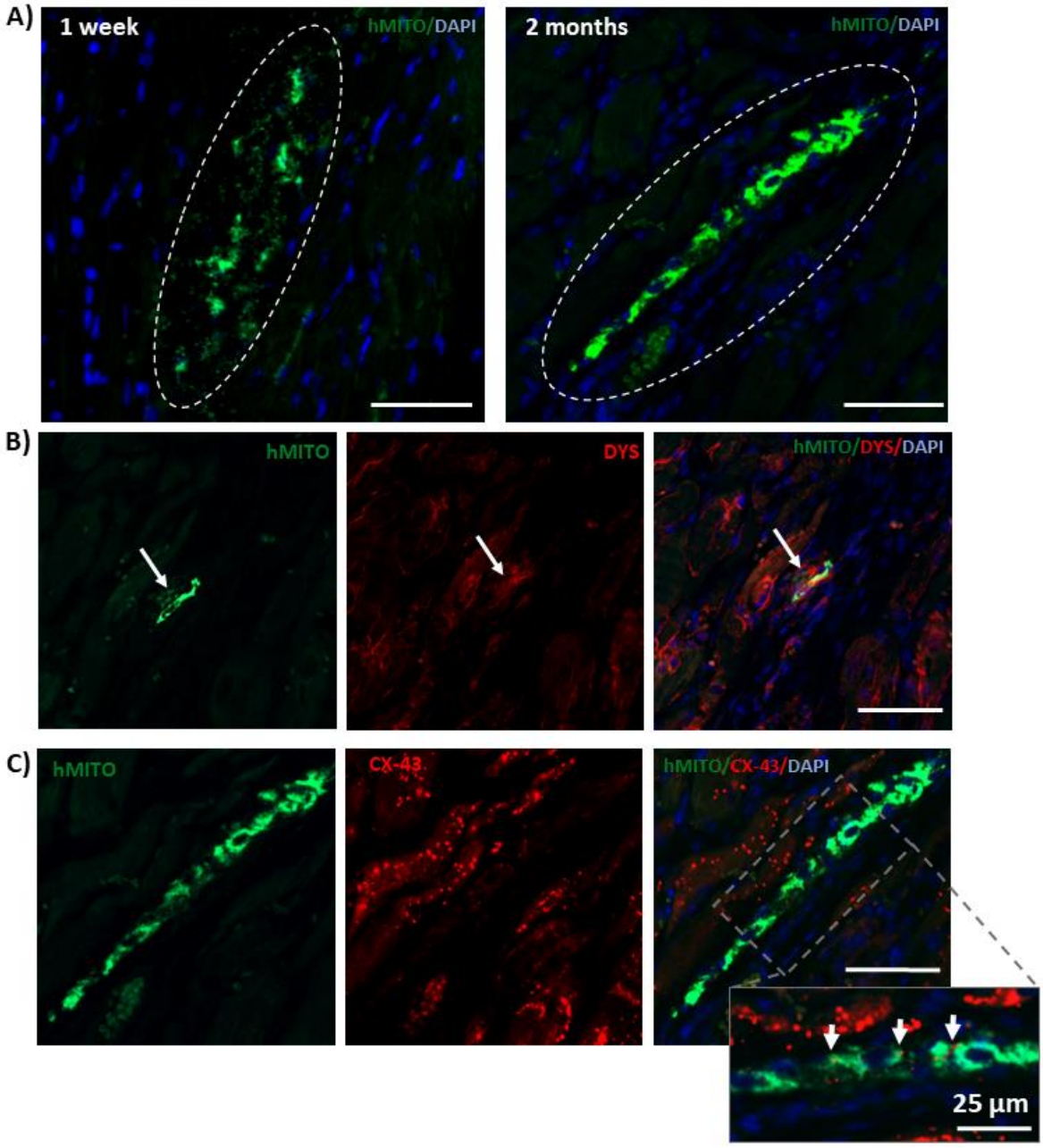

Figure 5. Fate of hiPSC-CMs after transplantation in the cardiac tissue. A) Analysis of cell engraftment and survival at 1 week and 2 months post-administration into the hearts of infarcted mice. Representative areas of infarcted myocardium showing hiPSC-CMs (h-MITO, green) engrafted in the tissue at both time points (area delimited by a discontinuous line). B) Expression of dystrophin (DYS, red) was observed in transplanted hiPSC-CMs after 2 months (arrow) suggesting that these cells maintained a cardiac phenotype. C) Engrafted hiPSC-CMs expressed the gap junction protein connexin-43 (CX-43, red) at the interface between cardiomyocytes suggesting electrical coupling 2 months post-administration (arrows). Scale bars: 50 µm.

4. Discussion

In this paper, we have effectively developed a strategy for cardiac repair based on the enhancement of hiPSC-CM therapy potential through combination with biomimetic MPs. Given that the low survival of cells in the cardiac tissue critically limits the clinical application of cell therapy, the main goal of this study was to implement a system to improve long-term cell engraftment. Specifically, *in vitro* studies revealed that the combination of hiPSC with biomimetic MPs enhanced cell survival compared to cells alone, indicating the potential of MPs to support cell functions. In consonance, the transplantation of hiPSC-CMs associated with biomimetic MPs in a mouse MI model notably increased cell survival up to 2 months. After this period, engrafted cells showed maturation and expression of Cx-43 gap-junction protein. Importantly, progress in cell delivery shown in this study correlated with a cardiac functional improvement, mainly attributed to a paracrine effect of cells. Overall, these results highlight the potential of MPs to overcome cell delivery issues, which could be translated into a greater stimulation of heart repair.

Numerous preclinical and clinical studies have been conducted aiming to elucidate the cell source that presents the largest potential to stimulate the repair of the necrotic myocardium (Yu *et al.*, 2017). In a previous study, our group demonstrated that the transplantation of adipose-derived stem cells combined with MPs enhances cardiac repair in a rat MI model (Díaz-Herráez *et al.*, 2017). However, when the fate of transplanted cells was analyzed, adipose-derived stem cells did not differentiate to CMs. Lack of stem cell differentiation is similarly a constant in other studies (Lin *et al.*, 2010). Transplantation of differentiated CMs could bring some advantages. Injection of cells similar to the native myocardium is related to the mechanical and functional coupling of cells, which reduces the appearance of arrhythmias (Pijnappels et al., 2010). Additionally, Tachibana et al. recently described that hiPSC-CMs salvage the myocardium more effectively than hiPSC through their differential paracrine secretion (Tachibana *et al.*, 2017). Nowadays, the only realistic CM source are hiPSCs. Furthermore, as it has been demonstrated in this paper and by other authors, it is possible to apply robust differentiation protocols to generate CMs that can be implemented in regenerative therapies (Wang *et al.*, 2016; Breckwoldt *et al.*, 2017).

One of the most interesting findings in the present study is the demonstration that MPs are powerful carriers to improve cell survival both *in vitro* and *in vivo.* Previous studies showed that the number of cells remaining in the cardiac tissue after a few hours is extremely low (Hou *et al.*, 2005; Terrovitis *et al.*, 2010). Interestingly, in a previous study where hiPSC-CMs were injected in a mouse MI model, 3.8% cells could be found at 7 days, 0.3% at 14 days and no cells were found at 1 and 2 months after administration (Iglesias-García *et al.*, 2015). In this paper, we were able to localize engrafted cells in the cardiac tissue after 2 months, which hugely encourage the potential of particles to support cell viability. The positive impact that the association with particles has on cell viability and engraftment

can be explained by different mechanisms. Although traditionally used for protein encapsulation (Pascual-Gil *et al.*, 2015; Garbayo *et al.*, 2016; Suarez *et al.*, 2016) rather than cell delivery, MPs also constitute suitable scaffolds to provide cells with a biomimetic tridimensional support (Díaz-Herráez *et al.*, 2013). Although the choice of biomimetic coating proteins tested was aimed at maximising cell attachment, it is widely recognised that extracellular proteins have a fundamental role in cell biology and mechanosensing (Crowder *et al.*, 2016). Future experiments will help clarify this issue. Furthermore, our group proved that MPs similar to those employed in this paper are retained in the myocardium for up to three months, avoiding mechanical washout of cells from the heart (Formiga *et al.*, 2013). With the same aim of overcoming the low engraftment of cells, other authors have explored the delivery of hiPSC-CMs encapsulated in hydrogels (Wang *et al.*, 2015; Chow *et al.*, 2017). Wang et al. found an increased survival of hiPSC-CMs in the heart after transplantation in the hydrogel matrix at 2 weeks, but survival rates were not studied at longer times (Wang *et al.*, 2015). On the other hand, despite the encouraging results in terms of cardiac repair, Chow et al. reported the absence of grafted cells after 10 weeks when transplanted in a synthetic PEG hydrogel (Chow *et al.*, 2017), a similar end-point to ours. In view of these results, we suggest that biomimetic MPs could constitute better cell delivery platforms than hydrogels. Further studies using natural hydrogels and hiPSC-CMs would help to compare results with both delivery systems.

Going a step further, our data demonstrates that hiPSC-CM grafts showed a certain degree of maturation in the cardiac tissue, with a larger size and elongated morphology after 2 months. In addition, clusters of cells could be found in an aligned disposition, which could reveal integration within the surrounding myocardium. We have also shown that cells expressed dystrophin after 2 months, which confirms that engrafted cells maintained their cardiac phenotype. In the literature there are only a few studies describing the *in vivo* engraftment, maturation and alignment of transplanted cells in the infarcted myocardium in the long term. One example is the work recently carried out by Liu et al. (Liu *et al.*, 2018), where authors found a similar *in vivo* maturation of CMs derived from embryonic stem cells. Integration in the native myocardium could be suggested by the expression of the gap junction protein Cx-43 observed in grafted cells. Expression of this protein could be related to the electrical coupling of cells and protection against ventricular arrhythmias (Roell *et al.*, 2007). Apart from electrical coupling, transplanted cells must achieve functional integration in order to avoid the appearance of arrhythmias. For this, cells must be electromechanically similar to the endogenous adult myocardium (Pijnappels *et al.*, 2010), which supports the transplantation of differentiated CMs instead of stem cells.

Finally, we were able to confirm that the long-term engraftment of hiPSC-CMs resulted in the repair of the damaged cardiac tissue, which is the final objective of this strategy. The enhanced cardiac function observed could result from a dual action of particles and cells, since MPs alone were able to improve cardiac output and stroke volume (data not shown). In line with this, no significant

differences were found between the MPs and hiPSC-CM-MPs groups in infarct size and left ventricular wall thickness. These results could be due to the mechanical reinforcement that particles provide to the ventricle wall, which increases wall thickness, prevents infarct expansion and therefore, enhances the function of the host myocardium. Besides that, cardiac repair is mainly attributed to the delivered cells. Interestingly, the significant prevention in the increase of ESV and EDV after 2 months, reflect that the combinatorial treatment avoided the enlargement of the left ventricle to compensate for the loss of function. The low engraftment of transplanted hiPSC-CMs evidences that cells stimulate cardiac repair by the paracrine secretion of growth factors/cytokines. It has been previously described that hiPSC-CMs secrete anti-apoptotic, pro-angiogenic and cell migration related factors (Tachibana *et al.*, 2017). These results are in agreement with recent experiments by Tachibana et al. (Tachibana *et al.*, 2017) and Zhu et al. (Zhu *et al.*, 2018) attributing to the paracrine effect the functional improvements observed after transplantation of cardiac myocytes or cardiovascular progenitors. Moreover, the lack of evident sarcomeric striations 2 months after transplantation further points to the paracrine capacity over contractile support as the driver of the functional benefit.

In summary, the findings of the present study confirm that biomimetic particles represent an excellent platform to improve long-term cell engraftment, due to their ability to create a favourable microenvironment that retains cells at the injection site and supports cell functions. Future studies should focus on the mechanisms responsible for the therapeutic response observed, such as characterization of the paracrine secretion of hiPSC-CMs, as well as its impact on the endogenous tissue. Furthermore, specific molecules could be incorporated into MPs to direct transplanted cells function, as shown in other studies (Mahoney and Saltzman, 2001). It should also be considered that in this study, cells of human origin were injected in a mouse MI model. The use of more representative models of human physiology (pig or non-human primate) could help to elucidate the potential of hiPSC-CMs combined with MPs to directly remuscularize infarcted tissue. Finally, this platform could be implemented not only in the field of cardiac repair, but also in other areas of regenerative medicine.

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Supplementary Data

Table 1. Primers used in RT-PCR analysis of hiPSC-CMs.

Gene	Forward Primer	Reverse Primer
GAPDH	TGGTATCGTGGAAGGACTCATGA	ATGCCAGTGAGCTTCCCGTTCAG
NANOG	GATTTGTGGGCCTGAAGAAA	CAGATCCATGGAGGAAGGAA
POU5F1	GGCTCGAGAAGGATGTGGT	GTTGTGCATAGTCGCTGCTT
SOX2	AACGGCAGCTACAGCATGA	ATGTAGGTCTGCGAGCTGGT
NKX2-5	ACCCAGCCAAGGACCCTA	TTGTCCGCCTCTGTCTTCTC
GATA4	GCGAGCCTGTGTGCAATG	CTGGTTTGGATCCCCTCTTT
MEF2C	CCACCAGGCAGCAAGAATAC	TGGGGTAGCCAATGACTGAG
MYH6	GGAGGGAGGCAAGGTCAT	GGTTCTGCTGCAACACCTG
MYH7	ATGCATTCATCTCCCAAGGA	GAAGCCCAGCACATCAAAAG
MYL2	CAACGTGTTCTCCATGTTCG	GTCAATGAAGCCATCCCTGT
MYL7	CCCATCAACTTCACCGTCTT	AGGCACTCAGGATGGCTTC
HCN4	CGGCCGGATTTTGGATTAT	AATCAGGTTTCCCACCATCA

CHAPTER 2

SHEDDING LIGHT ON THE ISOLATION OF EXTRACELLULAR VESICLES FOR CARDIAC REPAIR

SHEDDING LIGHT ON THE ISOLATION OF EXTRACELLULAR VESICLES FOR CARDIAC REPAIR

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ABSTRACT

Since the discovery of the key role of extracellular vesicles (EVs) in the beneficial effects of cell therapies, these agents have been attracting great interest as next generation therapies. EVs are nanosized membrane bodies secreted by all types of cells that mediate cell-cell communication. Although classification of different subpopulations of EVs can be complex, they are broadly divided into microvesicles (MVs) and exosomes, based on their origin and size. As this is an emerging field, current investigations are focused on basic aspects such as the more convenient isolation method or EV characterization. In the present paper, we used cardiac progenitor cells (CPCs) to study and compare different cell culture conditions for EV isolation as well as two of the most employed purification methods: ultracentrifugation (UC) and size-exclusion chromatography (SEC). MVs and exosomes were separately analysed. We found that serum starvation of cells during the EV collecting period led to a dramatic decrease in EV secretion and major cell death. Regarding the isolation method, our findings suggest that similar EV recovery was obtained with UC and SEC, but the former involved a larger co-purification of other serum proteins. Separation of MV- and exosome-enriched subpopulations was efficiently achieved with both protocols although a certain sample heterogeneity was observed. Noteworthy, while calnexin and CD63 were abundant in MVs, alix was mainly found in exosomes. Finally, when the functionality of EVs was assessed on cardiac fibroblasts, we found that EVs were taken up by these cells, which resulted in a pronounced anti-fibrotic effect. Specifically, a tendency towards a larger functionality of SEC-related EVs was observed. No differences could be found between MVs and exosomes. Altogether, we believe that this work contributes to establish the basis for the use of EVs as therapeutic platforms, in particular in regenerative fields.

CHAPTER 3

DEVELOPMENT OF AN INJECTABLE ALGINATE-COLLAGEN HYDROGEL FOR CARDIAC DELIVERY OF EXTRACELLULAR VESICLES

DEVELOPMENT OF AN INJECTABLE ALGINATE-COLLAGEN HYDROGEL FOR CARDIAC DELIVERY OF EXTRACELLULAR VESICLES

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ABSTRACT

Extracellular vesicles (EVs) are increasingly being investigated as therapeutic agents for various medical applications, including heart repair. However, as is the case with cell therapy, the efficacy of direct delivery of EVs into the infarcted myocardium is severely hampered by poor retention. As an alternative, the combination of EVs with drug delivery vehicles would contribute to defeat present delivery limitations. In this sense, hydrogels offer the most advantageous properties for EV delivery, allowing for controlled release and retention in the desired area, which prolongs EV tissue exposure and therefore therapeutic outcomes. In this work, we aimed to develop a novel injectable *in situ*-forming hydrogel that exerts a dual function: stimulation of cardiac repair and sustained delivery of EVs. Different concentrations of alginate and collagen crosslinked with calcium gluconate were tested. Based on injectability studies, 1% alginate, 0.5 mg/ml collagen and 0.25% calcium gluconate hydrogel was selected as the most adequate combination for cardiac administration using catheter-based systems. Next, rheological examination revealed that the selected hydrogel possessed an internal gel structure, weak mechanical properties and a low viscosity, which facilitate easy administration. Finally, EVs were successfully incorporated and homogeneously distributed in the hydrogel matrix. Besides EV delivery, the developed hydrogel could represent a useful platform for cardiac delivery of multiple therapeutic agents.

CHAPTER 4

CARDIOPROTECTIVE EFFECT OF SQUALENE-ADENOSINE NANOPARTICLES IN AN ISCHEMIA/REPERFUSION MYOCARDIAL INFARCTION MODEL

CARDIOPROTECTIVE EFFECT OF SQUALENE-ADENOSINE NANOPARTICLES IN AN ISCHEMIA/REPERFUSION MYOCARDIAL INFARCTION MODEL

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ABSTRACT

Protection from reperfusion injury after a myocardial infarction (MI) remains a great challenge in clinical practice. The reopening of an occluded coronary artery represents the most effective therapeutic approach, but it also triggers several damaging processes that finally result in an increased infarct size and a larger incidence of heart failure. Adenosine represents a promising cardioprotective drug but its potential has been restricted in clinical trials due to its rapid metabolization in blood (half-life of up to 10 sec). Here we report on the conjugation of adenosine to squalene (SQAd), a natural and biocompatible lipid, and the formation of nanoparticles (NPs) to enhance adenosine efficacy by positively influencing its degradation rate, biodistribution and bioavailability. In a first study, fluorescent SQAd NPs were intravenously injected in a rat ischemia/reperfusion MI model and their accumulation in the heart and other organs was analysed. We found that SQAd NPs accumulated in the ischemic myocardium for at least 24h. Later, we investigated the efficacy of SQAd NPs and multidrug SQAd NPs in combination with antioxidant vitamin E (SQAd/VitE NPs) to provide cardioprotection, and determined the optimal timing of treatment administration (pre- or post-reperfusion). Both SQAd NPs and SQAd/VitE NPs improved cardiac functional performance after 3 months while free adenosine and SQ/VitE NPs failed. However, only SQAd NPs (and not SQAd/VitE NPs) were able to prevent infarct expansion and adverse left ventricular remodelling. No differences were found between pre- or post-reperfusion treatment administration. Overall, chemical encapsulation of adenosine into NPs by its conjugation to squalene could serve as a novel strategy for the treatment of reperfusion injuries with enhanced therapeutic effects and reduced side effects.

GENERAL DISCUSSION

I. CARDIOVASCULAR DISEASES

Cardiovascular diseases: a global problem caused by an inadequate lifestyle

Modern society is experiencing incessant and frenetic changes in lifestyle driven by a social, economic and cultural transformation. While scientific advances are undeniably bringing medicine to an incredibly high level, a demanding and stressful pace of life is gravely affecting our health [1,2]. Fortunately, not only life expectancy but also life quality are better than they have ever been. However, in parallel with these outstanding improvements, the incidence of cardiovascular diseases (CVD) is markedly increasing, mirroring the adoption of bad behavioural habits in our society [3,4]. Physical inactivity, unhealthy diet, smoking and alcohol abuse are the main factors that are exacerbating CVD morbidity, as published in the latest report of the World Health Organization [5]. Effects of these in individuals can be seen in the appearance of hypertension, obesity, high blood lipids and glucose [5]. Reversal of these habits as well as drug treatment of such effects have been proven to reduce CVD events [6]. Resolution of the increasing incidence of CVD episodes is not the same across different countries and it is clearly subject to the level of economic development. Whereas three quarters of deaths associated with CVD occur in low- and middle-income countries with limited access to healthcare system, in high-income countries mortality is falling as CVD cases increase [7,8]. Nowadays, 5-10% of acute myocardial infarctions (MI) culminate in in-hospital death in developed countries [9,10]. However, each day a larger number of patients have to deal with serious consequences that threaten their lives and reduce their quality of life. Rehospitalisation become frequent as heart damage chronifies and global function worsens [11]. Beyond the personal scope, this means a heavy economic burden for many countries, which are unable to face such consequences [8]. In view of this situation, modern scientific communities should focus their efforts on targeting chronic heart disease, preventing progressive heart deterioration and reverting its fatal dysfunction.

Cardiac therapies throughout the years

Over the last 100 years, knowledge about CVD has evolved greatly, allowing the implantation of medical algorithms and more effective interventional protocols. This has been possible thanks to the joint efforts of many professionals coming from different areas of knowledge, such as cardiologists, researchers, pharmacists or physicists. Treatment has progressed from a passive approach recommending several days of bedrest after a MI over a century ago (1912-1961), through the establishment of specialized coronary care units with specific resources destined to treat patients (1961-1974), to the current practice of myocardial reperfusion therapy using fibrinolytic therapy or percutaneous coronary intervention (1974-present) [12]. Nowadays, we are running through an era where resources are allocated to limiting the extension of reperfusion injury as well as regenerating the injured heart [12,13], two aims addressed throughout this thesis.

II. CELL THERAPY FOR CARDIAC REGENERATION

Acute MI, the most predominant form of CVD together with stroke, is characterized by the acute myocardial injury produced by the sudden death of cardiomyocytes due to ischemia [14]. As early as 10 min after the beginning of the ischemic insult, abnormalities can be appreciated in the mitochondria, which after some hours lead to myocyte death [15]. As described by E. Braunwald in the 1970s, "time is muscle", which implies that the extent of the MI is tightly shaped by the duration of ischemia [16]. It is estimated that approximately 1 billion cardiac cells are irreversibly lost following a MI [17]. In view of this, the logical idea that first emerged aiming to restore cardiac tissue was the administration of stem cells with potential to differentiate into new cardiac cells and mechanically integrate in native heart to replace dead muscle. First, non-directed stem cells were proposed as the optimal cell source, constituting the first generation of cell therapy [18]. Encouraged by the promising but heterogeneous clinical outcomes of these cells, a rapid shift to cardiac-committed cells was produced, known as the second-generation cell therapy [19]. The rationale behind this movement was the hypothesis that cardiac-specific cells could better match the requirements of target organ. Furthermore, earlier studies had shown that administration of first-generation stem cells stimulated the recruitment of cardiac resident stem cells, which boosted a regenerative response [20]. Finally, thanks to the inconsistencies that were still present, as well as the recently postulated "paracrine hypothesis", the next generation of cell therapies entered the picture. This hypothesis holds that the functional benefits achieved by cell therapies are a result of the release by injected cells of a biologically active cocktail of growth factors, cytokines and other molecules, rather than their differentiation to cardiac cells [21]. On this basis, emerging research lines are investigating the therapeutic potential of cell-released agents, specifically extracellular vesicles (EVs).

What do biomaterials have to offer to cell therapies?

It was soon after all the hype surrounding the birth of cell therapies and the initial animal studies that the main problem hampering the therapeutic application of stem cells was manifested: cell survival and engraftment in the cardiac tissue. On deeper investigation, some reports used radiolabelling for *in vivo* tracking of administered cells [22,23]. The findings were somewhat disappointing, since only a small proportion of cells remained in the ischemic heart a few hours post-administration [24,25]. In this context, **Chapter 1** of this doctoral thesis focused on an innovative approach that links the benefits of cell therapy with the potential of biomaterials. The objective was to evaluate how the combination of stem cells with microparticles (MPs) could help to enhance the long-term survival of transplanted cells and finally cardiac performance. Following the evolution of cell therapies over the years explained in the section above, we first delivered adipose-derived stem cells (ADSCs, first-generation cell therapy) adhered to biomimetic MPs, and then we used a similar strategy for the

delivery of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs, second-generation cell therapy). In **Chapter 3**, the main objective was heart retention and sustained delivery of EVs (next-generation cell therapy); hence, these were combined with a novel injectable hydrogel.

Microparticles (**Chapter 1**)

Predominantly, the drug delivery systems of choice for the administration of stem cells have been hydrogels (reviewed in the **Introduction**). However, in this thesis we proposed the use of biodegradable and biocompatible poly(lactic-co-glycolic acid) (PLGA) MPs as the ideal candidates for cardiac delivery of stem cells (Fig. 1). In favour of the use of MPs, several aspects should be considered. MPs postulate themselves as superior vehicles exerting a dual function by delivering both therapeutic cells and proteins. On the one hand, biomimetic functionalization of the MP surface enables the adhesion of cells providing a tridimensional support. In addition, our group have proved that MPs remain in the cardiac tissue for at least 3 months after injection, which prolongs cell retention [26]. This is of great importance, since washout of cells from the myocardium could be explained by the lack of a supportive surface causing a poor engraftment [27]. Indeed, we not only provided a physical support for cells, but the outstanding properties of the proposed strategy rely also on the use of a native mimicking attachment surface made of collagen. As previously described, collagen is the major component of the heart extracellular matrix and stimulates a biological response in cells [28]. Although not addressed in this thesis, it would be very interesting, especially in the current "paracrine era", to analyse the paracrine secretion of ADSCs and hiPSC-CMs after adhesion to MPs. Previous studies investigated the paracrine secretion of these cells [29,30] but it would be of great importance to shed light on how adhesion to biomimetic MPs affects their secretion and how it contributes to tissue healing. In line with this, our group is presently working on a revealing study that will help to clarify this issue. Paralleling cell support, MPs offer the possibility of boosting the therapeutic capacity of this strategy by encapsulating proteins, as we did by encapsulating neuregulin (NRG) (Fig. 1). As previously shown, MPs protect labile molecules from rapid degradation and ensure their sustained release in the target area [31,32].

Practical factors work also in favour of the use of MPs for cell delivery. Whereas manufacturing of injectable hydrogels needs a precise tailoring of their composition and mechanical properties, MPs are compatible with an easy and non-invasive administration using current catheter technologies, as proved by our group [33]. Indeed, some hydrogels require an open-chest procedure for injection or they are designed as patches that are sutured to the epicardium [34]. This advantage could make the difference for bench to bedside translation. Linked with the described benefits, another advantage that could facilitate clinical progress is the manufacturing material used, i.e. PLGA. PLGA is a synthetic polymer that was approved by the Food and Drug Administration of the United States for its use in humans and thus it has been widely explored for biomedical applications. Due to PLGA

being constituted by the copolymerization of lactic and glycolic acid, modification of these monomers ratio enables easy tailoring of biomaterial properties such as biodegradation, controlled release profile or encapsulation efficiency of different therapeutics [35].

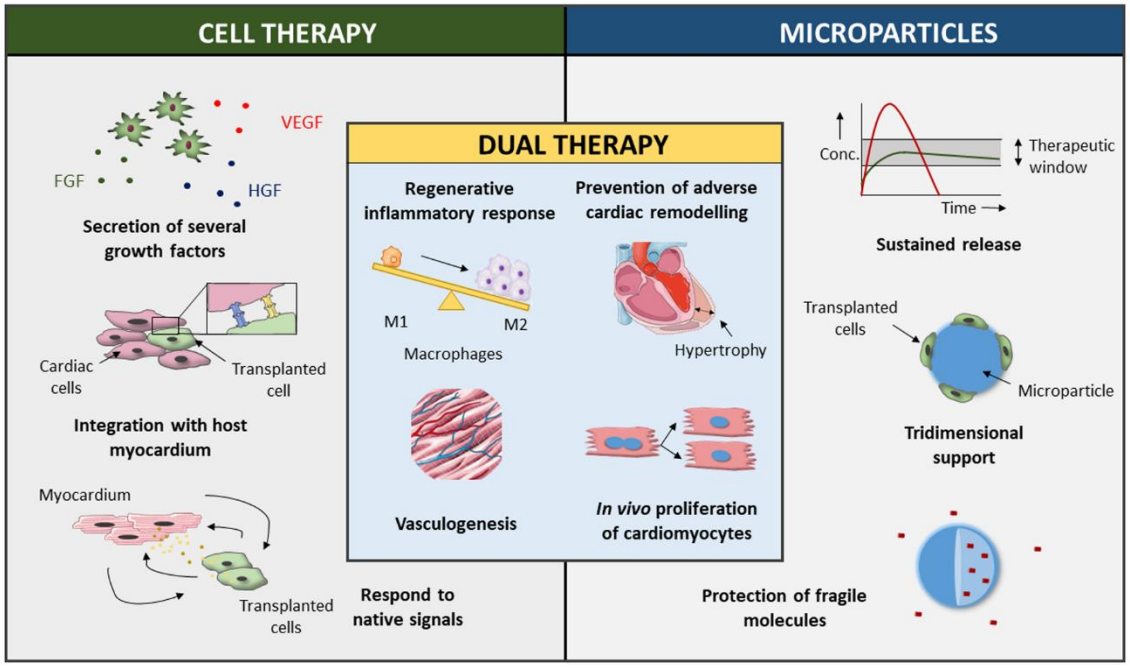

Figure 1. Combinatorial therapy of stem cells and MPs. Advantages associated with cell therapies and MP-based drug delivery systems as well as therapeutic outcomes observed when both are combined and administered in a preclinical MI model.

Hydrogels (**Chapter 3**)

In **Chapter 3**, the objective was to enhance the cardiac retention of EVs and achieve a sustained release. Therefore, this was the optimal situation to implement hydrogels. Hydrogels have been largely investigated in preclinical studies for the delivery of therapeutics and some clinical trials have even been launched for investigation of these systems as mechanical support for the weaken myocardium [36,37]. Although hydrogels have been used as vehicles for stem cells, their use as reservoirs for proteins is debatable. The main reason is that hydrogels are characterized by their capacity to absorb large amounts of liquids, especially water, which becomes their main component. As a result, diffusion of small therapeutics out of the hydrogel matrix is a huge concern and calls into question their use as protein reservoirs. Chemical modification of their structure to achieve a high binding affinity with delivered molecules could help to overcome this problem [38]. By contrast, some recent studies have demonstrated the utility of hydrogels for the sustained delivery and cardiac retention of nanosized bodies, such as EVs [39,40].

Given the importance of providing non-invasive administration, we focused on developing a novel hydrogel that could be easily injected. The ideal hydrogel should meet several requisites: i) biocompatibility with host tissue, ii) biodegradability, iii) responsiveness to native stimulus to form

in situ and iv) mechanical and biological properties that alleviate cardiac workload and stimulate regeneration. Thus, we intended to take advantage of the inherent characteristics of the heart microenvironment to synthetize an *in situ*-forming hydrogel with natural materials. From this starting point, we selected alginate, a natural polysaccharide that forms gels in response to the calcium concentration of the infarcted heart [41], and collagen, the most abundant protein of the cardiac extracellular matrix that gels at 37ºC [28]. Both are biocompatible materials that constitute biodegradable hydrogels. While collagen may be rapidly degraded by collagenases, alginate remains for longer periods in the heart and progressively dissolves by the exchange of calcium ions with the microenvironment [41]. Therefore, we hypothesized that the combination of alginate and collagen could be favourable and not only serve as an exceptional platform for EV delivery but also act as a thermo- and ion-responsive hydrogel in the heart. This should ensure its non-invasive administration. As an indicator of alginate potential, it should be remarked that two different clinical trials were undertaken in recent years, so that this is the only biomaterial (along with decellularized extracellular matrix) that has reached the clinical arena for ischemic applications in the heart [36,42].

First-generation cell therapies

The first attempts to repair the diseased heart with the administration of stem cells arose in the beginning of the 21st century [43]. This novel group of therapies made use of non-committed multipotent stem cells such as ADSCs, bone marrow-derived stem cells or endothelial progenitor cells (Fig. 2). Subsequently, the field experienced its golden age with the initiation of many animal studies in mice, rats and pigs, whose encouraging results led to several clinical trials [44,45]. Lessons learnt from those investigations confirmed the safety and feasibility of cell therapies with no induction of teratomas. On the other hand, these investigations revealed limited efficacy [18]. As mentioned before, the main challenge was poor, inefficient cell delivery. Especially complex is heart delivery because of this organ's incessant contraction, which causes the ejection of injected cells to other organs [25]. In this sense, the main contribution of our work (first article in **Chapter 1**) was the long-term engraftment and survival of transplanted ADSCs by the use of biomimetic MPs as delivery vehicles. Interestingly, this was the first study that achieved the retention of these cells in the heart for as long as three months. Previous studies did not succeed in prolonging ADSC survival beyond 4 weeks [46–48]. We could probably state that this was the crucial factor explaining the beneficial effects observed.

Inefficient cardiac differentiation and paracrine secretion

Beyond delivery issues, there was initial excitement in the scientific community when the first studies showed transdifferentiation of first-generation stem cells to cardiomyocytes [49]. However, cardiac commitment of undifferentiated stem cells was later discarded due to a lack of consistency between

studies. In consonance, in this thesis we found that transplanted ADSCs did show a certain degree of differentiation to smooth muscle cells but they failed to give rise to endothelial cells or cardiomyocytes. However, ADSCs induced a shift in macrophage polarization and enhanced left ventricular wall thickness and vasculogenesis. This case represents a clear example where lack of cardiomyocyte transdifferention is not necessarily associated with a lack of therapeutic efficacy. Although ADSCs are not efficiently repopulating the defective myocardium, they are sending a reparative stimulus to the host tissue. A great number of studies have consistently come to similar outcomes [50,51]. Until now, the most widely accepted hypothesis to explain these observations is the paracrine hypothesis. The secretome of ADSCs, through a synergistic effect of EVs and soluble factors, has been shown previously to induce skeletal muscle regeneration [52]. The study of the molecules involved in the regenerative process is highly complex. In particular, the strong pro-angiogenic effect of these cells has been described to be mediated by the secretion of several cytokines and microRNAs: vascular endothelial growth factor, hepatocyte growth factor, transforming growth factor-β, as well as miR-23 and miRNA-126 [53–55]. Furthermore, ADSCs secrete other miRNAs such as miR-let7b and miR-let7c, which have been reported to alter macrophage polarization towards an anti-inflammatory M2 phenotype [56,57].

Cell therapy versus protein therapy

The complex paracrine secretion of ADSCs, and in general of stem cells, means that cell therapy offers a superior and more complete approach for tissue regeneration than protein therapies. Protein therapies are based on the administration of therapeutic growth factors or cytokines [58]. Up until now, few studies have exploited the combination of two or more different molecules in a single strategy due to difficulties providing the appropriate dose and kinetics for each molecule. Instead, these therapies frequently investigate the injection of only one agent, limiting the scope of the endogenous response. Biological processes involved in the regenerative response are regulated by the interplay of multiple complex and multifactorial mechanisms [59]. Stimulation of endogenous cell signalling pathways with a single molecule could be insufficient to orchestrate timely complete tissue regeneration. By contrast, cell therapies are largely more effective activating simultaneously many native pathways thanks to their cytokine-enriched secretome. In addition, we should note the importance of crosstalk between transplanted cells and the native tissue to maximize cardiac healing, an issue that has previously been described for similar mesenchymal stem cells (MSCs) [60].

Role of NRG in the strategy

Besides the broad variety of therapeutic agents secreted by ADSCs, in the first article of **Chapter 1** we incorporated NRG in the strategy, a growth factor not released by these cells, to foster cardiac repair. By a multiple emulsion solvent evaporation method using Total Recirculation One-Machine

System, it was possible to efficiently encapsulate NRG in the MPs in an easy and reproducible way maintaining NRG bioactivity [31]. NRG is a key mediator involved in cardiac development [61–63]. In fact, it was recently published that NRG "makes heart muscle" [63]. This was explained by the importance of NRG in stimulating cardiomyocyte proliferation and hence cardiac regeneration in zebrafish and mammal animal models, through the activation of Erbb2 and Erbb4 receptors [64–66]. Such receptors are expressed in cardiac muscle cells in newborns and in the adulthood, which makes NRG the ideal candidate for heart treatment [61,62,67]. In line with this, we found a strong stimulation of cardiomyocyte proliferation when NRG was incorporated in the formulation, which was associated with a reduction in infarct size. In addition, our group successfully demonstrated that NRG is not only useful for triggering cardiomyocyte proliferation, but it also acts on other pathways stimulating neovascularization [31,33]. Finally, special attention should be paid to NRG delivery, as the overexpression of the NRG-Erbb2 pathway in off-target organs could be associated with cancer. Administration of NRG encapsulated in MPs could avoid such risks by the retention of MPs in the heart with no leakage to other organs [26].

Second-generation cell therapies

After the safety and feasibility of cell therapies had been proven, the potential of transplanting cells from the cardiac lineage that better match organ biological environment brought second-generation therapies into the spotlight. In this group, different subpopulations of cardiac progenitor cells (CPCs), iPSC-CMs or cardiosphere-derived cells were used (Fig. 2).

Comparison between ADSCs and hiPSC-CMs cell therapies

Given the divergent study designs, a good comparison of first-generation and second-generation cell therapies is often difficult. For example, this is the case with both studies in **Chapter 1,** where different animal models (rat versus mouse), cell origins (rat ADSCs versus human iPSC-CMs) or administration times (1 week versus 15 mins post-MI) were used. Due to the human origin of hiPSC-CMs, it was mandatory to use immunodeficient animals and therefore a MI model in mice was chosen. Regarding administration time, it should be noted that the course of the disease over time differs a lot between larger or smaller animal models and even between different species of the same animal, making it difficult to establish comparisons. While the ADSC article was focused on the cardiac repair of a chronic infarcted heart by administering the treatment at 1 week post-MI, in the hiPSC-CMs article treatment was injected at 15 mins post-MI, representing an earlier therapeutic approach. On the one hand, developing treatments for addressing chronic heart failure is of vital importance as the mortality after a MI has declined in recent years but long-term consequences have increased [68]. On the other hand, an earlier cardiac intervention could maximize therapeutic options. Besides that, endpoints of both studies also differ in their global scope. The first article related to

ADSCs aimed at providing more mechanistic insights with the analysis of cardiomyocyte and ADSC proliferation and macrophage polarization. On the contrary, the hiPSC-CM study conceded greater importance to functional outcomes, assessing the evolution of cardiac performance.

Despite the above-mentioned differences, the completion of both studies allowed us to elucidate some issues related to cell therapies and specifically cell delivery. For example, the functionalization of MPs by a mixture of collagen and poly-D-lysine (PDL) favoured the attachment of cells. In the first approach, a 1:1 ratio of collagen and PDL, which provided an almost neutral zeta potential, was selected as the most appropriate for ADSC adhesion. However, in the second approach, we found no differences in the adhesion of hiPSC-CMs when the proportion of collagen in the mixture was decreased. Thus, the collagen:PDL ratio was shifted to 1:3.3, yielding a more positive zeta potential as a result. In consonance with the ADSC study, the main finding of the second article was the enhanced *in vivo* cell survival and engraftment. In a previous paper, transplanted hiPSC-CMs could no longer be found in the ischemic tissue after 14 days [69], whereas combination with MPs improved cell survival up to two months. In favour of the use of these cells, we found that injected hiPSC-CMs maintained a cardiac phenotype and formed gap junctions with other hiPSC-CMs or native cardiac cells, which could favour tissue electromechanical integration. Nonetheless, this last issue remains to be clarified. Finally, infarct size was only improved by the incorporation of NRG in the strategy of the first article, while neither ADSCs nor hiPSC-CMs produced a significant infarct size reduction. This finding leaves the door open for a potential incorporation of therapeutic proteins in the strategy.

Comparison between first-generation and second-generation cell therapies in other studies

Comparison between unspecific stem cells and cardiac-guided cells has also been investigated by a few authors [70–73]. Citro *et al*. observed decreased fibrosis when hiPSC-CMs were used over MSCs [71]. Zheng *et al*. found that cardiac stem cells are superior to MSCs in modulating electrophysiological aberrations and ventricular fibrillation after a MI [72]. As these authors suggested, superior properties of cardiac stem cells could be a consequence of the integration with host myocardium, reflected in the expression of connexion-43, a marker mostly absent in MSCs. Finally, comparison of cardiosphere-derived cells with bone marrow-derived MSCs and ADSCs led Li *et al*. to determine that the former provide the largest improvement in cardiac performance, angiogenic effect, ischemic tissue preservation and cardiomyocyte protection after a MI [73]. Interestingly, these authors found that a more potent paracrine secretion explained the superior regenerative capacity. Globally, all these studies concluded that cardiac-directed cells repair the heart more efficiently than their undifferentiated counterparts, suggesting the importance of tissue specificity for cell therapies.

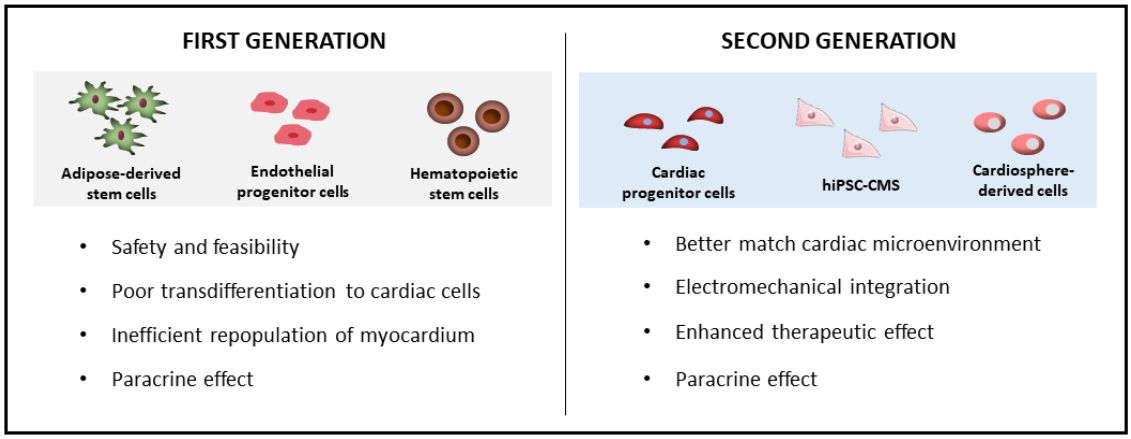

Figure 2. Evolution of cell therapies. First-generation cell therapy involves undifferentiated stem cells. This group proved the safety and feasibility of cell therapies. However, these cells do not transdifferentiate to cardiac cells or repopulate damaged myocardium, but exert a therapeutic effect by paracrine secretion. By contrast, second-generation cell therapy includes cardiac-committed cells. These cells better match organ requirements and thus favour an electromechanical integration and an enhanced therapeutic effect via a paracrine effect.

Anversa's controversy

To conclude, the cell therapy field was deeply affected by the controversy surrounding the recent retraction of several articles from P. Anversa's laboratory alleging data fraud. The 21st century started with the dogma that the heart lacked self-renewal capacity. Unexpectedly, this belief was shaken by the publication by Anversa that bone-marrow derived MSCs could regenerate heart muscle. The subsequent finding revealed the existence of c-kit^{+} resident cardiac stem cells that were clonogenic, multipotent and with self-renewal capacities [74,75]. After giving rise to a large body of publications based on that dogma and the initiation of several clinical trials, difficulties encountered in replicating Anversa's results led to reasonable doubts in the scientific community. Finally, 31 publications were retracted due to unreliable data. Nowadays, the existence of true cardiac stem cells that generate cardiomyocytes is still open to debate. The next few years will surely determine the real impact of these retractions, as well as the future of cell therapies in the post-Anversa era [74,75].

Next-generation cell therapies

The ascent of EVs

Nowadays, we could declare that we are witnessing the era of the EVs. The first evidence of the existence of some kind of cell-derived vesicles appeared in the middle of the last century with the finding of platelet-derived pro-coagulant particles in plasma [76]. Since then, several studies have described the presence of cell-derived vesicles involved in different biological or pathological processes [77–79]. However, lack of knowledge prevailed concerning the relevance, origin or function of these bodies, and they were frequently regarded as cellular "dust". It was not until 2006

that EVs were found to contain biological cargo and even to transfer this cargo to recipient cells, which increased the interest of scientists in these vesicles as possible mediators of intercellular communication [80]. Subsequently, they were found in all body fluids and acknowledge to be secreted by most cell types, which boosted their importance even more [81]. During the last few years, EVs have been mainly explored in the cancer field as prognostic and diagnostic biomarkers or as therapeutics capable of targeting specific cells [82,83]. The growing interest in EVs led in 2012 to the foundation of the International Society of Extracellular Vesicles (ISEV) aiming at standardizing the knowledge about EVs and fostering advances in the field. In regenerative medicine, the parallel expansion of the EV field, along with findings suggesting a therapeutic paracrine secretion of transplanted cells, brought the first studies exploiting the use of EVs. As shown in Fig. 3, the first article addressing the use of EVs for cardiac regeneration was published in 2011 [84]. In the following years, the number of studies that investigate EVs as therapeutic tools for cardiac healing has been progressively increasing. However, this is still a quite unexplored area with a long way to go. Considering the impressive amount of related research that is currently being developed, it would not be a surprise that the number of publications increases exponentially during the next few years.

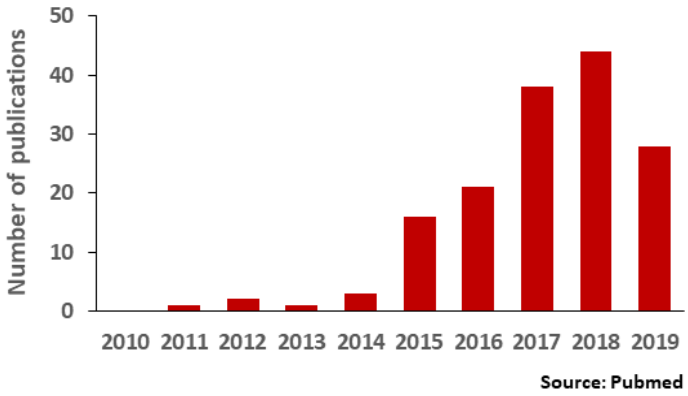

Figure 3. Number of publications per year related to "extracellular vesicles" and "cardiac regeneration" as referenced by Pubmed.

EVs as cell substitutes

In accordance with the latest research findings, from **Chapter 1** we could deduce that transplanted ADSCs and hiPCS-CMs exerted reparative effects by paracrine secretion. Therefore, a novel therapeutic strategy was developed in **Chapter 2** that relied on the potential of EVs for cardiac repair. Recent encouraging results revealed that EVs are not only more effective than cells, but they also represent a safer alternative [85]. As proved by Adamiak *et al*., iPSC-derived EVs led to a better cardiac recovery than iPSC and avoided the formation of teratomas observed after cell transplantation [85]. Furthermore, EVs have been found to be less immunogenic than their parent cells, mainly due

to a lower presence of transmembrane proteins, and to prevent the risk of transmission of viral pathogens [86]. As opposed to living cells, EVs may be stored for long periods before their use facilitating clinical application. Overall, the above-mentioned advantages pave the way for expanding their use instead of cells.

Ultracentrifugation versus size-exclusion chromatography

Because of the incipient state of the EV field, several challenges remain to be solved prior to their widespread use as therapeutic agents. Our first question contemplated whether we should use ultracentrifugation (UC), the gold-standard method, for EV isolation or by contrast, we should implement size-exclusion chromatography (SEC), a novel method that has been shown to provide a superior EV yield, purity and functionality in recent studies [87,88]. Overall, our work revealed certain similarities between both methods. However, in view of the higher purity and slightly enhanced anti-fibrotic effect observed after SEC, along with multiple studies providing evidence in favour of the use of this method against UC or other methods [89], we considered SEC to be an advantageous isolation method. In fact, we suggest that the use of longer columns than that used in our work could provide a better separation of EVs specific subpopulations and less serum contamination. Besides, SEC represents an easy method for implementation in the laboratory, since it does not require specialized equipment, as it occurs with UC, and allows EV isolation from larger volumes, which could facilitate process scaling-up.

EV subpopulations

Although commonly divided into MVs and exosomes due to their origin (Fig. 4), it is now being shown that EV subpopulations are much more complex with the existence of biological and functional differences among them. As recently described by Smith *et al*., even single exosomes isolated from the same cell type exhibit a high variability, mainly associated with membrane composition but also linked to other components [90]. In another example, it was reported that MSCs secrete three different EVs with unique composition of lipids, proteins and RNA [91]. Due to this large variety, only a small proportion of EV population might be responsible for the therapeutic effects. However, discrepancies between studies result in uncertainties about the specific population analysed and therefore about how to interpret the data [92]. Even current EV nomenclature and classification into MVs and exosomes is in flux, and it will probably evolve in parallel with new methods and findings. In order to better guide their therapeutic application, it would be illuminating to progress in the study and characterization of the diverse EV subpopulations.

Importantly, current purification methods are not effective for the isolation of completely pure EVs or for the accurate separation of the different EV subpopulations. In fact, the certain overlap

concerning MVs and exosomes size complicates their separation with current protocols. As reflected in our study, it is only possible to obtain preparations enriched in a certain subpopulation. Furthermore, it is important to implement methods to assess EV purity as it could influence outcomes. For example, the tendency towards a higher anti-fibrotic effect observed with SEC-derived EVs in this work could be in part influenced by the reduced co-precipitation of serum proteins that we also found.

Mechanisms of EVs

In our work in **Chapter 2**, we observed a strong anti-fibrotic effect and we could attribute this effect to EVs, as we confirmed EV internalization in the same cells. For exerting their function, EVs need to be first internalized by receptor cells (Fig. 4). This process involves the fusion of the vesicle membrane with the cell membrane or endosomal membrane after endocytic uptake [93]. There are multiple routes implicated in EV internalization. EV membrane may directly fuse with cell membrane to release their cargo in the cytosol. However, few studies report this mechanism [94]. Alternatively, the most described mechanism is based on the implication of a wide variety of endocytic pathways, which could be clathrin-dependent or independent. Clathrin-dependent endocytosis is a well-known internalization pathway, whose inhibition has been associated with the prevention of EV internalization [95]. In addition, a plethora of clathrin-independent pathways has also been found to be essential [96]. Among these, macropinocytosis, which involves the uptake of large quantities of extracellular fluid, has been reported in many studies as a major participant in EV cell entry [97,98]. Another determining route could be caveolin-mediated endocytosis. Notably, this pathway has been found to participate in EV uptake, but paradoxically it also behaves as a negative modulator of this process [99]. Membrane lipid rafts have been described in some studies to play some part but the exact role of these elements remains to be elucidated. Finally, phagocytic cells such as macrophages have been shown to take up EVs by phagocytosis [100,101]. Possibly several cellular internalization pathways coexist in the same cell and the preference for one specific route could be determined by cell type and proteins found in the EV surface.

Following internalization, EVs must reach the intracellular site of action in order to stimulate a biological action in recipient cell (Fig. 4). The above-mentioned endocytic pathways lead to the formation of intracellular vesicles that enter the endosomal system by fusing in early endosomes. From that point, several uncertainties govern the EV pathway. Most endosomal bodies are fated to enzymatic degradation, including those containing EVs [102]. Interestingly, recent studies suggest that EVs are capable of partially escaping degradation, reaching targeted organelle and releasing their cargo [101,103,104]. However, further studies are needed since nowadays these mechanisms remain enigmatic.

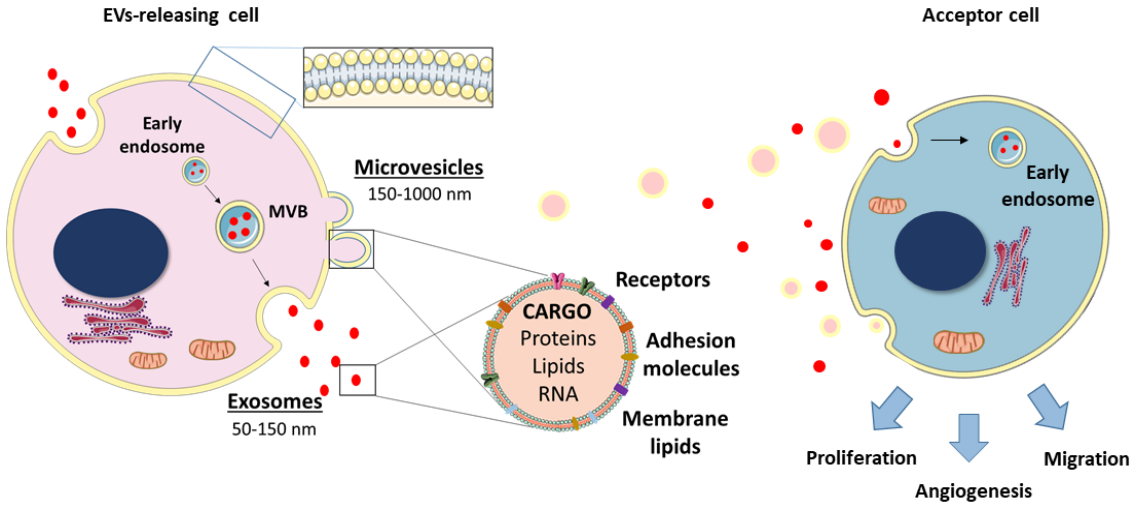

Figure 4. EV secretion and uptake. Schematic representation of major EV routes. Exosomes are released to the extracellular space when multivesicular bodies (MVB) membrane fuses with cell membrane. By contrast, MVs are formed by the budding of the cell membrane. Both subpopulations contain a cargo comprised of proteins, lipids and RNA, that reflects secretory cell cytosolic composition. When taken up by a recipient cell, EVs fuse in early endosomes, which could in part avoid degradation and modulate cell behaviour.

Administration strategies

Harnessing EVs for therapeutic purposes requires understanding how exogenously administered EVs behave *in vivo*. For that reason, over the last few years EVs have been explored in different disease preclinical models. In all these studies, the route of administration and the dose play an important part as they determine EV biodistribution pattern, tissue exposure and therefore final therapeutic index [82]. Both local and systemic administration routes have been explored with debatable results. On the one hand, intravenous delivery of 400 µg of EVs resulted in mouse asphyxiation due to heavy accumulation in the lungs [105]. When a lower dose was injected, EVs were rapidly cleared from blood circulation and taken up by the liver and spleen. In another study, blood residence time of intravenously injected exosomes was found to be only 2 mins [106]. Besides, exosomes derived from cardiosphere-derived cells proved to be effective for reducing scar size, preventing adverse remodelling and improving cardiac function in a pig MI model, but only when exosomes were intramyocardially injected. Unfortunately, intracoronary administration of exosomes was ineffective [107].

In view of all these findings, it could be inferred that local administration fosters EV therapeutic potential by an increased tissue-specific concentration and avoidance of safety and stability issues associated with systemic injection. Nevertheless, advantages of local administration could be counteracted by a poor retention of EVs in the heart, similar to what occurs after direct protein and cell administration [107]. Here, drug delivery systems could stand out by enhancing EV retention

and providing a sustained release kinetics over time. Among them, hydrogels represent excellent candidates for cardiac delivery of therapeutic agents, as described before. Hydrogel manufacturing materials should be carefully chosen as hydrogels have shown to improve and support heart performance on their own [108,109]. Manufacturing materials also determine possible administration routes. Therefore, it is highly recommended to select materials with shear-thinning or *in situ*-forming properties, which could be compatible with a non-invasive administration procedure using catheter systems. It is with that in mind that in **Chapter 3** we selected alginate and collagen for hydrogel manufacturing, which meet all the mentioned criteria. Both materials form hydrogels after heart injection, mimic native cardiac extracellular matrix and guide cardiac repair [110,111]. Recently, a few hydrogels have been investigated for cardiac delivery of EVs [39,40,112]. Encouragingly, these studies showed an enhanced retention of EVs, which turned into a reduced apoptosis, polarization of macrophages, increased angiogenesis and improved cardiac function [40,112].

To conclude, **Chapters 2 and 3** constitute the first steps towards the development of a new therapeutic approach for cardiac repair. After identifying signs suggesting a paracrine therapeutic effect following cell transplantation in **Chapter 1**, we suggested the investigation of EV potential. Therefore, in **Chapter 2** CPCs were selected and EV purification protocols were examined. Next, in **Chapter 3** we developed a novel injectable hydrogel for cardiac delivery of the isolated EVs. Future studies will analyse the efficacy of the designed strategy on a MI animal model.

The future of cell therapies

A few months ago, a surprising finding was published in *Nature* involving the mechanistic basis behind the therapeutic effects of cell therapy [113]. In this study, authors found similar heart rejuvenation after injecting two different types of adult stem cells in the injured area, dead cells or a chemical that induces inflammation. This impressive discovery led authors to conclude that benefits of cardiac cell therapy are due to an acute immune response [113]. The corroboration of this hypothesis in other studies would signify a change of paradigm within current cell therapies and it would be necessary to reconsider ongoing preclinical and clinical investigation lines. Whether this finding opposes the actual “paracrine hypothesis” or by contrast both could be complementary remains elusive. What is undeniable and consistent with other studies is the key role that modulation of inflammation has in cardiac regeneration [114,115]. In view of that, next era will probably be defined by the devotion of large efforts to the understanding and management of the inflammatory process.

III. CARDIOPROTECTION OF SQUALENE-ADENOSINE NANOPARTICLES FROM REPERFUSION INJURY

Reperfusion injury

In **Chapter 4**, we focused on a different therapeutic scenario from earlier chapters and exploited the advantages of nanomedicine to halt reperfusion injury in the heart. Perhaps one of the major advances in the treatment of acute MI is the implantation of early reperfusion therapy in clinical routine. In the first years, administration of fibrinolytic agents was the eligible strategy to achieve reperfusion. However, during the last decade percutaneous coronary intervention became the preferred option due to its superior efficacy and patient prognosis [116,117]. While the benefits of reperfusion to impede the extension of irreversible tissue necrosis are undeniable, the sudden return of blood to non-perfused areas is responsible for 50% of the final infarct size (Fig. 5) [118]. The pathophysiology of reperfusion injury is characterized by transient arrhythmias, myocardial stunning (i.e. dysfunctional contraction), microvascular obstruction and cardiomyocyte death. Mechanisms behind these phenomenon include mechanical damages such as haemorrhage from injured vessels, which increases interstitial pressure, cardiomyocyte swelling as well as molecular misbalances in cardiac myocytes [118]. In fact, reoxygenation produces greater oxidative stress than the ischemic period itself, intracellular calcium overload, neutrophil activation and myocardial accumulation, platelet aggregation and a complex inflammatory process [119].

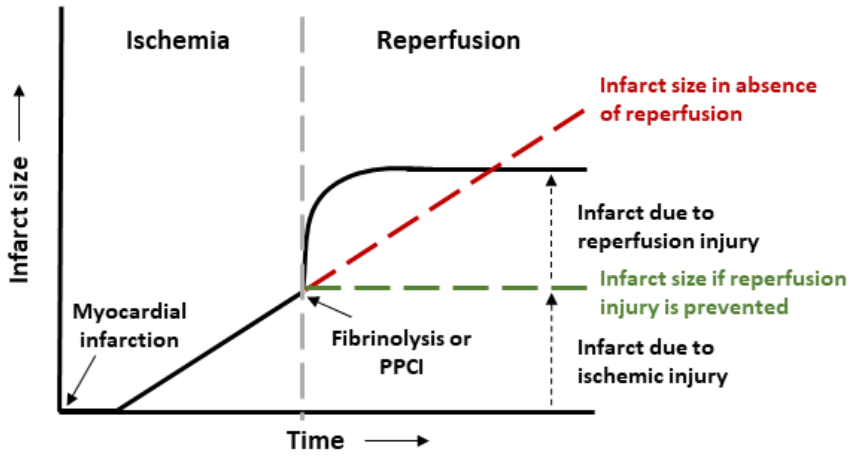

Figure 5. Evolution of infarct size and reperfusion injury. After a MI, infarct size progressively increases over time. Clinical reperfusion of the ischemic area by fibrinolysis or primary percutaneous coronary intervention (PPCI) halts infarct expansion. However, reperfusion itself is responsible for 50% of final infarct size.

The rationale behind the use of adenosine and vitamin E

After the discovery that reperfusion is associated with secondary damages, the search for a therapeutic solution started. Until now, different strategies have been tested to mitigate myocardial reperfusion injury. For example, it was shown that ischemic conditioning based on the induction of short periods of ischemia followed by reperfusion was useful to decrease reperfusion injury [120]. However, most efforts have focused on the development and evaluation of cardioprotective drugs that could act as adjuvants to reperfusion therapy.

Adenosine, an endogenous purine nucleoside produced through the metabolism of adenosine triphosphate, has been investigated with the aim of reducing the impact of reperfusion in preclinical and clinical studies [121,122]. However, its success has been hindered by its short half-life. Its rapid degradation in blood circulation obligates to administer high doses, which induces bradycardia, hypotension and tachycardia. Adenosine signalling is mediated by the activation of four ubiquitous G protein-coupled receptors (A_1, A_{2A}, A_{2B} and A_3). Interestingly, these receptors are expressed in multiple cardiac cells including cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells [123], which could make them a suitable target for post-MI healing. Whether the activation of one or more receptors is needed for cardioprotection and the specific protective mechanisms requires further investigation. However, it has been shown that the activation of these receptors attenuates many of the reperfusion injury mechanisms: it preserves microvasculature, inhibits neutrophils, restores calcium homeostasis and inhibits reactive oxygen species production lowering inflammation, among other advantages [124].

Due to the enhanced production of reactive oxygen species after reperfusion, it was proposed that the incorporation of a natural antioxidant as it is vitamin E (vitE) could help to reduce oxidative stress and restore normal cell functions. In the long-term, we failed to observe any beneficial effects associated with vitE. The reasons could be an inefficient incorporation of vitE in the formulation or a low dose administration. A recent study by Wallert *et al*. confirmed that the administration of vitE 2h prior to reperfusion, at reperfusion and twice a day for three consecutive days, improved cardiac function [125]. Therefore, a careful adjustment of vitE dose and administration routine should be performed in future studies.

Adenosine ´squalenoylation´

Adenosine meets the most important criteria for its combination with drug delivery systems. It is a fragile molecule with a very short half-life in the blood circulation, the infusion of high doses is needed to reach cardioprotective effects and it induces side effects by the activation of adenosine receptors in off-target areas [126]. In view of the limited potential of adenosine due to difficulties posed by delivery, in the last decade some authors proposed the encapsulation of this molecule into

NPs [127,128]. First, Takahama et al. found that the delivery of adenosine encapsulated in PEGylated liposomes to reperfused rats significantly reduced MI size and negative hemodynamic effects comparatively to free adenosine [127]. In a later study, adenosine was adsorbed in silica NPs and administered in transiently infarcted rats. The proposed treatment decreased infarct size and attenuated hypotension produced by free adenosine [128]. These nanomedicines made use of two of the most common methods for the incorporation of molecules into particulate systems: physical encapsulation and adsorption, which are often related to a burst release. In this study, we proposed the easy and reproducible manufacturing of NPs by the conjugation of adenosine to squalene (SQAd), a natural lipid. SQAd bioconjugates had an amphiphilic nature and self-assembled into spherical NPs of 100-130 nm when nanoprecipitated in an aqueous solution [129]. Besides avoiding initial burst release, chemical linkage to squalene allows for a high drug loading of 37% [130] and favourably modifies its biodistribution, metabolism and therefore therapeutic effect [131].

Possible mechanisms of NPs accumulation in the ischemic heart

In the first study in **Chapter 4**, we found that NPs accumulated in the ischemic myocardium following intravenous administration. Several mechanisms could participate in this biodistribution. Some of the most probable are summarized below:

1. Enhanced permeability and retention effect (EPR)

In a healthy microenvironment, the extravasation of NPs to the myocardium across the tight vascular endothelium is not feasible. However, as discovered in the cancer field, tissue injury damages the tight junctions of endothelial cells, turning the restrictive endothelium into a leaky barrier for NPs [132]. This phenomenon, known as the EPR effect, has also been described in the heart [133]. On that basis, several strategies rely on this pathological environment to easily deliver nanomedicines to the heart and other organs, assuring an enhanced accumulation in the desired area [134]. Whether SQAd NPs cross the compromised vasculature on their own or conjugated with low-density lipoproteins (LDL) is still open to question. In any case, previous studies supported the design of NPs with a mean size of 100-200 nm for passive targeting to the left ventricle [135].

2. Interaction with plasma lipoproteins

After administration in the bloodstream, SQAd NPs interact with a plethora of different biomolecules that bind to their surface, conferring on them a biological identity and affecting final residence time, biodistribution and efficacy. In particular, the hydrophobic, lipid and cholesterol-related nature of squalene favours the interaction of NPs with endogenous lipoproteins, prolonging their residence time in the vascular compartment, which is probably a key mediator of the enhanced cardiac localization. Furthermore, in a previous study, squalene-based NPs were observed to interact mainly with LDL in plasma [136], giving them LDL receptor-targeting properties. Interestingly, some LDL

receptors have been described to be upregulated in the heart after ischemia [137], which could also favour NP accumulation in the heart.

Besides such passive targeting approaches, other authors have made use of active targeting to enhance heart accumulation by the incorporation in the nanoformulation of cardiac specific ligands, such as angiotensin II type I receptor (AT1) ligand [138] or mitochondria-targeted peptides [139].

In this study, a large number of SQAd NPs ended up in the liver and spleen. Although this is not the most desirable finding, some research by P. Couvreur's group (University of Paris-Sud) demonstrated that SQAd NPs induced no markers of hepatic or renal stress, nor did they alter the white blood cell count, red blood cell count or food intake (unpublished results). These preliminary results support the safety of the treatment. Further studies would be required to obtain more detailed evidence.

Mechanisms of SQAd NPs mediated cardioprotection

In the second part of **Chapter 4**, we correlated the accumulation of SQAd NPs in the heart with an improved cardiac performance and limited adverse left ventricular remodelling after 3 months. The delivery of SQAd NPs had already proved its effectiveness in the management of other pathologies [130,140]. However, it was not until very recently that the mechanism of action of SQAd NPs was elucidated. Whether these bioconjugates directly activate adenosine receptors or by contrast, it is free adenosine, which after SQAd intracellular processing, activates such receptors, remained unknown. Several techniques performed by Rouquette *et al*. such as radioligand displacement assays or assessment of SQAd NPs cell uptake confirmed the second hypothesis (Fig. 6) [141]. The deep interaction of SQAd complexes with LDL molecules in the blood could be the reason why direct activation of adenosine receptors is hampered. As a summary, it could be stated that SQAd NPs act as prodrugs that are efficiently taken up by cells through interaction with LDL receptors. In accordance with the clathrin-dependent endocytosis induced by these receptors, it is probable that SQAd NPs follow the same pathway. In that case, bioconjugates would be degraded in endosomes and lysosomes. Adenosine would be slowly cleaved from squalene and released into the extracellular medium, acting in an autocrine and paracrine manner [141]. Finally, we should take into account that the above-mentioned studies were performed in hepatic cells due to the large accumulation of SQAd NPs in the liver, and further experiments would be needed to confirm this hypothesis. In fact, current studies in our group focus on the mechanisms responsible for the cardioprotective effects discovered in this thesis, with special emphasis on cardiomyocyte apoptosis and inflammation modulation.

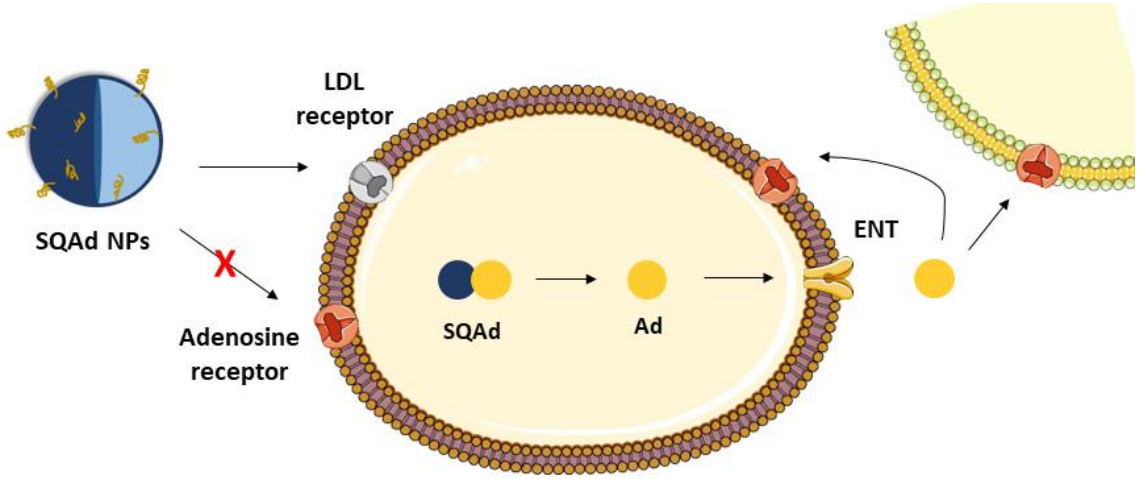

Figure 6. SQAd NPs mechanism. SQAd NPs do not activate adenosine receptors. By contrast, NPs are internalized through interaction with LDL receptors and intracellularly processed. Next, adenosine is released into the extracellular space via equilibrative nucleoside transporter (ENT) and activates adenosine receptors.

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CONCLUSIONS

The main objective of this work was to address cardiac injury after a myocardial infarction by promoting heart repair or providing cardioprotection from reperfusion injury. Based on all the work carried out throughout this doctoral thesis, the following can be concluded:

1. Poly(lactic-co-glycolic acid) microparticles with a size compatible with intramyocardial injection were successfully synthetized by multiple emulsion solvent evaporation method using TROMS® technology. Neuregulin was encapsulated into microparticles obtaining a high encapsulation efficiency. Biomimetic functionalization of microparticles with collagen type I and poly-D-lysine induced the adhesion of both adipose-derived stem cells and human induced pluripotent stem cells-derived cardiomyocytes.

2. Microparticles represent excellent vehicles for cell delivery. Administration of adipose-derived stem cells and human induced pluripotent stem cell-derived cardiomyocytes adhered to microparticles in the infarcted cardiac tissue increased notably long-term cell survival and retention. Interestingly, this was the first time that such prolonged survival was described.

3. Combination of adipose-derived stem cells and neuregulin-loaded microparticles stimulated cardiac repair after a myocardial infarction by inducing cardiomyocyte proliferation, macrophage polarization, vasculogenesis and preventing adverse remodelling. However, these cells showed poor transdifferentiation to cardiac cells. Similarly, human induced pluripotent stem cell-derived cardiomyocytes combined with microparticles enhanced cardiac performance. The therapeutic effects induced by both cell types might be due to a paracrine secretion of cytokines.

4. The culture of cardiac progenitor cells in presence of extracellular vesicles-free serum is unavoidable for an adequate production of extracellular vesicles. Regarding isolation protocols, ultrafiltration combined with size-exclusion chromatography is as reliable as ultracentrifugation for the isolation of extracellular vesicles. Indeed, size-exclusion chromatography resulted in a reduced co-purification of serum major proteins. Both microvesicles and exosomes subpopulations exerted similar anti-fibrotic effects, arising as potential candidates for cardiac repair.

5. A novel hydrogel constituted by alginate, collagen type I and crosslinked with calcium gluconate was designed for a potential cardiac delivery of extracellular vesicles. The hydrogel showed a low viscosity and good injectability, suggesting its compatibility with catheter-based administration. When incorporated in the hydrogel, extracellular vesicles showed a homogeneous distribution throughout the scaffold matrix.

6. Squalene-adenosine nanoparticles were successfully prepared by nanoprecipitation, obtaining a homogeneous population with a mean size of 100-130 nm, a negative zeta potential and a spherical shape. After intravenous administration in a murine ischemia/reperfusion myocardial infarction model, nanoparticles accumulated in the ischemic myocardium for at least 24h.

7. Reperfusion injury was prevented by the intravenous administration of squalene-adenosine nanoparticles before or after reperfusion in a preclinical ischemia/reperfusion myocardial infarction model. As a result, ejection fraction and fractional shortening were significantly improved after three months. In parallel, left ventricular adverse remodelling was prevented and infarct size was decreased. The incorporation of vitamin E in the formulation did not enhance the therapeutic effects. By contrast, administration of free adenosine failed to improve cardiac performance.

CONCLUSIONES

El objetivo principal de este trabajo fue abordar el daño cardiaco producido tras un infarto de miocardio, mediante la estimulación de la reparación del corazón y/o proporcionando cardioprotección frente a la lesión miocárdica ocasionada por la reperfusión. Los estudios realizados permiten extraer las siguientes conclusiones:

1. Mediante emulsión múltiple y evaporación del disolvente utilizando la tecnología TROMS, se formularon micropartículas de ácido poli(láctico-co-glicólico) de tamaño compatible con la administración intramiocárdica. La neuregulina fue encapsulada en dichas micropartículas, obteniéndose una alta eficacia de encapsulación. La funcionalización biomimética de las micropartículas con colágeno tipo I y poli-D-lisina permitió la adhesión de las células madre derivadas de tejido adiposo así como de los cardiomiocitos derivados de células madre pluripotentes inducidas.

2. Las micropartículas son excelentes vehículos para la administración de células. La inyección en el tejido cardiaco isquémico de células madre derivadas de tejido adiposo y de cardiomiocitos derivados de células madre pluripotentes inducidas en combinación con micropartículas aumentó considerablemente la supervivencia y retención celular a largo plazo. Es la primera vez que se describió la supervivencia de dichas células en el corazón tres meses y dos meses después de su administración, respectivamente.

3. La combinación de células madre derivadas de tejido adiposo con micropartículas cargadas con neuregulina promovió la reparación cardiaca tras un infarto de miocardio, mediante la estimulación de la proliferación de cardiomiocitos, la polarización de macrófagos hacia un fenotipo regenerador, la vasculogénesis y la prevención del remodelado adverso del ventrículo. Sin embargo, estas células mostraron un bajo índice de diferenciación a células cardiacas. Por otro lado, la combinación de cardiomiocitos derivados de células madre pluripotentes inducidas con micropartículas mejoró la función cardiaca. Los efectos terapéuticos observados en ambos casos podrían deberse a una secreción paracrina de citoquinas.

4. La presencia de suero en el medio de cultivo de las células progenitoras cardiacas fue indispensable para una producción adecuada de vesículas extracelulares. Por otro lado, el método de ultrafiltración combinado con cromatografía de exclusión por tamaño resultó tan

eficaz como la ultracentrifugación para el aislamiento de vesículas extracelulares. Además, la utilización de la cromatografía de exclusión por tamaño permitió una menor co-purificación de proteínas mayoritarias del suero. Las subpoblaciones de microvesículas y exosomas mostraron efectos anti-fibróticos similares, lo que las convierte en posibles candidatas para la reparación cardiaca.

5. Se desarrolló y caracterizó un hidrogel constituido por alginato, colágeno tipo I y gluconato cálcico con el objetivo de optimizar la administración cardiaca de vesículas extracelulares. El hidrogel presentó una baja viscosidad y una adecuada inyectabilidad, lo cual implica su compatibilidad con la administración mediante catéter. Tras incorporar las vesículas extracelulares en el hidrogel éstas mostraron una distribución homogénea en la matriz del sistema.

6. La preparación de nanopartículas de escualeno-adenosina utilizando el método de nanoprecipitación permitió obtener una población homogénea de nanopartículas esféricas con un tamaño medio de 100-130 nm y potencial zeta negativo. Tras su administración en un modelo murino de isquemia/reperfusión, las nanopartículas se acumularon en el miocardio infartado durante al menos 24h.

7. La administración intravenosa de las nanopartículas de escualeno-adenosina antes o después de la reperfusión previno el daño cardiaco asociado a dicha intervención. Como consecuencia, la fracción de eyección y la fracción de acortamiento mejoraron significativamente a los 3 meses. De igual manera, estas nanopartículas disminuyeron el remodelado adverso del ventrículo izquierdo y el tamaño de infarto. La incorporación de vitamina E a esta formulación no mejoró la eficacia terapéutica. Por otra parte, la administración de adenosina libre no mejoró la función cardíaca.