



Development of pharmacologically active microcarriers transporting stem cells and releasing growth factors for cardiac tissue-engineering

Jean-Pierre Karam

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Development of pharmacologically active microcarriers transporting stem cells and releasing growth factors for cardiac tissue-engineering

PHYLOSOPHICAL DOCTORATE

Speciality Cellular Biology

Ecole doctorale Biologie Sant 

Presented in public

The 05th of July 2012

At Angers

By **Jean-Pierre Karam**

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Development of pharmacologically active microcarriers transporting stem cells and releasing growth factors for cardiac tissue-engineering

This thesis is a human story about human

To my Parents

To my grand parents especially in loving memory of Paul Dorville and Floriane Karam

“And, if I do not carry the price, I will have at least the honor to have undertaken it.” Jean de
La Fontaine

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Abbreviations

ADSC:	Adipose-derived stem cells
BMP:	Bone morphogenetic protein
CX43:	Connexin 43
CPC:	Cardiac progenitor cells
cTnI:	Cardiac troponin I
ECM:	Extracellular matrix molecules
EPC:	Endothelial progenitor cells
ESC:	Embryonic stem cells
FGF:	Fibroblast growth factor
FN:	Fibronectin
GATA4:	GATA binding protein 4
GF:	Growth factors
HG:	Hydrogel
HGF:	Hepatocyte growth factor
IGF-1:	Insulin-like growth factor 1
IL-6:	Interleukin 6
iPSC:	Induced pluripotent stem cells
LIF:	Leukemia inhibitory factor
LM:	Laminin
LVEF:	Left ventricular ejection fraction
MEF2C:	Myocyte enhancing factor 2

MI:	Myocardial infarction
MLC-2v:	Myosin like chains 2
MSC:	Mesenchymal stem cells
NKX2.5:	NK2 homeobox 5
P188:	Poloxamer 188
PAM:	Pharmacologically active microcarriers
PEG:	Poly (ethylene) glycol
PLGA:	Poly D, L (lactic-co-glycolic) acid
SCNT:	Somatic cell nuclear transfer
SERCA2a:	calcium-transporting ATPase sarcoplasmic reticulum type 2
SM:	Skeletal myoblast
SVF:	Stromal vascular fraction
TE:	Tissue engineering
TGF β :	Transforming growth factor
VEGF:	Vascular endothelial growth factor

Preamble

The story started in my third year of university on the benches of the Faculty of Science at Angers where I made knowledge with the biosignalisation and the biology of the development and started thus to discover a little more in-depth the cell. The cell, this small organism a little bit complex became finally interesting. Most interesting was that from one only among them; it could give rise to a tissue, an organ, a perfectly organized living thing. More interesting was the fact that this tedious organism could be handled and modified under the influence of signals and give a cell more differentiated or make another thing completely different, proliferating in an anarchistic way and giving rise to a cancer cell. There was this duality in the classes where in one part the cell represented the evil to fight through the development of tumors and cancers and in the other hand it represented the hope, the small thing from which everything “birth/grow” from which we could repair/replace/cure to give again a normal life. I made my first training class in clinical research in oncology on the “Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor targeted therapies in colorectal cancer”. All this consolidated/reinforced my desire for evolving to a more applied research, a preclinical research at the interface between the clinical field that I had met and the fundamental one. My second experience in research was a third month training classes about the “Conception of an in vitro system to study the direct effect of a protein released from biodegradable microspheres”. I thus discovered a new domain, a part the pharmaceutical world which was that of the formulation and galenic. The goal of this training classes was to develop a system that ensure that the quantity of released Nerve Growth Factor from microspheres was sufficient to allow the survival and the differentiation of the cells. This first step was more or less a pharmaceutical approach of tissue engineering for neurological disorders of the central nervous system. Then I continued my training classes of Master on the “Adherence of human neural stem cells on Pharmacologically Active Microcarriers and their detection in rat brain“. It was during this stay that my interest for the cellular therapy really developed. It was at a conference of Prof. Marc Péschanski with this video of patients with Huntington disease before and after the graft! It was just impressive, amazing, and incredible and although the effects were not permanent, the cellular therapy worked!

I have then started my PhD in “cotutelle” between the university of Angers and the university of Bologna. Finally it was a more “ambitious” project with the Italian national institute for

cardiovascular research (INRC), a consortium of several research groups such as the university of Roma, Parma, Chieti, Turin, Florence and Bologna. The global subject was “cardiac regeneration by implantation of pharmacologically active microcarriers (PAM) transporting stem cells and releasing growth factors. The aim was the formulation of PAMs releasing different growth factors (GF) implied in cardiac repair and combined with different cell population used in each laboratory. I thus started to work in Angers for 6 months to develop some PAMs-GF and then I left to Bologna for 18-20 months. The difficulty relies on my teaching activity as a monitor at the university of Angers. So, I returned approximately every 2 months to Angers for periods from 1 to 2 weeks to ensure my lesson at the superior institute of health and bio-products of Angers (ISSBA) and the faculty of pharmacy of Angers and/or for microspheres formulations. The last part of my thesis and the last experiments were performed in Angers. The manner of working was different and I have tried to combine these two methods that finally gave rise to this work of thesis that I present below.

General introduction

1. Myocardial infarction

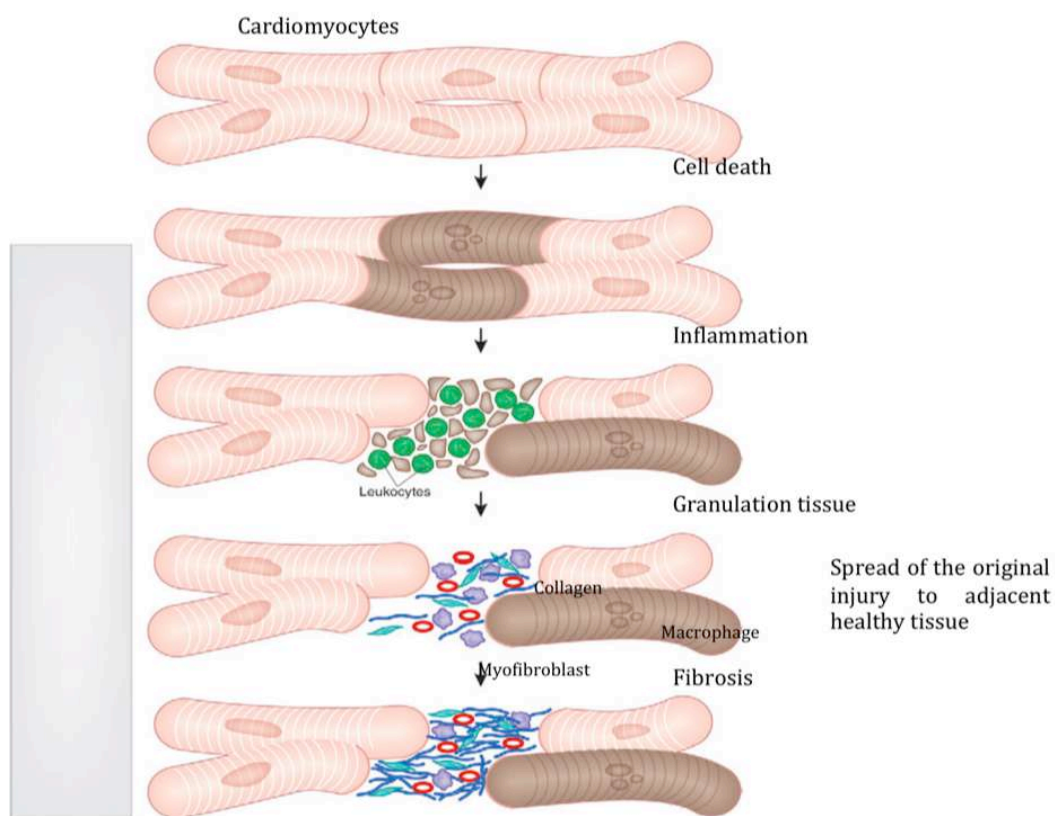
1.1. Epidemiology

Myocardial infarction (MI) constitutes the first cause of morbidity and mortality in developed countries with an annual incidence rate of approximately 600 cases per 100,000 individuals in USA, where approximately 500,000-700,000 deaths are caused by ischemic heart disease. In France, MI has an annual incidence rate of 120,000 cases, similar to the Italian annual incidence rate around 118,000 cases. According to data of the World Health Organization (WHO), of 50 million annual deaths in the world, ischemic heart diseases are the first causes of death with 7.2 million deaths of coronary origin. In France, its prognostic remains severe since MI is still responsible for 10-12% of the annual total mortality in adults. With this mortality, an important socio-economic repercussion should be added as the related spending on this disease represents more than 1% of the total medical expenditure.

1.2. Physiopathology

The launching of the MI is dependent, in a majority of cases, on the rupture of a coronary atheroma plaque, involving the formation of an occlusive thrombosis. The disruption of the plate of atheroma will break the thrombo-resisting endothelial barrier and will expose the extracellular matrix (ECM) molecules (fibronectin, collagen, vitronectin) to the circulating platelet. That will allow platelet aggregation and lead to the formation of an intracoronary occlusive platelet thrombus. The brutal occlusion of the coronary artery involves an imbalance between the tissue requirements in oxygen and the contribution of arterial blood beyond the occlusion site. The consequences at the cellular level are generally associated to a reduction of the energy level by activation of the anaerobic pathways of oxidation, a tissue acidification by accumulation of cellular metabolism products such as lactate, an ionic imbalance between the intra and extracellular medium and an increase in intracellular calcium. Consequences on the whole cardiac muscle are a reduction of the contractility, modifications of the electrocardiogram and hyper excitability of the myocardium. If ischemia exceeds 30 minutes, an irreversible process of necrosis occurs and causes cardiomyocyte death within few hours. An immediate and massive infiltration of circulating leucocytes into

the ischemic core occurs, due to endogenous surrounding cells secreting cytokines and chemokines such as tumor necrosis factor alpha, monocyte chemoattractant protein 1, interleukin 1 (IL-1), IL-6 and IL-8 (Boudoulas and Hatzopoulos 2009). This process is followed by the recruitment of pro-inflammatory immune cells due to up-regulation of cell adhesion molecule secretion by endothelial cells, such as E-selectin, intercellular adhesion molecules and vascular adhesion molecules. The immune cells remove cellular debris from the injured area allowing myofibroblast infiltration, depositing collagen and other ECM proteins leading to scar formation. In this way, a fibrotic scar gradually replaces dead cells, which disrupt the electromechanical continuum of the ventricular muscle and compromises its pumping capacity (cf. Fig 1.). The effects on the whole cardiac muscle are an evolution of necrosis from the “under-endocardium to the under-epicardium and from the non-perfused area towards the periphery. Moreover, the irreversible damage leads to heart remodeling to try to compensate the deficient pumping capacity.



Konstantinos D. Boudoulas and Antonis K. Hatzopoulos. *Disease Models & Mechanisms* 2, 344-358 (2009)

1.3. Treatment

Myocardial revascularization by angioplasty or coronary bypass, adjunction of inhibitors of the angiotensin-converting enzyme and beta-blocker association constitute the current MI treatments. Recently, electrical treatment has allowed important progress for MI treatment. However, in severe forms the only treatment remains heart transplantation but it can only be proposed to a restricted number of patients. In addition, the reduced availability of grafts that is worsening in occidental countries, as well as the need for subsequent immunosuppression further decreases the possibilities of transplantations. Several innovating alternative therapeutic strategies are under exploration to increase quality of life and life expectancy, hence, in recent years cell based therapies have attracted considerable interest.

2. Cell therapy in human myocardial infarction

Cell therapy consists in the transplantation of cells with the aim of preventing, treating or attenuating a disease. The cells will repair, replace or improve the failing biological function of the damaged tissue. There is growing evidence that heart muscle has the ability to regenerate through the activation of resident cardiac stem cells (CPCs) or through recruitment of a stem cell population from other tissues (Beltrami, Barlucchi et al. 2003). However, this regenerative capacity of the heart cannot compensate for the large-scale tissue loss after MI. Cell therapy tries to ensure cell colonization but also to restore the viability and the functional capacity of the damaged myocardium. Stem cell-mediated cardiac repair follows two main strategies to improve regeneration of the damaged heart area. The first, aims at recruiting or promoting the homing of endogenous or circulating stem cells at the periphery or inside the damaged zone with locally injected factors such as growth factors (GF), cytokines and ECM molecules (Post, Laham et al. 2001; Kastrup, Ripa et al. 2006; Anderson, Heydarkhan-Hagvall et al. 2008; Srinivas, Anversa et al. 2009). The second is based on the local transplantation of stem cells to replace the dead cells, therefore building a new tissue with a new population of functional and beating cardiomyocytes, with cell-cell communications, secreted factors and induction of neoangiogenesis.

2.1. Cells used for cardiac repair

Recent studies have focused largely on committed myogenic cells including skeletal myoblasts (Menasche 2008; Menasche, Alfieri et al. 2008; Seidel, Borczyk et al. 2009), embryonic stem cells (ESCs) (Fujiwara, Hayashi et al. 2007; Laflamme, Chen et al. 2007; Tomescot, Leschik et al. 2007; Chen, Wu et al. 2008; Habib, Caspi et al. 2008; Leschik, Stefanovic et al. 2008; Puceat 2008; Vidarsson, Hyllner et al. 2010), bone marrow–derived hematopoietic cells (Orlic 2001; Orlic, Kajstura et al. 2001; Strauer, Brehm et al. 2002), endothelial progenitor cells (EPCs) (Urbich 2004; Krenning, van Luyn et al. 2009), multipotent mesenchymal stromal cells also called mesenchymal stem cells (MSCs) (Rangappa, Entwistle et al. 2003; Hattan, Kawaguchi et al. 2005; Antonitsis, Ioannidou-Papagiannaki et al. 2007; Yamada, Sakurada et al. 2007), resident cardiac progenitor cells (CPCs) (Beltrami, Barlucchi et al. 2003; Urbanek 2005; Barile, Messina et al. 2007; Kajstura, Urbanek et al. 2008; Di Nardo, Forte et al. 2010; Kuhn and Wu 2010) and more recently induced pluripotent stem cells (Zhang, Wilson et al. 2009; Zwi, Caspi et al. 2009; Das and Pal 2010) to improve cardiac regeneration.

The transplanted cells may induce tissue repair by cell replacement or by an indirect paracrine manner. They can either directly differentiate into endothelial cells to rebuild and vascularize the ischemic area (Chavakis, Urbich et al. 2008) or into cardiomyocytes to replace the fibrotic scar by a new functional tissue (Beltrami, Barlucchi et al. 2003) They can secrete several GFs and cytokines involved in neoangiogenic processes, cell survival, recruitment or differentiation.

We can divide the cells used for cardiac repair in two groups in function of their differentiation properties.

2.1.1. Pluripotent stem cells:

Pluripotent stem cells come from the embryo and can differentiate into all cell types of the organism except extra-embryonic cells. We called them embryonic stem cells (ESCs). They represent a theoretically unlimited source of cardiac cells for cardiac repair. Transplantation in rodent models of MI have shown that hESCs derived cardiomyocytes can graft and survive

in normal and infarcted animal after several weeks (Min, Yang et al. 2003; van Laake, Passier et al. 2007; Qiao, Zhang et al. 2010). Interestingly, transplantation of mESCs cultured on poly-(lactic-co-glycolic) (PLGA) biodegradable scaffolds into infarcted mouse myocardium significantly improved animal survival, blood pressure, and ventricular function (Ke Q 2005). Authors also report the presence of implanted cells in the infarcted area suggesting cell retention and possible myocardium repair (Ke Q 2005). However, their use is limited by ethical concerns and the risk of teratomas formation. Indeed, some studies utilizing injection of undifferentiated ESCs reported the formation of teratomas suggesting that this approach is not clinically relevant (Nussbaum, Minami et al. 2007; Chung, Kee et al. 2011; Stachelscheid, Wulf-Goldenberg et al. 2012). However, teratoma formation with pre-differentiated ESCs into cardiomyocytes is not documented (Behfar A., Perez-Terzic C. et al. 2007, Blin G., Nury D. et al. 2010). So, novel alternative sources for autologous stem cells employ the new technology of somatic cell nuclear transfer (SCNT) (Cervera and Stojkovic 2007; Cervera and Stojkovic 2008). SCNT cells are generated by removing the genetic material from a donated human oocyte and replacing it with genetic material from the cells of patients. They also have ESC pluripotency. However, this approach has met political, ethical and social obstacles.

Alternatively, another population of pluripotent stem cells called induced pluripotent stem cells (iPSCs) has been described recently. They are derived from somatic cells (generally fibroblast) that have been transformed into pluripotent cells by the forced expression of stem cell-associated genes. iPSCs express embryonic markers, such as Oct3/4, Sox2 and Nanog, and have the differentiation potential of ESCs, which allow them to be considered as interesting candidates for cardiac regenerative applications. Additionally, mouse iPSCs were demonstrated to be able to differentiate into cell derivatives of all 3 germ layers, including functional cardiomyocytes (Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007). Engraftment and differentiation of iPSCs was recently demonstrated in a rat model of acute myocardial infarction (Mauritz, Martens et al. 2011; Yan, Abdelli et al. 2011). Another interesting study using a cell-sheet of differentiated cardiomyocytes into a rat model of MI has recently shown improvement of systolic function accompanied by neovascularization (Masumoto, Matsuo et al. 2012). Thus, these cells constitute an interesting alternative to embryonic stem cells.

Interestingly, pluripotent stem cells give rise to multipotent stem cells that are able to differentiate into several cell type from the same embryonic layer.

2.1.2. Multipotent stem cells:

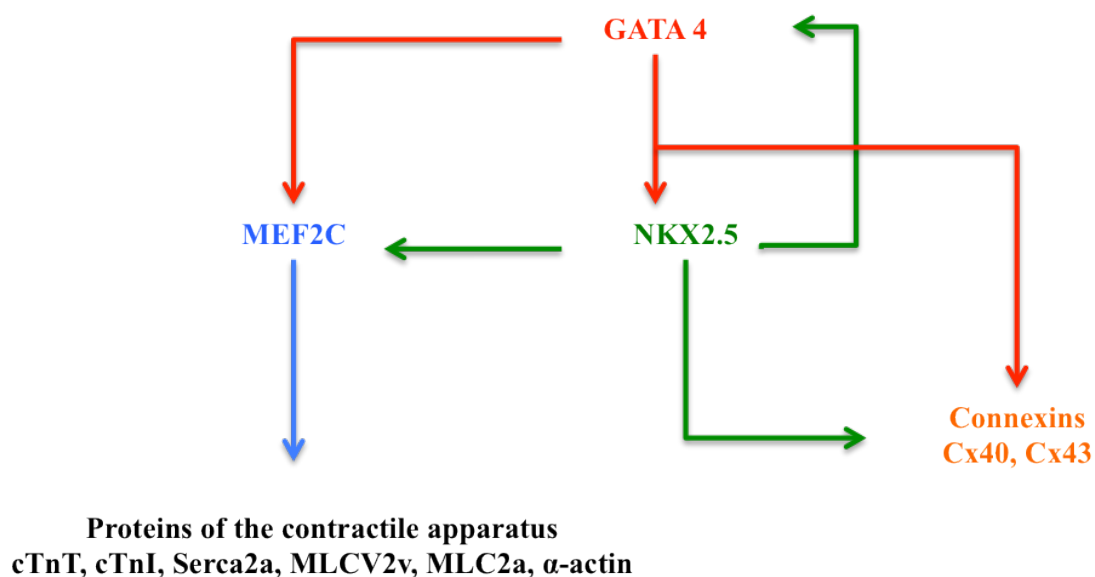
As well as pluripotent stem cells they can self-renew but they can only generate different cells from a specific tissue. Mesenchymal stem cells (MSCs) are a bone marrow stromal population of CD105⁺/CD45⁻/CD90⁺/CD73⁺/CD34⁻/CD133⁻ cells and give rise to adipocytes, chondrocytes, osteoblasts, and in some conditions to endothelial cells (Pittenger, Mackay et al. 1999). They have immunosuppressive properties and can be easily isolated and expanded in culture to obtain large numbers that are adequate for cell therapy. Transdifferentiation of MSCs, that is the differentiation into a cell type originating from a different germ layer, has been reported. Particularly, MSC transdifferentiation into cardiomyocytes and vascular lineage has been proposed as the principal mechanism of cardiac repair. Furthermore, due to their high secreting paracrine activity MSCs might be used as a platform to deliver specific soluble proteins to the site of injury (Caplan and Dennis 2006; Gneccchi, Zhang et al. 2008).

Another population of mesenchymal cells that can be easily isolated from the stromal vascular fraction (SVF) of adipose tissue is named adipose-derived stem cells (ADSCs). These cells are easy to amplify in culture and express CD34⁺, CD105⁺, CD45⁻, CD31⁻ cells. ADSCs subpopulations displaying the hematopoietic stem cell markers CD34 have been recently shown to contain progenitor cells, able to differentiate into mature endothelial cells and participate in blood vessel formation (Miranville, Heeschen et al. 2004; Planat-Benard, Silvestre et al. 2004; Madonna, Geng et al. 2009). Likewise, it has been found that cells positive for the kinase insert domain receptor (KDR), vascular endothelial growth factor receptor-2 (VEGFR-2), isolated from cultured ADSCs adopted an endothelial phenotype in the presence of VEGF (Martinez-Estrada, Munoz-Santos et al. 2005). They also express mesenchymal markers such as CD73 and CD90 and display a mesenchymal differentiation potential, being able to differentiate into chondrocytes, osteoblasts or adipocytes (Noël, Caton et al. 2008)

(Gimble, Katz et al. 2007; Meliga, Strem et al. 2007). Recent studies have also reported their immunoregulatory properties by cytokine secretion and through direct cell-cell contact by for example, the expression of co-stimulatory molecules at their surface (Puissant, Barreau et al. 2005; Yañez, Lamana et al. 2006; Technau, Froelich et al. 2011). Additionally, their secretion of angiogenic and antiapoptotic factors VEGF, HGF and TGFβ have been described in mice (Rehman J, Traktuev D. et al. 2004). Other cytokines such as granulocyte

and macrophage colony stimulating factors, interleukins (ILs) 6, 7, 8 and 11, tumor necrosis factor-alpha (TNF-alpha), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) have also been identified within the ASCs' secretome (Salgado AJ, Reis RL et al 2010).

To obtain functional cardiomyocytes, the scientific community tries to reproduce the cardiac marker pattern expression during cardiomyocytes development. Transcription factors such as the GATA binding protein 4 (GATA4), the NK2 homeobox 5 (NKX2.5) and the myocyte enhancing factor 2 (MEF2C), involved in the commitment into the cardiac lineage at the beginning of the differentiation, but also more mature proteins like the cardiac troponins I and T (cTnI and cTnT), the myosin like chains 2 (MLC-2v and MLC2a) and the calcium-transporting ATPase sarcoplasmic reticulum type (SERCA2a), implied in cytoskeleton organization and cardiac contraction are investigated. Finally gap junction proteins involved in the cell-cell contact and the electric signal conduction are also studied (Fig 2).



Representation of some factors involved in cardiomyogenesis

Figure 2: schematic representation of signaling events involving cardiomyocytes differentiation. The transcriptions factors GATA4 and Nkx2.5 are necessary for the commitment into the cardiac lineage. They are mutual cofactors that act on a third transcription factor MEF2C, which is involved in the expression of contractile proteins. Moreover, they are also involved in gap junction protein expression

ADSCs have been shown to exhibit also in vitro differentiation into the cardiomyocyte

lineage (Hoke NN, Salloum FN et al. 2009; Mazo M, Gavria JJ et al 2011; Song YH, Pinkernell K et al. 2011), particularly, adipose derived mice stromal cells, are also able to differentiate spontaneously into cardiomyocytes (Lee, Sepulveda et al. 2009; Leobon, Roncalli et al. 2009). Furthermore, under specific in vitro conditions human ADSCs could differentiate into cardiomyocytes-like cells. In summary the production of cells expressing contractile proteins such as cTnI was described using demethylating agent (Dijk, Niessen et al. 2008; Choi, Dusing et al. 2010), ECM molecules and GFs also. However, although the expression of late stage differentiation proteins like cTnI, MLC-2v and connexin 43 (CX43), are observed with ECM and GFs the differentiation remains incomplete (Dijk, Niessen et al. 2008). Interestingly, ADSC differentiation into beating cardiomyocytes using co-culture with cardiomyocytes has been reported (Gaustad, Boquest et al. 2004; Metzele, Alt et al. 2011), suggesting a role of a cell-cell contact and the importance of gap junctional coupling. However, this strategy appears insufficient to induce mature cardiomyocytes as recently described (Ramkisoensing, Pijnappels et al. 2012). In vivo transplantation of ADSCs has shown beneficial effects on cardiac function such as improvement of the left ventricular (LV) fraction, on wall thickness and on microvessel density (Leobon, Roncalli et al. 2009; van der Bogt, Schrepfer et al. 2009; Bai, Yan et al. 2010; Bayes-Genis, Soler-Botija et al. 2010; Mazo, Gavira et al. 2011; Bagno, Werneck-de-Castr et al. 2012; Mazo, Hernandez et al. 2012). These beneficial results can be partially attributed to the high secretion potential of many GFs and cytokines implicated in tissue repair (Cai, Johnstone et al. 2009; Li, Cheng et al. 2012) and their angiogenic potential (Cai, Johnstone et al. 2009; Mazo, Hernandez et al. 2012). Recently, an interesting study has reported that ADSCs growing as sphere clusters produced high levels of secreted angiogenic factors, essential to repair the damaged heart vasculature (Breant, De Francesco et al. 2009).

Another cell population cultured as floating spheres has been described, named cardiospheres-derived cells (CDCs). They constitute a population of cardiac progenitors isolated from adult heart by endomyocardial biopsy that form 3D-spherical cardiospheres in culture. CDCs represent a heterogeneous cell population with a core of c-kit-expressing proliferating cells surrounded by differentiated cardiomyocytes at the surface. These cells are capable of forming differentiated contractile cardiomyocytes in vitro (Smith, Barile et al. 2007; Davis, Kizana et al. 2010). Recently a phase 2 clinical trial (CADUCEUS) of CDCs in patients with heart failure after MI was started (Makkar, Smith et al. 2012). First results have shown reduction in scar mass, increase in regional contractility and regional systolic wall thickening.

However, changes in end-diastolic volume, end systolic volume and left ventricular ejection fraction did not differ between groups after 6 months (Makkar, Smith et al. 2012). Actually, the most adapted and specialized, but also the most seductive cells for myocardial regeneration are resident cardiac stem and progenitor cells (CPC). Indeed, many studies have identified the contribution of stem cells and progenitor cells that reside in the post-natal heart and that are capable of generating cardiomyocytes, but also smooth muscle and endothelial cells (Beltrami, Barlucchi et al. 2003; Messina, De Angelis et al. 2004; Wang, Hu et al. 2006; Barile, Messina et al. 2007). The adult heart contains a clonal c-kit positive cell population (c-kit⁺) that has been shown to regenerate all lineages of the heart, increase in number after myocardial injury, undergo self-renewal and generate cardiomyocytes in several animal models (Beltrami, Barlucchi et al. 2003; Oh 2003; Urbanek 2005; Wang, Hu et al. 2006; Bearzi, Rota et al. 2007). Recently, a phase 1 clinical trial of CPCs (SCIPIO) in patients with heart failure after MI was started (Bolli, Chugh et al. 2011). Preliminary results have suggested that intracoronary infusion of autologous CPCs is effective in improving left ventricular (LV) systolic function and reducing infarct size in patients after 12 months. However, due to the difficulty to obtain a biopsy, this strategy is principally reserved to heavy patients having undergone a coronary bypass. Moreover, several weeks are necessary to obtain a sufficient quantity of cells for transplantation due to the small biopsy size (1mm³).

We frequently speak about myocardial regeneration and often associate it to cardiomyocyte replacement. However, other cellular populations are touched as well and cells taking part in heart revascularization must be also used for a functional repair. MSCs and ADSCs are potential cell candidates, but the most interesting are undoubtedly endothelial progenitor cells (EPCs). They have been defined by their cell surface expression of the hematopoietic marker proteins CD133 and CD34 and the endothelial marker, VEGFR-2, as well as by their capacity to incorporate into sites of neovascularization and to differentiate into endothelial cells in situ (Jujo, Ii et al. 2008). In animal models of myocardial infarction, the injection of ex vivo expanded EPCs significantly improved blood flow and cardiac function and reduced LV scarring (Urbich 2004; Jujo, Ii et al. 2008; Krenning, van Luyn et al. 2009; Sekiguchi, Ii et al. 2009). Moreover, transplantation of EPCs significantly improved coronary flow reserve and LV function in patients with acute myocardial infarction. In addition to directly contributing to the vasculature required to deliver nutrients to new cardiomyocytes, endothelial cells can also provide paracrine survival signals to cardiomyocytes (Narmoneva 2004; Hsieh, Davis et al. 2006).

Finally, we can cite skeletal myoblast (SM) as another adult cells used for cardiomyocyte replacement. one of the first cells used for cardiac regeneration in the ischemic heart due to their myogenic lineage, reducing the risk of tumor formation, and particularly due to their high resistance to ischemia (Menasche, Hagege et al. 2001; Menasche 2007). Human trials of myoblasts in heart failure are ongoing; but, some have confirmed the arrhythmic risk (Siminiak, Kalawski et al. 2004; Laflamme and Murry 2005), and some have been terminated because of lack of efficacy (Hagege, Marolleau et al. 2006).

2.2. Limits of cardiac cell therapy

In spite of the benefits observed in cell therapy after MI, the cell retention and the short but also long-term survival and functional state of the cells after transplantation, still need to be ameliorated. Indeed, regardless of the type of cell used, the majority of the cells die within the first days following injection (Jonathan R. Beauchamp 1999). This death is a multifactorial phenomenon, first involving mechanical constraints and stress exerted on the cells during their preparation (conditioning in suspension without serum) or injection (pressure of injection), In addition, the ischemic conditions due to the injection process and the consecutive poor vascularization of the damaged heart, the loss of contact between the cells and the ECM molecules and the inflammatory reaction in response to the implantation, further contribute to cell death by apoptosis and necrosis. Increasing the number of grafted cells is not the appropriate solution, because besides the multiplication of injection sites due to an increase of the injected volume it amplifies the inflammatory reaction.

Cell differentiation is also to be taken into consideration when cells used for myocardial repair come from another tissue. The use of cells such as MSCs and ADSCs must be controlled in order to avoid their differentiation into cells from another completely different lineage that could involve serious complications. Although such complications are poorly documented, the use of committed/pre-differentiated cells is an appropriate and interesting strategy as their ability to differentiate into cardiomyocytes has already been evaluated. The efficiency of stem cell transplantation is also limited by low cell retention as cells are lost in the blood-flow following injection. Intracoronary cell delivery is convenient and widely used but exhibits particularly low cell retention rates. Indeed, a recent study reports a 5% retention rate of CPCs when injected in the infarcted myocardium.

The number of surviving functional cells following injection is proportional to the functional improvement observed and optimization of cellular survival, retention and differentiation appears crucial. The scientific community is thus attempting new strategies to improve cell engraftment in order to overcome the very hostile ischemic environment and the lacking signals, leading to these problems.

3. Tissue-engineering for cardiac repair:

3.1. Scaffold characteristics:

Tissue engineering strategies, combining cells with biomaterials often arranged as a scaffold, show encouraging results letting us foresee interesting perspectives for cardiac repair. Scaffolds were developed to provide to the cell the three-dimensional (3D) environment as well as the mechanical and signaling cues in order to improve their survival and function. It has indeed been reported that 3D support promotes cell survival, proliferation but also differentiation (Ingber 2002; Stupack 2002; Stupack and Cheresch 2003; Ingber 2006; Alavi and Stupack 2007; Ghosh and Ingber 2007; Parker and Ingber 2007). The ideal scaffold used for cell transplantation into the heart, should be biodegradable and biocompatible in order to minimize inflammatory and immunological responses. It should have the adequate size to allow the ongoing cell integration within the cardiac parenchyma without having to remove it by open-chest surgery. Its surface characteristics (topography, charge, roughness) are also to be tailored carefully as they regulate cell behavior. Furthermore, its elasticity, which also affects cell behavior, needs to be taken into account. It should also have enough flexibility to respond synchronically with the myocardium contraction and effectively transfer mechanical stimuli from the myocardium environment to the transported cells, providing cues to develop a contractile phenotype (Fig 3).

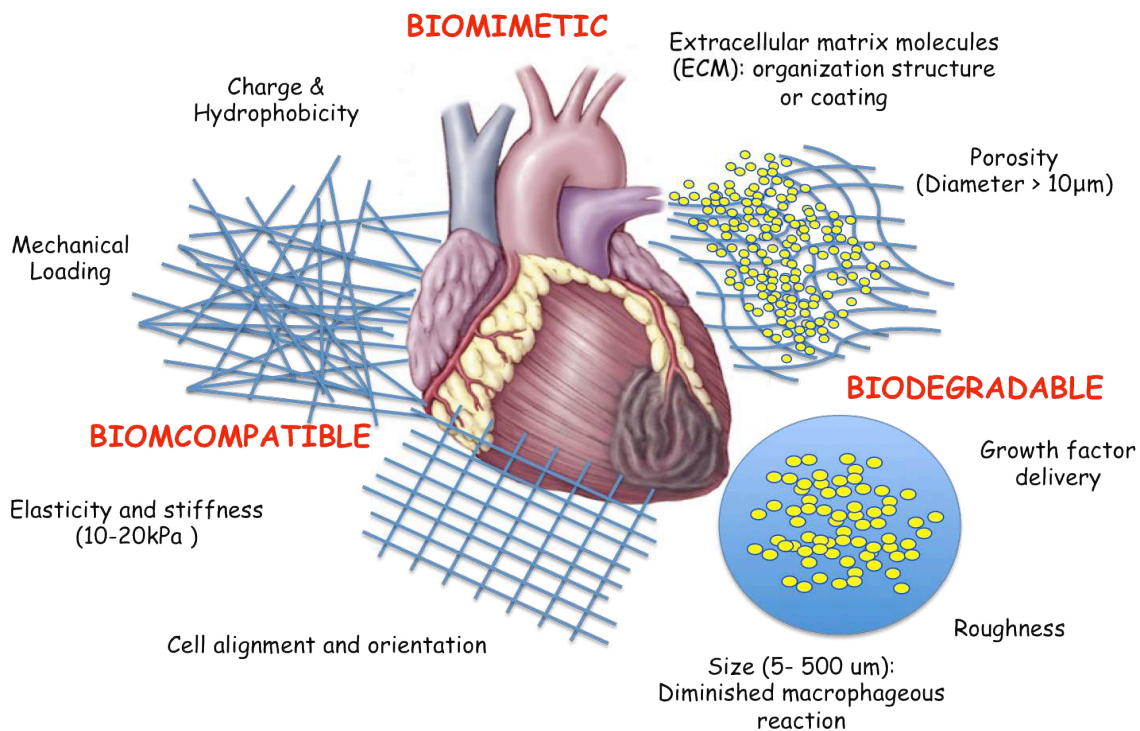


Figure 3: Scaffolds characteristics and properties for cardiac tissue repair. The first requirements for a 3-D scaffold for the heart are biocompatibility, biodegradability. Additionally, it should be biomimetic and have the adequate size to allow the ongoing cell integration within the cardiac parenchyma without creating inflammatory response. Its surface characteristics (topography, charge, roughness) are also to be tailored carefully as they regulate cell behavior. Furthermore, its elasticity, which also affects cell behavior, needs to be taken into account. It should also have enough flexibility to respond synchronically with the myocardium contraction and effectively transfer mechanical stimuli from the myocardium environment to the transported cells, providing cues to develop a contractile phenotype

3.2. Scaffold properties

Synthetic and natural materials could be used as 3D scaffolds for cardiac cell graft and in both cases it is very important to note that the scaffold must be adapted to the type of transported cells and their differentiation state. Commonly used injectable scaffolds may be produced from natural materials such as collagen, chitosan, gelatin, fibrin and alginate, but also from synthetic and biodegradable polymers including poly(lactic-co-glycolic) (PLGA), polycaprolactone (PCL), poly(glycerol–sebacate) (PGS), poly(ethylene glycol) (PEG) and self-assembling peptides (Wang and Guan 2010; Segers and Lee 2011). Materials from natural sources have the advantage of being produced without organic solvent or elevated temperatures. Moreover they possess inherent properties of biological recognition and are

composed of extracellular matrix components (collagen, fibrin) or of native tissue (decellularized matrix). However, they must be purified before use and it is difficult to control their homogeneity between batches. On the contrary, synthetic materials have a known composition and their structural and mechanical properties can be modulated (size, form, porosity, alignment, viscoelasticity) playing on their physico-chemical properties. In addition, biomaterial-based scaffolds have also been explored for use as cellular and acellular cardiac patches providing a structural support for the damaged ventricle and allowing cellular recruitment into the material (Segers and Lee 2008; Schussler, Chachques et al. 2010; Segers and Lee 2011). However, their implantation involves an invasive and massive open-chest procedure that limits a wide use in the clinical setting.

3.3 Bio-inspired scaffold

Many studies have developed very interesting scaffolds that promote survival of the cells or guide them into the cardiac lineage. It is evident that the first differentiating signal is the surrounding ECM that the cells sense directly. The physical but also the mechanical stimuli sensed by the cells induce their differentiation into the desired pathway (Ingber 2002; Engler, Sen et al. 2006; Ingber 2006; Stamenović, Rosenblatt et al. 2007; David Merryman and Engler 2010; Ingber 2010; Reilly and Engler 2010; Young and Engler 2011). But I think that these signals are just sufficient to induce the commitment but not to maintain or improve this differentiation into mature cells. Moreover, according to the type of cell and its differentiation state the response would be completely different. I often tend to compare tissue engineering to a boat trip. The cells, the very capricious passengers always need to be at ease and the first thing that will put them in this condition is the way in which they feel once on the boat, they must “feel at home.” Then, we will have to bring them to the desired destination and for this reason it will be necessary to exploit their environment, their comfort (using ECM coating, RGD peptides, GF) so that the trip really occurs as required. We thus propose the combination of GFs to the scaffold to potentiate or maintain/stabilize the cell differentiation and promote its survival.

The scaffolds can be thus associated with ECM molecules or functionalized with simple peptides (adhesion motif or RGD peptide) to advantageously guide survival, proliferation but also differentiation of the cells toward the desired phenotype, replacing and mimicking the

regulatory characteristics of the lacking natural ECM. This approach offers a biomimetic support for the cells.

In a first approach the scaffold allows endogenous cell colonization within the infarcted area. This kind of scaffold should possess the appropriate cues for endogenous cell adhesion (charge, adhesion molecules, porosity) as well as the signals to induce their survival and proliferation in situ (charge, alignment, ECM molecules). This scaffold is used to attract circulating endothelial progenitors or endogenous stem cell colonization to improve vascularization or colonizing cell secretion into the infarcted area.

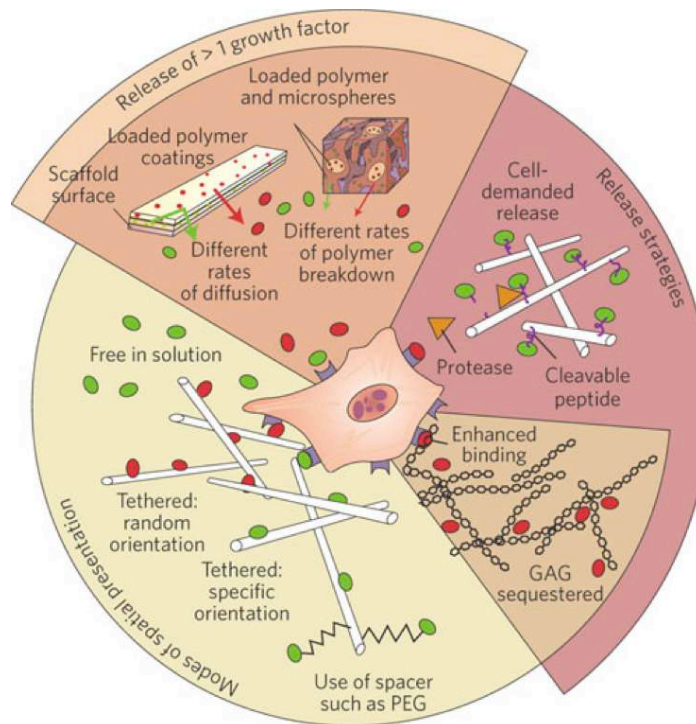
In a second strategy, the scaffolds are designed as a structural support for transported cells. This support fulfills the lacking properties of the surrounding ischemic tissue and partially mimics the structural architecture or organization of the myocardium. In this way, it provides to the graft a new 3D, communicative, mechanical and nutritional status, guiding the cell into the desired phenotype and acting as an instructive whole system. This system is mainly used as support for only one cell population. Transported cells may either serve for cardiomyocyte replacement or for vascularization of the ischemic area, using cells that may differentiate into one or the other phenotype (Davis 2005; Davis 2006; Frederick, Fitzpatrick et al. 2010; Tokunaga, Liu et al. 2010; Yu, Du et al. 2010; Ye and Black 2011; Hibino, Duncan et al. 2012; Masumoto, Matsuo et al. 2012). But it can also be used as support for several cell types that generally mobilize paracrine mechanisms of one population of grafted cells that acts on the other population. In such strategy, one of the cells is already committed/differentiated into cardiomyocytes or endothelial cell while the other stimulate its survival/proliferation through paracrine mechanism or angiogenesis.

3.4 Engineering bioactive scaffolds: becoming biomimetic combining GFs with scaffold

3D scaffolds with or without cells have also been designed combined to GF (Madonna R. and De Caterina R, 2011). An interesting study has demonstrated the necessity of 3-dimensionality and GFs to allow differentiation of CPCs into cardiomyocytes. Using 3D PLGA and PLLA scaffold with defined geometry, researchers observed the cells colonize only the PLLA scaffold with square pores and stiffness inferior to 100 kPa. Moreover alignment of these cell expressing cardiac markers sarcomeric myosin, α -sarc act and actinin was described on the scaffold in combination with a culture medium containing GFs (Forte,

Carotenuto et al. 2008), suggesting also a synergistic role of GFs in differentiation. GFs can be used very differently depending on the desired effect. On one hand, they can improve grafted cell survival or differentiation; in this way the GF acts directly on the grafted cells (ref). On the other hand, they can stimulate angiogenesis inside the scar tissue and indirectly favor engraftment of the cells ((Kawamoto, Murayama et al. 2004; Anitua, Sánchez et al. 2008; Lee and Lee 2009). Indeed, delivery of insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) induced reduction of the fibrotic area and apoptosis and increase cardiomyocyte proliferation compared to controls, when injected in the infarcted rat heart (Urbanek 2005). Another study using vascular endothelial growth factor (VEGF) and (IGF-1) has also shown reduction of cell death and neoangiogenesis in the rat infarcted heart (Cittadini, Monti et al. 2011). These sophisticated scaffolds can either present a GF to the cells to increase their engraftment or can deliver it in a prolonged and continuous manner that can improve grafted cell behavior and also modulate the surrounding environment. In fact, controlled-release strategies are frequently adopted to overcome the short half-life and residence of these GFs. For the ideal scaffold, different factors become active at different times and growth factor release that recapitulate these dynamics are likely to provide more effective cues for cell behavior.

In particulate or hydrogel (HG) scaffold, the GFs can be at the surface of the scaffold or within the polymeric matrix. Its release will depend on biomaterial composition and degradation but also on its interaction with the scaffold. HGs can be thermosensitive and offer the advantage to be used as in situ gelling process, while in particulate scaffolds cell–material interactions may be advantageously increased due to a large specific surface. Moreover, most of the time open-surgery is not required. Different release methods have been developed in order to have a rapid or prolonged released of the protein. The GF can be rapidly released from the scaffold, generally with HG scaffolds where a burst is observed followed by a short-time release (some days), or when the GF is immobilized onto the surface of the scaffold. Although these strategies have shown interesting effects, the protein release is not adequately controlled and the quantity of GF that can be fixed at the surface of such scaffolds is very low. Another strategy uses GFs embedded within the polymer and more or less homogeneously distributed. These scaffolds are generally particulate systems that can release a defined and controlled amount of protein continuously for several weeks, depending on the degradation of the scaffold (Fig 4).



Place ES, Evans ND, Stevens MM. *Complexity in biomaterials for tissue engineering*. Nat Mater. 2009;8(6):457-70.

Figure 4: Presentation and release of growth factors from tissue-engineering scaffolds. Anticlockwise, from top: growth factors within TE scaffolds may be loaded into polymers whose rate of degradation or diffusive properties can be modulated to tailor release rate, and which may be combined into systems releasing multiple factors with distinct kinetics. The exposure of cells to different growth factors with time may therefore imitate developmental pathways and healing responses. An alternative to presenting growth factors in soluble form is to bind them to a surface in either random or specific orientations, with the possible use of a spacer molecule. Non-covalent associations with matrix components, particularly glycosaminoglycans (GAGs), can affect slow release and in some cases may potentiate binding to membrane receptors. Cell-demanded release is based on the presence of protease-sensitive peptide sequences within the growth factor protein (Place, Evans et al. 2009).

3.5 Combining 3D, ECM and GFs: the future of smart scaffolds

Finally, new 3D scaffolds tend to combine GF and ECM to be more effective and better control grafted cells behavior. Recently, structural and electrical integration into host myocardium was demonstrated combining cardiomyocytes to an alginate scaffold containing angiogenic factors IGF-1, VEGF and stromal derived factor 1 in a biomimetic gel, Matrigel® (Dvir T, Kedem A et al. 2009).

An innovative vector for tissue engineering combining the biomimetic 3D approach with the GF release strategy and named Pharmacologically active microcarriers (PAMs), have been developed by our laboratory. These vectors are constituted of PLGA a “Food and Drug Administration” approved polymer (Tatard, Venier-Julienne et al. 2005). PAMs are biodegradable and biocompatible PLGA microspheres covered with ECM molecules, thus conveying cells on their biomimetic surface providing an adequate 3D microenvironment for the transplanted cells in vitro and in vivo. They have a mean size of 60 μm and can be easily implanted through a needle or catheter. They contain a GF encapsulated under a nanosolid state to preserve its structure and integrity within the polymer and can be delivered in a sustained and controlled manner for a prolonged period (Giteau, Venierjulienne et al. 2008). The delivered factor in combination with the 3D biomimetic surface of the PAMs act synergistically to stimulate the survival and/or differentiation of the grafted cells toward a specific phenotype, therefore enhancing their engraftment but also affecting the host microenvironment allowing better integration of the grafted cells. The total degradation of PAMs occurs approximately 2 month after transplantation, and allows thus optimal cell integration in the tissue without inducing notorious inflammatory reaction. The use of these PAMs for neurodegenerative disorders has been successfully validated in animal models of Parkinson’s disease with neuroprogenitor cells and MSCs (Tatard, Venier-Julienne et al. 2005; Tatard, Sindji et al. 2007; Bouffi, Thomas et al. 2010; Delcroix, Garbayo et al. 2011). They further potentiate the chondrogenic differentiation potential of MSCs (Bouffi, Thomas et al. 2010). Depending on the choice of the molecule used for the biomimetic coating and the encapsulated protein, PAMs constitute a multifunction tool usable in many contexts and with a whole variety of cells (Tatard, Venier-Julienne et al. 2005) (Fig 5).

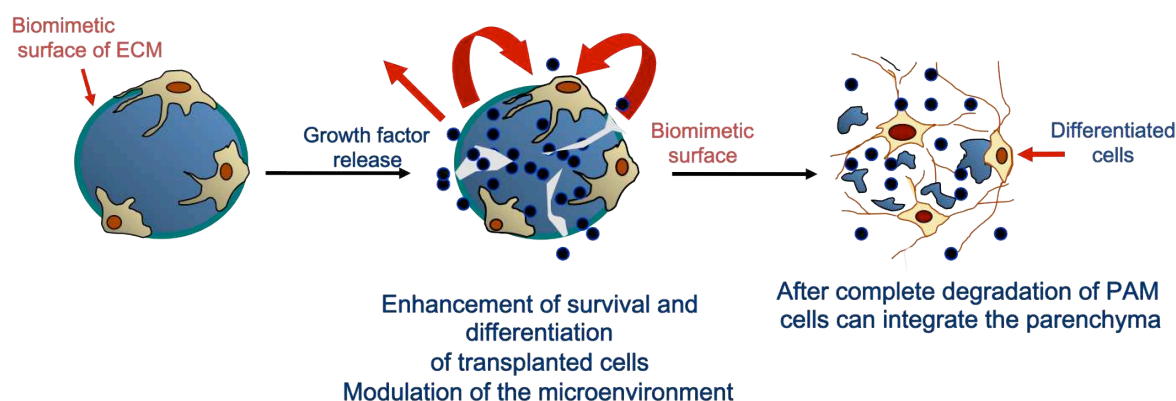


Figure 5: Concept of pharmacologically active microcarriers. PAMs are biocompatible and biodegradable microparticles, they have a biomimetic coating of extracellular matrix molecules and can favour cell adhesion

while supplying a three-dimensional structure for the transported cells; they can continuously release a protein that acts on the transported cells and on the surrounding tissue. Owing to the combination of these parameters, three-dimensional structure, biomimetic coating and controlled release of a growth factor, the PAMs can constitute a support to improve survival, differentiation, and integration of grafted cells to repair lesioned tissues.

Objectives of the Thesis

The implementation of a tissue engineering strategy for the repair of the ischemic heart raises many questions, for example, the choice of the cells that should provide the most important benefits with the less practical but also ethical constraints. ADSCs seem to be an attractive approach because, besides their large differentiating potential, they can be used as autologous cells for transplantation and overcome the immunological, ethical as well as availability concerns encountered with embryonic or foetal cells. The transplanted cells may induce tissue repair by cell replacement or by an indirect paracrine manner. In addition, the choice of the scaffold to be combined with the cells is also crucial to implement a tissue engineering strategy. Different scaffolds have been developed that can provide a 3D support to the transported cell, support that can further be coated or is constituted by ECM or ECM-like molecules or that can deliver a GF to the cells. A bibliographical review having for objective to pose the bases of the possible contribution of tissue engineering for cell therapy of the heart, is the object of the first chapter of this thesis.

The field of tissue engineering still poorly explores the combination of adult stem cells, biomimetic scaffolds and GF delivery, although this kind of strategy should be able to improve cell behavior in vivo. The work developed in this thesis aims at implementing the PAMs for tissue engineering of the infarcted myocardium. In the second chapter, is presented the first part of the study, using a set of recombinant factors to induce the commitment of ADSCs towards the cardiac lineage. What is particularly studied is the combination of a GF cocktail with two ECM molecules, fibronectin or laminin, to define the more appropriate surrounding coating for the 3D microcarriers to induce ADSCs differentiation into immature cardiomyocytes.

The third chapter concerns the production of PAMs, which may also continuously release a bioactive GF for several weeks, and their effects on ADSC cardiac differentiation. Three GF useful for cardiac regeneration, IGF-1, VEGF and HGF are encapsulated. The effect of PAMs with a biomimetic surface releasing IGF-1 and HGF on ADSCs behavior in vitro is then investigated. As hydrogel scaffolds may mechanically support the beating myocardium due to their elastic properties, the effect of these PAMs integrated within an injectable thermosensitive hydrogel on the adhered ADSCs behavior is also investigated.

The main technological challenge of this strategy consists in obtaining a prolonged release of the protein under its bioactive form (Giteau, Venier-Julienne et al. 2008). The use of PLGA is limited due to low and incomplete protein release from microspheres that are related to protein instability during the release period. The fourth chapter of the thesis relates a new approach, performed in collaboration with MC. Venier-Julienne, to enhance protein delivery by introducing hydrophilic segments of poly (ethylene glycol) (PEG) into the hydrophobic polyesters, PLGA, to obtain a more hydrophilic polymer, PLGA-PEG-PLGA (A-B-A). In this study, the impact of different combinations of (B) and (A) segments on the protein-release profile of the model protein, lysozyme, as well as the ECM coating and MSCs behavior has been investigated.

A general discussion comparing the existing strategies will come to close this work and to open new prospects. Moreover, the effect of PAMs releasing VEGF combined with MSCs in an *in vitro* model of ischemia/reperfusion, as well as their effects on eEPCs adhesion, proliferation and survival *in vitro* have been investigated by our Italian collaborators. The regenerative potential of ADSCs adhered onto PAMs releasing HGF and IGF-1 has also been investigated in a rat model of ligation of the coronary artery. The results of these studies are commented in the discussion and the articles are included as annexes at the end of the thesis.

Chapter 1

Combining adult stem cells and polymeric
devices for tissue-engineering in infarcted
myocardium

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Review

Combining adult stem cells and polymeric devices for tissue engineering in infarcted myocardium

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ABSTRACT

An increasing number of studies in cardiac cell therapy have provided encouraging results for cardiac repair. Adult stem cells may overcome ethical and availability concerns, with the additional advantages, in some cases, to allow autologous grafts to be performed. However, the major problems of cell survival, cell fate determination and engraftment after transplantation, still remain. Tissue-engineering strategies combining scaffolds and cells have been developed and have to be adapted for each type of application to enhance stem cell function. Scaffold properties required for cardiac cell therapy are here discussed. New tissue engineering advances that may be implemented in combination with adult stem cells for myocardial infarction therapy are also presented. Biomaterials not only provide a 3D support for the cells but may also mimic the structural architecture of the heart. Using hydrogels or particulate systems, the biophysical and biochemical microenvironments of transplanted cells can also be controlled. Advances in biomaterial engineering have permitted the development of sophisticated drug-releasing materials with a biomimetic 3D support that allow a better control of the microenvironment of transplanted cells.

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1. Introduction

Myocardial infarction (MI) constitutes the first cause of morbidity and mortality in developed countries with an annual incidence rate of approximately 600 cases per 100,000 individuals in USA, where approximately 500,000–700,000 deaths are caused by ischemic heart disease. MI continues to represent a significant problem for health and economy in industrialized countries and is now becoming a serious concern even in developing countries. Concerning the pathological process, it usually results from coronary artery occlusion owing to acute atherosclerotic plaque rupture and platelet aggregation, which leads to thrombosis within the vessel [1]. Severe ischemia downstream from occluded arteries causes cardiomyocytic necrosis and apoptosis within few hours. There is growing evidence that heart muscle has the ability to regenerate through the activation of resident cardiac stem cells (CPCs) or through recruitment of a stem cell population from other tissues [2]. However, this regenerative capacity of the heart cannot compensate for the large-scale tissue loss after MI [3]. Following

the ischemic insult, an immediate and massive infiltration of circulating leucocytes into the ischemic core occurs, due to secretion of cytokines and chemokines such as tumor necrosis factor, monocyte chemoattractant protein 1, interleukin 1 (IL-1), IL-6 and IL-8 by the endogenous surrounding cells, and cell adhesion molecule (E-selectin, intercellular adhesion molecules and vascular adhesion molecules) up-regulation by endothelial cells. Myofibroblast infiltration also occurs, depositing collagen and other extracellular matrix proteins leading to scar formation, mechanical dysfunction, electrical uncoupling and loss of structural integrity. This irreversible process reduces cardiac performance compromising the pumping capacity of the heart, leading to ventricular remodeling and cardiac failure.

Various drugs and surgical interventions for patients with heart failure have been developed. However, current drug therapies can increase their life expectancy by only a few years [4]. Other conventional treatments such as medical management or mechanical circulatory assistance devices can reduce post-myocardial infarction mortality, but they are unable to restore cardiac function [5]. Several alternative strategies are being investigated to complement the current pharmacological therapies for myocardial diseases, including reactivation of cardiomyocyte cell cycle activity and reduction of myocardial cell death [6–10]. Whole-organ transplantation is limited by the inadequate supply of

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donor hearts and the need for subsequent immunosuppression, hence, in recent years cell-based therapies have attracted considerable interest as an alternative way of achieving cardiac repair. Recent work has focused largely on committed myogenic cells including skeletal myoblasts [11], embryonic stem cells (ESC) [for review 12,13], bone marrow-derived hematopoietic cells [14–17], endothelial progenitor cells (EPCs) [18], multipotent mesenchymal stromal cells also called mesenchymal stem cells (MSCs) [19–21] and resident cardiac progenitor cells (CPCs) [2,22].

Stem cell-mediated cardiac repair follows two main strategies to improve regeneration of the damaged heart area. The first, aims at recruiting or promoting the homing of endogenous or circulating stem cells at the periphery or inside the damaged zone with locally injected factors such as growth factors (GF), cytokines and extracellular matrix molecules [for review 23]. The second is based on the local transplantation of stem cells to replace the dead cells, therefore building a new tissue with a new population of functional and beating cardiomyocytes, with cell–cell communications, secreted factors and induction of neoangiogenesis. New human myocardium has been formed recently in infarcted rodent hearts after injection of human embryonic stem cell (hESC)-derived cardiomyocytes, but small graft size due to cell death currently limits the benefits of this therapy [24,25]. Adult stem cells are interesting candidates that have been tested in preclinical models of cardiac injury, and most have been reported to mediate at least some functional benefits when delivered either at the time of, or within the first week following experimentally induced MI [for review 26–28]. However, stem cell transplantation still faces many problems related to cell survival and control of cell fate, for e.g. maintenance of differentiated state and proper cell engraftment after transplantation. Indeed, it has been reported [29–31] that cell survival is one of the most challenging technical issues as only a small percentage of implanted cells survive within the following weeks after transplantation. These results indicate that the infarcted area is a very hostile microenvironment for good cell integration after transplantation, due its poor vascularization, fibrotic and acidic characteristics. Unfortunately, for these reasons remarkably few studies have shown convincing evidence for electrical or mechanical activation of grafted cells within the infarct.

An alternative strategy is to associate cells and biomaterials providing an adequate 3D support for transplanted cells, thereby increasing cell survival and even guiding cell differentiation and fate *in vivo*. In recent years it has become evident that going biomimetic by combining integrins and extracellular matrix molecules with 3-dimensionnal (3D) structures will be the way to deliver cells in tissue-like structures that preserve cellular integrity (attachment, stress,...). This approach may increase cell delivery efficiency and reduce cell death, optimising engraftment efficacy and transplanted-cell function. On the other hand, cells can also be associated with *in situ* controlled drug delivery systems that will mimic a suitable cell microenvironment favoring their homing inside the infarcted area. Growth and differentiating released factors may improve survival and differentiation of the cells, and also affect the immediate environment, thus allowing better graft integration. The focus of this review is to provide an overview of what solutions tissue engineering may provide for adult cell therapy of the infarcted myocardium.

2. Adult stem cells for cardiac repair

Adult stem cells seem to be an attractive approach because, besides their large differentiating potential, they can be used as autologous cells for transplantation and overcome the immunological, ethical as well as availability concerns encountered with embryonic or fetal cells. The transplanted cells may induce tissue

repair by cell replacement or by an indirect paracrine manner. They can either directly differentiate into endothelial cells to rebuild and vascularise the ischemic area [32] or into cardiomyocytes to replace the fibrotic scar by a new functional tissue [28; for review 33]. They can secrete several GFs and cytokines involved in neoangiogenic processes, cell survival, recruitment or differentiation. However, the cell retention and the short but also long-term survival and functional state of the cells after transplantation, still need to be ameliorated [29,33].

2.1. Skeletal myoblasts

They were one of the first cells used for cardiac regeneration in the ischemic heart [34] due to their myogenic lineage, reducing the risk of tumor formation, and particularly their high resistance to ischemia [for review 34]. Differentiation of skeletal myoblast into myotubes but not into cardiomyocytes was found *in vivo* [11,35]. Myotubes formed by differentiated myoblast do not integrate electrically with surviving cardiomyocytes [35] and thus do not beat in synchrony with the surrounding myocardium. Human trials of myoblasts in heart failure are ongoing; but, some have confirmed the arrhythmic risk [36,37], and some have been terminated because of lack of efficacy [38]. However, two experimental studies have reported that mouse skeletal muscle harbors a population of cells that have the capacity to acquire some key phenotypic features of cardiac cells [39,40]. In animal models of MI, these cardiac-committed cells yielded greater engraftment rates and better functional outcomes than non-purified skeletal myoblasts. Nevertheless, no equivalent population of cardiac-committed cells in human skeletal muscle has been described.

2.2. Resident cardiac stem and progenitor cells (CPC)

Many studies have identified the contribution of stem cells and progenitor cells that reside in the post-natal heart and that are capable of generating cardiomyocytes, but also smooth muscle and endothelial cells [2,41–43]. A clonal c-kit positive cell population (c-kit⁺) has been shown to regenerate all lineages of the heart, increase in number after myocardial injury, undergo self-renewal and generate cardiomyocytes in rat [2] and in human [44]. Other studies have shown that Sca-positive (Sca⁺) cells can differentiate into cardiomyocytes [45]. Resident Sca⁺/CD31[−] murine cardiac progenitors have been reported to increase in number after acute MI. These Sca⁺/CD31[−] cells are capable of differentiating into endothelial cells and cardiomyocytes *in vitro* and *in vivo* in mouse heart [43,46].

2.3. Cardiospheres

A population of cardiac progenitors isolated from adult heart by endomyocardial biopsy forms 3D-spherical cardiospheres in culture. They represent a heterogeneous cell population with a core of c-kit-expressing proliferating cells surrounded by differentiated cardiomyocytes at the surface, and are capable of forming differentiated contractile cardiomyocytes *in vitro* [47]. Injection of cardiospheres after MI resulted in cardiac function improvement in several rodent models but also in swine [47–52]. Moreover, autologous transplantation of cardiospheres into the post-injured pig heart induced repair and regeneration [52].

2.4. Endothelial progenitor cells (EPCs)

EPCs are a subset of hematopoietic cells found in the bone marrow that have the potential to differentiate into endothelial cells. They have been defined by their cell surface expression of the

hematopoietic marker proteins CD133 and CD34 and the endothelial marker, vascular endothelial growth factor receptor-2, as well as by their capacity to incorporate into sites of neo-vascularization and to differentiate into endothelial cells *in situ*. The cell surface antigen CD133 is expressed by early hematopoietic stem cells and EPCs, both of them collaborate to promote vascularization of ischemic tissues. EPCs have not been shown to differentiate into cardiomyocytes *in vivo*, but they have a role in promoting angiogenesis [53,54]. In addition to directly contributing to the vasculature required to deliver nutrients to new cardiomyocytes, endothelial cells can also provide paracrine survival signals to cardiomyocytes [55]. Their regeneration potential has been described in acute MI [56–59].

2.5. Mesenchymal stem cells/marrow stromal cells (MSCs)

MSCs are a bone marrow stromal population of CD45[−]/CD34[−]/CD133[−] cells. They can be easily isolated and expanded in culture to obtain large numbers that are adequate for cell therapy. MSCs are multipotent and give rise to adipocytes, chondrocytes, osteoblasts, and in some conditions to endothelial cells [60,61]. MSCs have become really interesting candidates for cardiac therapy for their ability to differentiate into cardiomyocyte-like cells under appropriate conditions *in vitro* and *in vivo* but only with low efficiency [19,62,63]. In addition, MSCs have local immunosuppressive properties, which promote their use in allogeneic recipients [64–66]. Studies have shown that injection of MSCs into infarcted myocardium improved cardiac function, resulting from MSC transdifferentiation into cardiomyocytes and endothelial cells. However, despite enhanced cardiac function, the differentiation of MSCs to cardiomyocytes is limited [66–69]. It has then been suggested that these effects may be related to paracrine effects [67,70], that influence neovascularization of the ischemic tissue and the protection of resident cells [71,72].

2.6. Adipose-derived stem cells (ADSCs)

ADSCs can be isolated from the stromal vascular fraction of adipose tissue. They are CD34⁺, CD105⁺, CD45[−], CD31[−] cells that also express mesenchymal markers such as CD73 and CD90. They display a mesenchymal differentiation potential and are able to differentiate into chondrocytes, osteoblasts or adipocytes [73–75]. ADSCs have angiogenic potential [76] and are also able to differentiate into cardiomyocytes [77,78]. Indeed, their *in vitro* differentiation into cardiomyocytes has been reported using demethylating agent [78–80] or co-culture with cardiomyocytes [81] or their protein extracts [82]. The differentiation of ADSCs into cardiomyocytes seems to be dependent on the autocrine/paracrine action of vascular endothelial growth factor (VEGF) [83]. They also have been shown to differentiate into endothelial cells *in vitro* [84,85]. *In vivo* transplantation of ADSCs has shown benefits on cardiac function [86–88]. But the low percentage of cells that remain in the tissue [77,86] and the limited rate of differentiation [88] are problems to take in consideration and to overcome.

2.7. Induced pluripotent stem cells (iPS) cells

iPS cells are derived from somatic cells (generally skin fibroblast) that have been transformed into pluripotent cells by the forced expression of stem cell-associated genes. The cells express embryonic markers, such as Oct3/4, Sox2 and Nanog, and have the differentiation potential of embryonic stem cells, which allow them to be considered as interesting candidates for regenerative medicine applications [for review 89]. As a matter of fact, iPS cells have been differentiated into beating cardiomyocytes [90]. Recently,

prior induction of pluripotency, (iPS cells), has been indicated as unnecessary to generate beating cardiomyocytes from fibroblasts as deciphering the pathways or factors that will directly convert the cells to cardiomyocytes may be more simple. In this regard, forcing the expression of three developmental transcription factors (Tbx5, Gata4 and MEF2C) reprogrammed murine cardiac fibroblasts into cardiomyocyte-like cells [91–93].

3. Adult stem cell therapy for cardiac ischemia: improvements with tissue engineering

The majority of transplanted cells die within the first days following injection. This death is a multifactorial phenomenon involving an inflammatory reaction in response to injection, apoptosis, ischemic conditions (consecutive to its poor vascularisation) and the loss of contact between the cells and the extracellular matrix (ECM) molecules. To overcome this problem, the scientific community is attempting to supply the lacking signals with new strategies to improve grafted cell retention and survival. Tissue-engineering strategies, combining cells with biomaterials often arranged as a scaffold, show encouraging results letting us foresee interesting perspectives for cardiac repair. Scaffolds were developed to provide to the cell the 3D environment as well as the mechanical and signaling cues in order to improve their behavior and stimulate the cells to express, for example, cardiac molecules in the context of cardiac ischemia (Fig. 1).

3.1. Scaffold properties for cardiac repair

The design of an appropriate scaffold for cardiac repair must be adapted to the organ requirements. Indeed, size, physical properties and topography of the desired scaffold must comply with the architecture of the heart, its mode of action and also provide the most pertinent signals, thus guiding the cell throughout the regeneration process. The myocardium has the ability to contract naturally and in a rhythmic way. The cardiomyocytes are spontaneously excitable and are organized out of short, ramified and strongly striated fibers. These cells are connected between them by the collagen network contained in conjunctive tissue. Moreover they are united end-to-end by specialized junctions called intercalated discs, which confer a mechanical bond between the cells. At this level, specific molecular connections are localised such as the fascia adherens, desmosomes and communicating junctions (gap junctions). Therefore, to ensure that the new generated cardiomyocytes can function properly, provision of the appropriate regenerative environment is critical. We will next describe general parameters that must be taken into consideration in scaffold tailoring for tissue engineering of the ischemic heart.

3.1.1. Non-cytotoxicity/biodegradability

The basic requirements for scaffolds are non-cytotoxicity and biodegradability. Indeed, scaffold non-cytotoxicity after transplantation is crucial to minimize the inflammatory and immunological response and avoid further tissue damage. Biodegradability is also important as it permits the ongoing integration of the transplanted cells into the cardiac parenchyma without removing the scaffold by surgery operations. These two requirements go hand in hand with the choice of the material used for the fabrication of the scaffold. Materials from natural sources are advantageous because of their inherent properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remodeling. However, they must be purified before use and it is difficult to control the homogeneity of product between batches. Synthetic materials in contrast, have a known composition and can be

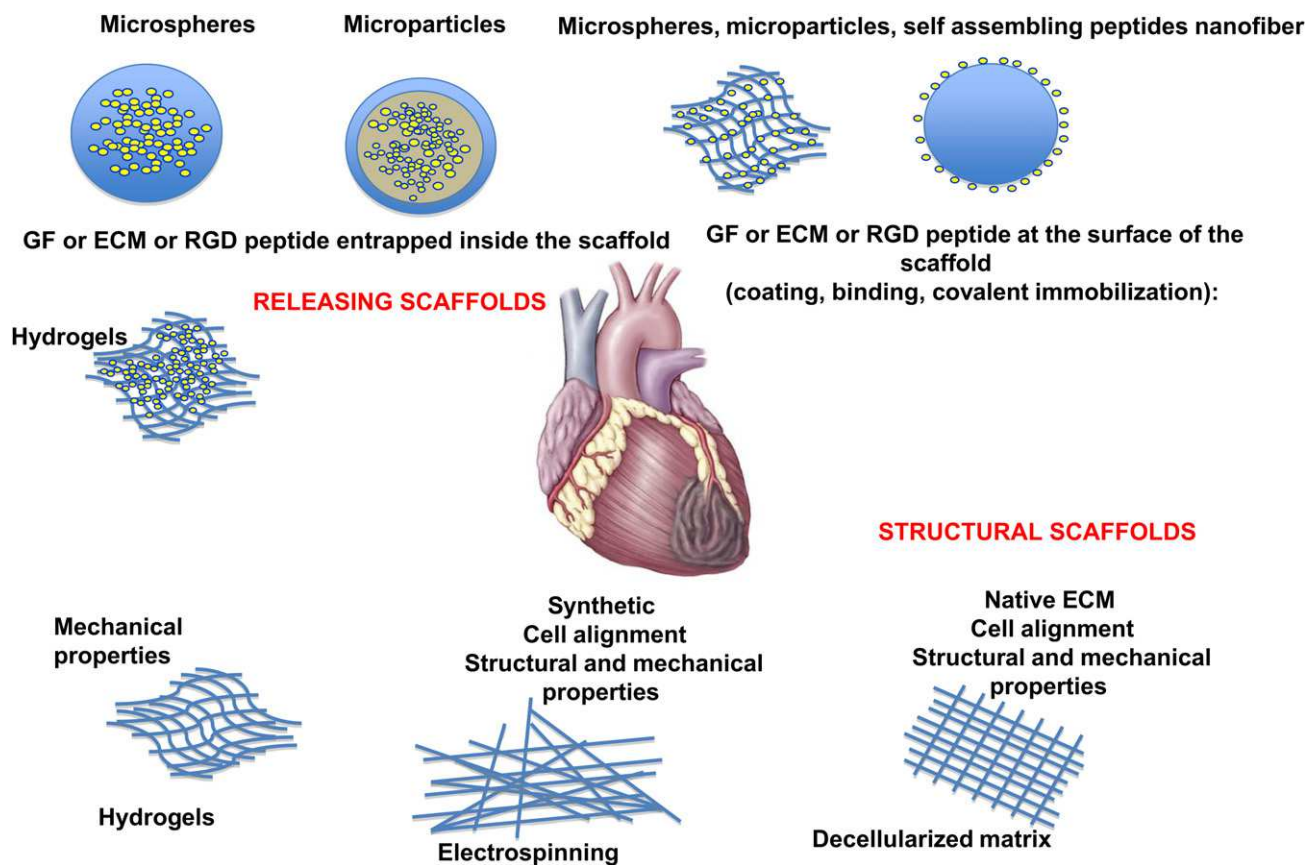


Fig. 1. Representation of ECM scaffolds used for cardiac repair.

designed to trigger cellular events, minimize the immune response and control the degradation rate, allowing thus cell integration and cardiac function improvement for a prolonged period. Different implantable biodegradable scaffolds based on gels or particulate systems encapsulating various molecules and/or cells have been studied for heart regeneration [for review 94–96].

3.1.2. Size and texture

Both scaffold size and texture have also to be taken into consideration. The cellular support must be porous and thin enough, i.e. less than 0.5 mm, to permit adequate oxygenation and nutrition within the tissue-like structure formed by the scaffold/cells complexes. Moreover, small-sized microstructured and/or nanostructured particles, should necessarily be small enough to be easily implanted in the myocardium, rendering repeated injections possible in discrete and precise areas, i.e. by minimally invasive surgery using catheter injection, closed chest echocardiography or fluoroscopy guided injection methods [97–100]. Recently, the importance of scaffold size for intra-myocardial injection has been reported for scaffold/cell complex retention in the beating heart [101]. For instance, augmenting from 10 μm to 400 μm the size of alginate-poly-L-lysine-alginate microspheres encapsulating MSCs, a fourfold increase in cell retention rate was observed [101].

Hydrogels with an *in situ* gelling process have also been extensively studied these last years due to the possibility of a direct injection in the ischemic area using a catheter/ needle [102,103]. Commonly used injectable scaffolds include natural materials such as collagen, chitosan, gelatin, fibrin and alginate, but also synthetic and biodegradable polymers including poly(lactide-co-glycolide) (PLGA), polycaprolactone (PCL), poly(glycerol-sebacate) (PGS),

poly(ethylene glycol) (PEG) and self-assembling peptides [for review 96,104]. In addition, biomaterial-based scaffolds have been explored for use as cellular and acellular cardiac patches providing a structural support for the damaged ventricle and allowing cellular recruitment into the material [105,106]. However, their implantation involves an invasive and massive open chest procedure that limits a wide use in the clinical setting.

3.1.3. Surface characteristics

Topography, charge, roughness, hydrophilicity and hydrophobicity, are also of great importance for cell therapy purposes. Indeed, transplanted-cell attachment critically depends on the polymer surface, but also cell differentiation and survival and consequently tissue healing around the implanted biomaterial. Hydrophobic/hydrophilic properties play an important role in protein adsorption kinetics and their folded conformation, which in turn influence cellular activities [107,108]. When biomaterials are exposed to biological environments, proteins and ECM molecules are non-specifically adsorbed on the surface of nearly all the biomaterials. The cells then indirectly interact with the biomaterial surface through the adsorbed proteins via their integrin receptors [109]. An interesting study has demonstrated that the charge and hydrophilicity of the substrate surface can also have an impact on apoptosis. Using an *in vivo* model of rat cage implant system, it was shown that the proportion of adherent cells undergoing apoptosis was increased significantly on anionic and hydrophilic surfaces when compared to hydrophobic surfaces. Indeed, the hydrophilic and anionic surfaces promote low level of adhesion and increase percentages of apoptosis [110], confirming surface property importance.

3.1.4. Porosity

Approaches in scaffold design must be able to create hierarchical porous structures to attain desired mechanical function, permeability and diffusion properties. For bigger scaffolds, porosity not only affects the biomaterial degradation but also cellular infiltration [111,112]. Pores should be interconnected with a diameter $> 10 \mu\text{m}$ to allow for cellular and vascular infiltration as well as for diffusion of nutrients [113]. Biochemical composition and porosity of scaffolds are thus crucial for the revascularization of the ischemic region.

3.1.5. Elasticity

Growing evidence now identifies scaffold elasticity as another important parameter. Most of the biomaterials used for cardiac cell therapy are developed to improve grafted cell retention and survival without considering biomechanical scaffold properties. The pertinent scaffold should have enough flexibility to respond synchronically with the myocardium contraction and effectively transfer mechanical stimuli from the myocardial microenvironment to the transported cells, providing appropriate cues to develop a contractile phenotype and communicate with other cells. The mechanical properties of a scaffold can also exert significant influence on seeded stem cell behavior. By exerting traction forces on a substrate, many mature cell types such as fibroblasts, muscle cells, and neurons sense the stiffness of the substrate and show dissimilar morphology and adhesive characteristics [for review 114]. The cells transduce the sensed signal via integrin receptors, localized at the cell surface, and in this way the behavior of the cell is regulated by a mechanism named mechanotransduction [115–118].

Hematopoietic cells cultured alone on a tropoelastin elastic support show increased progenitor expansion that is further enhanced by an additive treatment with cytokines. Inhibition of mechanotransduction abrogated these effects, suggesting that substrate elasticity and tensegrity are important mechanisms influencing hematopoietic stem and progenitor cell subsets [119]. In another study, the effect of matrix elasticity in directing MSCs lineage specification has been demonstrated. Soft type matrices, therefore mimicking brain, committed the cells toward the neurogenic lineage while slightly stiffer matrices appeared to be myogenic and stiff substrates osteogenic. MSCs initiated myogenesis on collagen-coated gels that mimic the elasticity of muscle ($E \sim 10 \text{ kPa}$) [120]. In addition, it has been shown that myoblasts or cardiomyocytes differentiate and beat synchronously on a matrix with an intermediate stiffness of 8–11 kPa, but they do not beat on a matrix with lower or higher stiffness, such as that observed in scar (20–40 kPa) [121,122]. These results highlight the importance of matrix stiffness not only to dictate differentiation into cardiomyocytes but also to spread the electric contraction. More recently, the same author has demonstrated the importance of dynamic material cues in guiding cell maturation. When cardiomyocytes are grown on collagen-coated thiolated-hyaluronic acid (HA) hydrogels crosslinked with poly-ethylene glycol diacrylate creating a stiffness gradient mimicking temporal stiffness during heart development, mature cardiac-specific markers increase and form up to 60% more maturing muscle fibers *in vitro* [123]. Matrix stiffness as well as stiffness gradients also has effects on cell migration and survival [124,125].

Stiffness can be directly linked to another phenomenon strongly implied in cell behavior, mechanical load. Cardiac cells, vascular smooth muscle cells (SMCs) and endothelial cells in blood vessels are all subjected to large mechanical forces, or loads, and are referred to as mechano-responsive cells. The cellular responses to loads depend on loading conditions that are, the type, magnitude, duration, and frequency of loading; they also depend on

surrounding matrix proteins as well as soluble factors [126–128]. For example, SMCs increase myosin expression in response to mechanical loading when cultured on laminin (LM) or collagen matrix but not on a fibronectin (FN) matrix [129]. Recently, an interesting study has investigated the response of hES hiPS-derived cardiomyocytes to mechanical load in a 3-dimensional collagen matrix [130]. They have shown that uniaxial mechanical stress conditioning promotes a 2-fold increase in cardiomyocyte and matrix fiber alignment and enhances myofibrillogenesis and sarcomeric banding while a 2.2-fold increase in cardiomyocyte hypertrophy and proliferation rates was observed with a cyclic stress conditioning compared to unconditioned constructs *in vitro*. Furthermore, co-culture with endothelial cells enhances cardiomyocyte proliferation and addition of stromal supporting cells enhances formation of vessel-like structures by approximately 10 fold *in vitro*. Finally, vascularisation and coronary circulation are observed 1 week after their transplantation into athymic rat hearts [130].

3.1.6. Cell orientation

It also appears to be an important parameter for enhancing cell-to-cell contact in order to achieve an implant with increased contractility and electrical stability. A really interesting study has shown that orientation of a neonatal rat cardiomyocyte preparation increases the conduction velocity as well as electrical integration of MSCs undergoing cardiomyogenic differentiation in co-culture with these cardiomyocytes [131]. Another study has shown that scaffold stiffness and geometry are crucial to enhance the differentiating effect of GF. Indeed, they have shown that CPC and MSC ability to adhere was geometry dependent and higher in the presence of square than hexagonal pores. They have also demonstrated that CPC aligned and acquired a cardiac phenotype on 3D scaffolds with square pores [132].

3.2. Biomimetic approach in tissue engineering

The first consideration in scaffold tailoring for heart after cardiac infarct is the choice between vascularisation and cardiomyocyte replacement of the ischemic area, by mobilizing endogenous or grafted cells. Indeed, endothelial cells exert trophic and inotropic effects on myocytes and also form blood vessels, provide oxygen and nutrients [130,133], and constitute a necessary part of myocardium. The physical and biochemical cues supplied to the graft must guide the cells into one or the other phenotype naturally occurring in the heart.

To better regulate grafted cell behavior, new strategies tend to implement the different signals that biological molecules, mainly ECM molecules and GF, will provide the cells when presented within a scaffold. We will first briefly describe cell–ECM molecule interactions and the resulting cell behavior particularly within the context of adult stem survival and cardiogenesis and then report some studies evaluating the benefits of such “biomimetic scaffolds”.

In a biomimetic approach the scaffolds are designed to mimic the regulatory characteristics of natural ECM and ECM-bound GF (Table 1). In a first approach ECM molecules can be used as a coating system in which the cell can sense it and interact directly through integrin receptors. In this strategy ECM can cover a scaffold to allow cell attachment to this scaffold which supply/replace *in vivo* the lacking ECM. In a second approach, they can be used as a structural system partially mimicking the structural architecture and/or organisation of the myocardium. ECM, used as a structural “scaffold/support” would possess the appropriate stiffness and elasticity to differentiate the transported cells into cardiomyocytes. Finally, ECM delivery through a drug-releasing system such as hydrogels or

Table 1
Representative biomimetic scaffolds used for MI tissue engineering.

Scaffolds	Properties	Cells	Animal	Results	Ref.
RGD-modified alginate microsphere	Modified RGD surface Cell retention Injectable	MSC	Rat	<i>In vitro</i> cell attachment, growth and angiogenic growth factor expression <i>In vivo</i> cell survival, maintenance of LV geometry, preservation of LV function, reduction of infarcted area and enhancement of arteriole formation	[100]
Collagen cardiac patch	Cell retention Natural ECM	BMMNC	Human	Cell retention Improvement of LVED volume and scar thickness compared to cell alone injection	[158]
Fibrin	Cell retention Natural ECM	ADSC	Rat	Improvement of ejection fraction (EF), LVED diameter, LVES diameter compared to fibrin alone or cell alone	[159]
Fibrin	Cell retention Natural ECM	Marrow-derived CSC	Rat	Improved EF in all groups compared to control	[160]
Hyaluronan derivative	Cell retention Esterified fibers	MSC	Rats	Ex vivo MSC adhesion, survival and ECM production MSC migration to the peri-infarcted area. Increased vascularization	[161]
Decellularized matrix	Injectable Cell retention Natural ECM	CM, EC, SMC	Rats	<i>In vitro</i> neonatal cardiomyocytes adhesion and survival Endothelial and smooth muscle cell migration <i>in vitro</i> and <i>in vivo</i> . Increase in arteriole formation	[162]
Fibrin hydrogel applied onto decellularized myocardium	Cell retention Natural ECM	Mesenchymal progenitor cells	Rat	Secretion of paracrine factors, migration into the ischemic area, recovery of ventricular systolic dimension and contractility.	[163]
Decellularized pericardium	Cell retention Natural ECM	MSC		Higher FS and greater LV ES pressure and lower LVED pressure	[164]
Peptide nanofibers	Cell retention Cell alignment Mimic the natural ECM	BMMNC	Mini-pigs	Improvement in LVES volume and LVED volume in all groups compared to control Increased transplanted-cell retention Increased capillary density in peri-infarcted area	[169]
Alginate/Matrigel	Factor binding Sustained release Cell retention	Neonatal CM	Rat	Structural and electrical integration of the grafted cells Improvement of scar thickness	[177]

particulate systems could be an interesting strategy to release it within the infarcted area to favor direct cell adhesion within the tissue. In the following sections we will discuss about these and other strategies employing ECM or mimicking ECM in cardiac tissue engineering.

3.2.1. Cell–ECM molecule interactions

It is now evident that any device fashioned for cardiac tissue engineering should be biomimetic. The transplanted cells should receive the appropriate cues from the scaffold, which fulfills the lacking properties of the surrounding ischemic tissue. In this way it provides to the graft a new 3D, communicative, mechanical and nutritional status, guiding the cell into the desired phenotype and acting as an instructive whole system. In this sense, many studies have used biomaterial scaffolds associated with ECM molecules, which interact mostly with integrins, the receptor at the cell surface [134,135]. The constant interaction with the environment positions integrins as a source of information to influence cell fate decisions. Although widely appreciated for their role in mediating cell anchorage, integrins are also responsible for initiating many signaling events. Most of these signaling cascades regulate cell survival pathways and have been directly implicated in cellular resistance to apoptosis [136]. They mediate cell–ECM and cell–cell communications in a bidirectional manner, that is, extracellular events affect nuclear activity, while signals that originate in the nucleus affect cell surface protein expression and function [116]. They are critical for cell behavior, specifically cell migration and

survival, during virtually all developmental stages, as well as pathological remodeling.

The list of integrin/ligand couples is expansive [137]. Integrins are essential for ECM interactions with cardiac myocytes and fibroblasts, and overall structure and cell communication in the heart [138]. Early studies have shown a role of FN and collagen in heart development [139,140] and collagen and LM for myocyte survival and proliferation [141]. LM and FN are the most extensively studied for their importance in cell adhesion, survival and differentiation [79,142–145]. They are expressed in the normal heart and increase after MI [146–148]. FN levels increase from 12 h onwards after MI in the human heart [79]. Further, LM is described to increase from 3 days post-MI [149]. Recently, the role of integrins in cardiac differentiation and engraftment of committed MSCs into cardiomyocyte-like cells was investigated in a rat *in vivo* model of MI. They have shown that collagen V, but not collagen I matrix, promoted attachment, proliferation and cardiac differentiation of committed MSCs. They also demonstrated that inhibition of $\alpha_v\beta_3$, but not $\alpha_2\beta_1$ integrin, selectively attenuated troponin-T and sarcomeric α -actin expression in the cells [20]. In another study, ADSC commitment was induced on an LM or FN coating, and the results showed that LM more efficiently differentiated the cells toward the cardiomyocyte lineage [79].

3.2.2. ECM as a biomimetic surface

Scaffolds have been functionalized with simple peptides such as RGD to promote adhesion [100,150] (Table 1). However, it is known

that more complex, combinatorial signals promote significant changes in cell behavior [151] indicating that the biochemical composition of a scaffold is critical to instruct cells. Thus, the surface of scaffolding materials is important in tissue engineering, because it can directly affect cellular response and ultimately the tissue regeneration. Although a variety of synthetic biodegradable polymers have been used as tissue-engineering scaffolding materials, they often lack biological recognition. The surface modification of biomaterials with bioactive molecules is a simple way to provide a biomimetism. The early work has used proteins such as FN, vitronectin (VN), collagen type I and LM for surface modification of scaffolds [152]. In a recent study, the expression of contractile marker levels was increased in smooth muscle cells (SMCs) adhered to PEG-based hydrogels coated with RGD peptides, LM and FN [153].

ECM molecules can also be incorporated into the biomaterials and in this way, the resulting recognition sites are present not only on the surface but also in the bulk of the materials. In this way, adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) were increased *in vitro* in an RGD peptide/alginate complex, as well as arteriole density and angiogenic response *in vivo* compared to a non-modified alginate group. Moreover, heart response was improved *in vivo* in ischemic rats compared to the control without the scaffold [150]. These results reveal that a biomimetic material not only promotes cell adhesion but also potentially serves as artificial ECM providing suitable biological cues to guide new tissue formation through survival mechanisms, angiogenesis and myocytes formation.

ECM used as a secreted molecule also constitutes an interesting approach. In such strategy, the ECM can be released by the grafted cells or by the scaffold to favor not only grafted cell adhesion but also endogenous cell colonization within the tissue or within the scaffold. This strategy could be interesting, but at the moment very few studies have been performed. It would be probably more interesting to use it in combination with other secreted molecules within the first hours following the infarct.

3.2.3. ECM as a structural molecular complex

The interest to provide to the cells the appropriate biomimetic coating [154,155], as well as the necessity to have the good elastic property by plating cells on hydrogels [123,155–157] has been mentioned here above. In this regard, integrating cells within an injectable hydrogel with the appropriate elastic modulus, containing or not ECM/RGD peptides is a really seductive approach to dictate and improve stem cell behavior [102]. Another strategy has employed collagen cardiac patches with bone marrow mononuclear cells (BMMNC) in 10 patients. The results have shown cell retention but also improvement of left ventricular end-diastolic volume (LVED) and scar thickness compared to cell injection alone, demonstrating the feasibility of such clinical studies [158]. Other studies using 3D fibrin scaffolds have shown similar results [159,160] (Table 1).

Recently the high retention rate and viability of MSCs cultured on a non-woven hyaluronan-based scaffold HYAFF® was demonstrated *in vitro*. Their adhesion to the scaffold through focal contact was shown after 7 days as well as ECM molecule secretion such as collagen and proteoglycans. An increase in vascularization and cell migration to the peri-infarcted area was demonstrated 2 weeks after MSC/HYAFF® transplantation in an MI model in the rat [161].

3.2.4. ECM from decellularized tissue

Recently, focus has been placed on the utilization of decellularized ECM for cardiac repair (Table 1). Indeed, cardiac-specific decellularized tissue that conserves a variety of proteins, peptides, and glycosaminoglycans could be the appropriate choice

to replace the damaged myocardial ECM. In a recent study, myocardial matrix was shown to reassemble *in vitro* at physiologic pH and temperature into a nanofibrous and porous structure and vascular cell infiltration with the formation of arterioles was promoted in a swine ischemic cardiac model *in vivo* [162]. Two really interesting strategies have employed MSCs with a decellularized human myocardium combined in rat model of MI [163,164]. Results have shown cell secretion of paracrine factors, their migration into the ischemic area but also the recovery of ventricular systolic dimension and contractility.

3.2.5. Mimicking ECM organisation with synthetic systems

In the myocardium, ECM proteins are mainly in fibrous form with diameters of several hundreds of nanometers. Scaffolds with similar structures would better approximate the fibrillar extracellular components compared to conventional nonfibrillar scaffolds. Electrospinning is a widely accepted technique for processing nanofibrous scaffolds. The scaffolds often have fiber diameters ranging from 10 to 1000 nm, within the range of the fibrous proteins in the myocardium. One of the advantages of electrospinning is that it can fabricate highly aligned scaffolds. Utilization of such scaffolds for cardiac scaffold tissue engineering is advantageous as they mimic not only the nanofibrous morphology but also the structural and mechanical anisotropy of the cardiac muscle [165,166]. Recently, attachment, alignment and survival of rabbit cardiomyocytes and MSCs was demonstrated *in vitro* with a polycaprolactone/gelatin (PG) composite nanofibrous scaffolds and a poly (glycerol sebacate) (PGS) nanofibrous scaffold, respectively [167,168]. Another similar study was performed using BMMNC and self-assembling peptide nanofibers in an MI model in mini-pigs and has demonstrated an increase of transplanted-cell retention but also of capillary density in the peri-infarcted area as well as improvement in left ventricular end systolic volume (LVES) and LVED volume compared to controls sham operated and cells alone while ventricular remodeling was prevented with the scaffold alone [169].

3.3. Going biomimetic using growth factors

Several factors play a role in early cell death after engraftment in the ischemic myocardium. Among them and of considerable importance is the absence of survival factors in the transplanted heart. The tissue repair process is a complex cascade of biological events controlled by numerous cytokines and GF that provide local signals at sites of injury; these signals regulate the mechanisms and pathways that govern wound healing and tissue regeneration. In addition, GFs control the production of extracellular matrix and cell behaviour. GFs are implicated in cell survival, growth, differentiation, and mobility as well as cell–cell communication processes. The particular hostility of the infarcted area for cell survival and homing, and the poor regeneration capacity of the myocardium led the scientific community to hypothesize that *in situ* injection of a growth factor would be an exciting and useful strategy for endogenous or grafted cells to integrate, proliferate or differentiate within the infarct area. For these reasons, the use of GF to regenerate the heart and to overcome the complexity of the ischemic environment has been extensively investigated.

Many studies have been performed using pleiades of GF as therapeutic agents to regenerate the ischemic heart including insulin-like growth factor-1 (IGF-1), VEGF, hepatocyte growth factor (HGF), erythropoietin (EPO), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), stromal cell-derived factor-1 (SDF-1), transforming growth factor beta 1 and granulocyte colony stimulating factor [for review 23]. In this regard, CPCs recruitment was observed after HGF and IGF-1 bolus injection

in the infarcted rat heart. Moreover, arterioles, capillaries, and functionally competent myocytes, which with time increased in size, were observed and ventricular performance was improved [170]. However, this model presents some limits for a therapeutic use, it requires high doses of GFs to have an effect due to their short half-life but also it requires a frequent administration of the active ingredients which could be an inconvenience for the patients with MI.

Sophisticated scaffolds can either present a GF to the cells to increase their engraftment or can deliver it in a prolonged and continuous manner that can improve grafted cell behavior and also modulate the surrounding environment. GFs can be used very differently depending on the desired effect. On one hand, they can improve grafted cell survival or differentiation; this way the GF acts directly on the grafted cells. On the other hand, they can stimulate angiogenesis inside the scar tissue and indirectly favor engraftment of the cells. In the past few years, with the growing evidence and the exciting perspectives of the presence of CPCs inside the heart, strategies using scaffolds releasing GF that can recruit them to the infarcted site have started to be investigated.

Different release methods have been developed in order to have a rapid or prolonged release of the protein. Rapid released methods generally use hydrogels or protein immobilization on the scaffold surface. For the hydrogel, a burst is observed within the first hours and the protein is released within a few days. Concerning protein immobilization, the delivery mechanism principally depends on scaffold hydrolysis properties. To obtain a prolonged release (several weeks) hydrogel properties can be modified, but particulate scaffolds, such as microspheres seem to be more appropriate for a prolonged release. Another strategy tends to covalently immobilize the GF on the scaffold in order to have it present locally for a prolonged period, corresponding generally to the time of degradation of the scaffold. Finally, the protein can be incorporated within the scaffold, and especially particulate scaffolds, which will then act as a delivery system that will release a growth factor within the tissue for a prolonged period. In this section we will review the main growth factors used in combination with scaffolds for the three approaches mentioned in cardiac tissue engineering.

3.3.1. Immobilization of GF for rapid release

Many attempts have been made to enhance the stability of growth factors associating them to devices including microspheres, scaffolds and hydrogels. One interesting study showed sustained release of bFGF, characterised by an important burst release during the first hours by immobilization of heparin onto the surface of porous PLGA [171]. In this study bFGF was loaded into the heparin functionalized (PLGA-heparin) microspheres by a simple dipping method and angiogenesis was observed in mice 1 week after subcutaneous injection. However, this strategy is limited due to the small amount of GF that can be loaded onto the scaffold and the control of its release kinetics into the ischemic area. Indeed, generally a rapid release occurs or a more sustained one but presenting an important burst during the first hours of release. Moreover the GF remains only in the injection site due to the small amounts and the limited diffusion of proteins used, thus reducing the efficacy within the biomaterial itself rather than the surrounding tissue.

3.3.2. Covalent immobilization of GF

The GF can be used as complement to the scaffold, acting directly on the grafted cells. In a first strategy, the GF can be covalently immobilized on the scaffold surface to localise the desired effect within the biomaterial rather than the surrounding tissue. This method offers the advantage to have a high loading

efficiency using dilute solutions with no risk of protein denaturation. Covalent immobilization of VEGF to a porous collagen patch/scaffold enhanced endothelial and bone marrow cell growth *in vitro* after 28 days. *In vivo*, both cell recruitment and proliferation increased but also blood vessel density was enhanced 28 days after implantation in the infarcted rat heart [172]. Another study using covalently immobilized VEGF and angiopoietin-1 onto a three-dimensional porous collagen scaffold with H5V cells derived from murine embryonic heart endothelium has shown similar effects *in vitro*, increasing endothelial cell proliferation, cell metabolism (glucose consumption and lactate production), capillary-like tube formation and cell infiltration after 7 days of culture conditions [173]. However, the limit of this strategy lies in the protection of the GF against degradation, since it is in direct contact with the surrounding microenvironment.

3.3.3. Releasing the GF to stimulate endogenous repair mechanisms

The protein is blended inside the scaffold matrix and a slow, continuous and controlled release can occur during several weeks. This drug delivery system would protect the protein from inactivation and guarantee the preservation of its structure and bioactivity during the whole release. The entrapped protein should be delivered in a time and dose-controlled manner in a specific area at a pre-programmed rate. This system has the advantage of delivering an appropriate physiological dose to the damaged area, which is particularly important in the case of GFs as they act in a dose-dependant manner. The method by which a drug is released can have a significant effect on therapeutic efficacy. The mode of drug delivery is especially relevant using GFs because the dose and spatio-temporal release is crucial in achieving a successful outcome. In this sense, various strategies were developed, in particular the targeted administration of these GFs in order to optimize their effectiveness, to limit the effects due to their pleiotropic action in the untargeted zones, to avoid their fast degradation and also to reduce the quantities injected.

Delivery devices commonly used are particulate or hydrogel-based scaffolds modified to retain the GF and deliver it in a prolonged manner. Hydrogel scaffolds are advantageous as they could mechanically support the beating myocardium due to their elastic properties, and they act simultaneously as a scaffold and a controlled delivery platform. Protein encapsulation within particulate micro- and nano-carriers is also regarded as a powerful tool to protect the biological activity and sustain the release over long time frames. Moreover, these particles offer the advantages of excellent size and morphology control, and their surface can be functionalized to enhance their interaction with the cells and the cardiac tissue. The dual delivery of IGF-1 followed by HGF in a sequential manner from affinity binding alginate hydrogels has demonstrated interesting results [174]. This system induced cardiomyocyte cycle activation and exhibited cytoprotective properties *in vitro*. When injected in the infarcted rat heart, significant reduction of the fibrotic area and apoptotic cells was observed along with an increase in vessel density and proliferating cardiomyocytes compared to controls. Biodegradable gelatin microspheres releasing VEGF and IGF-1 have also shown interesting results [175]. Briefly, complementary effects were found when both VEGF and IGF-1 microspheres were injected in the rat infarcted heart. While left ventricular (LV) remodeling, LV systolic and diastolic functions were attenuated and cell death reduced with IGF gelatin microspheres, neoangiogenesis was observed with VEGF-gelatin microspheres. In another study, crosslinked albumin-alginate microcapsules that sequentially release fibroblast growth factor-2 (FGF-2) and HGF were formulated. Both released proteins were bioactive and were able to induce angiogenesis in a mouse Matrigel plug model, but also stimulate angiogenesis and

arteriogenesis and prevent cardiac hypertrophy and fibrosis in a rat model of MI [176]. Cardiac perfusion was improved, remodeling was reduced and the left ventricular function was also improved, as observed 3 months after injection.

3.4. Combining cells, scaffolds and GF delivery systems

In cell therapy of the ischemic myocardium, the massive cell death occurring due to the lack of anchorage matrix, the poor cell retention of the grafted cells and the ischemic microenvironment have led to consider the scaffold as a new drug-releasing system capable of localised and sustained release of the GF or cytokines during the degradation of the polymer, combined to the three-dimensionality effect to better control cell behavior.

A recent study has used hMSCs in combination with an injectable thermosensitive hydrogel copolymer releasing continuously bioactive IGF-1 during 2 weeks *in vitro*. The cells were found to grow inside the hydrogel during a 7-day culture period and the IGF-1 loading significantly accelerated their growth *in vitro*. The scaffold also exhibited a high flexibility with elongation higher than 1000%, tensile stresses ranging from 10 to 17 kPa and a Young modulus ranging from 63 to 120 kPa. It showed good oxygen permeability and could thus be used as GF and cell carrier [102].

Recently, an interesting study has used neonatal cardiomyocytes combined with a mixture of pro-survival and angiogenic factors IGF-1, VEGF and SDF-1 into an alginate scaffold capable of factor binding. These patch were prevascularized for 7 days into the rat omentum before their injection into the infarcted rat heart. The

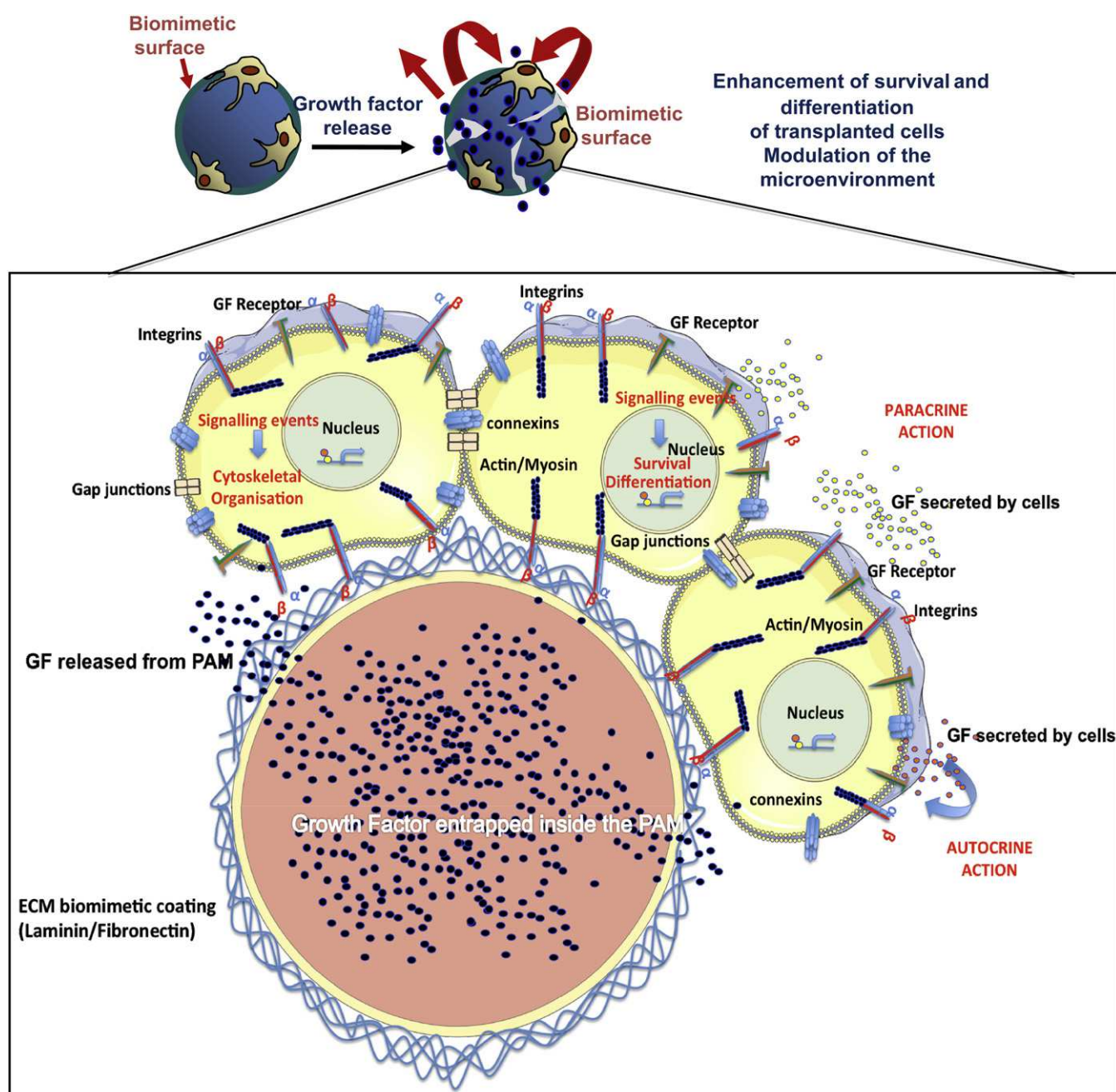


Fig 2. PAMs with a surrounding biomimetic coating of ECM and releasing pro-survival and differentiating GF can improve grafted ADSCs behavior, enhancing their differentiation and favoring grafted cell interaction.

vascularized cardiac patch showed structural and electrical integration into host myocardium compared to the *in vitro*-grown patch [177]. Although this study did not use adult cells the approach is quite interesting and could be implemented with adult stem cells.

3.4.1. Combining cells, ECM molecules and GF

Our group has an expertise in the development of drug delivery devices to repair damaged tissues [178–181] and in stem cell therapy combined to biomimetic drug delivering devices [182–184]. Within this line, we have developed an innovative vector for tissue engineering named pharmacologically active microcarriers (PAMs) [182]. These PAMs are biodegradable and non-cytotoxic PLGA microspheres covered with ECM molecules, thus conveying cells on their biomimetic surface providing an adequate 3D microenvironment for the transplanted cells *in vitro* and *in vivo*. They have a mean size of 60 μm and can be easily implanted through a needle or catheter. They contain a GF encapsulated under a nanosolid state to preserve its structure and integrity within the polymer and can be delivered in a sustained and controlled manner for a prolonged period [180]. The delivered factor in combination with the 3D biomimetic surface of the PAMs act synergistically to stimulate the survival and/or differentiation of the grafted cells toward a specific phenotype, therefore enhancing their engraftment but also affecting the host microenvironment allowing better integration of the grafted cells [185]. The use of these PAMs for neurodegenerative disorders has been successfully validated in animal models of Parkinson's disease with neuro-progenitor cells and MSCs [183–186]. They further potentiate the chondrogenic differentiation potential of MSCs [187].

We believe PAMs are an interesting candidate for cardiac cell therapy (Fig 2). Thus, PAMs releasing HGF and IGF-1 with a biomimetic surface of LM and conveying committed stem cells into a cardiomyocyte phenotype may represent an interesting strategy to repair the damaged heart. In one hand, the HGF released from PAMs could promote adult stem cell cardiomyocyte differentiation and CPCs recruitment while promoting a local neoangiogenesis around the graft [170,188–190]. In the other hand, the IGF-1 released from the PAMs could improve the grafted cells survival while inducing at the same time proliferation and survival of the CPCs [104,191–193]. Finally the LM surface as suggested by van Dijk et al. [79] can constitute the appropriate surrounding biomimetic coating of the PAMs. Preliminary results from our group showed that PAMs releasing IGF-1 and HGF and presenting the biomimetic coating promotes the expression of cardiac proteins whereas no protein expression was found in 2D. Combination of these PAMs with a thermosensitive hydrogel, which could provide appropriate mechanosensitive cues by controlling the stiffness of the material to modulate the differentiation profile of hADSCs, could further enhance stem cell survival and retention *in vivo*.

4. Conclusion

Adult stem cell therapy, with the possibility to repair the damaged tissue, holds tremendous promise for the treatment of MI. However, the major problems of cell survival, cell fate determination and engraftment after transplantation, still remain. With the recent development of new technologies intelligent biomaterials combining drug delivery and biomimetic support for the cells allow the modulation of their immediate microenvironment to favor cell engraftment. In the future, delivery of multiple growth and differentiation factors in a spatially and temporally controlled manner by a scaffold transporting stem cells with the appropriate mechanical properties and biomimetic ECM molecules will be needed for successful cardiac regeneration.

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Chapter 2

Adipose-derived stem cell adhesion on
laminin-coated microcarriers improves
commitment towards cardiomyogenic
lineage

Scientific context

It is known that the microenvironment plays an important role to determine cell fate and the molecular signals provided by cytokines and GFs are one of the principal actors (Anitua, Sánchez et al. 2008; Discher, Mooney et al. 2009; Srinivas, Anversa et al. 2009). The few studies performed with GFs have described ADSC cardiac commitment in vitro especially in the presence of transforming growth factor beta 1 (TGFβ1) (Gwak, Bhang et al. 2009). Recent studies using MSCs and a GF cocktail have shown that the combination of GFs permits to obtain a more mature cardiac phenotype, with cells expressing cardiac transcription markers and late differentiated stage proteins (Behfar, Faustino et al. 2008; Hahn, Cho et al. 2008; Behfar, Yamada et al. 2010). Despite the fact that they are very similar, ADSCs seem to be more capable to differentiate into cardiomyocytes than MSCs (Zhu Y, Liu T et al. 2008) and we can then hypothesize that ADSCs may display an enhanced differentiation profile in response to such growth factor stimuli.

However, the poor cell retention and the low survival of grafted cells remain a challenge for an efficient myocardial repair even if the appropriate cells are used. Providing to the cells ECM molecules as an instructive surrounding microenvironment that favor direct cell adhesion, survival and/or differentiation can be an interesting strategy to fill the lacking ECM signals due to scar formation in the ischemic area. In vitro and in vivo experiments have described the major role of integrins and their ligands LM and FN in myocardial development and ADSCs induction into the cardiomyocyte lineage. Such ECM molecules could be used as a direct biomimetic signal to further ADSC cardiac commitment in combination with a GF cocktail. In addition, three-dimensional support has been described to enhance cell retention and survival within the host tissue (Schussler, Chachques et al. 2010; Schwartz, Danoviz et al. 2010; Lee, Wei et al. 2011) but also to be involved in cell fate (Dutta and Dutta 2009; Martino, Mochizuki et al. 2009; Tibbitt and Anseth 2009; Kenar, Kose et al. 2011).

We have therefore investigated the in vitro effect on ADSCs cardiac differentiation of a GF cocktail enriched or not with TGFβ1, in combination or not with an ECM biomimetic substrate that could also be provided by the PAMs. We evaluated the role of each parameter, i.e. GF cocktail, ECM molecules and 3D, by following the expression of specific cardiac markers, transcription factors and contractile proteins that appear in a coordinated manner

during the differentiation process. This study may allow the development of an appropriate microcarrier for the heart.

Abstract

For tissue-engineering studies of the infarcted heart it is essential to identify a source of cells that may provide cardiomyocyte progenitors, that is easy to amplify, accessible in adults, and allowing autologous grafts. Recently, preclinical studies have shown that human adipose-derived stem cells (ADSCs) can trans-differentiate into cardiomyocyte-like cells and improve heart function in acute myocardial infarction. However, the poor cell retention, survival and differentiation need to be ameliorated for a successful therapy. We have developed pharmacologically active microcarriers (PAMs), which are biodegradable and biocompatible poly (lactic-co-glycolic acid) (PLGA) microspheres conveying cells on their biomimetic surface, therefore providing an adequate 3-dimensional (3D) microenvironment in vivo. Moreover, they can release a growth factor in a prolonged manner. These combined properties act together to stimulate survival and/or differentiation of the grafted cells toward a specific phenotype. In order to implement ADSCs and PAMs for cardiac tissue engineering we first defined the biomimetic surface by studying the influence of matrix molecules laminin (LM) and fibronectin (FN) in combination with a cocktail of growth factors (GF) present in the cardiogenic niche, to further enhance the in vitro cardiac differentiation of ADSCs. We demonstrated that LM increased the expression of cardiac markers GATA4, MEF2C and cTnI by ADSCs after 2 weeks in vitro. Interestingly, the 3-D support provided by PAMs with a LM biomimetic surface (LM-PAMs) further enhanced the expression of cardiac GATA4, MEF2C, cTnI and CX43 compared to the FN-PAMs and the 2D differentiating conditions after only 1 week in vitro. Finally we observed that an enrichment of the GF cocktail with TGF β -1 potentiate the expression of the cardiac markers in both 2D and 3D culture conditions. These results suggest that the use PAMs offering a LM biomimetic surface may be efficiently used for applications combining adult stem cells in tissue-engineering strategies of the ischemic heart.

Key words: Adipose stem cells, laminin, Growth factors, pharmacologically active microcarriers, myocardial infarction.

Adipose-derived stem cell adhesion on laminin-coated microcarriers improves commitment towards cardiomyogenic lineage

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Introduction

Myocardial infarction (MI) constitutes the first cause of morbidity and mortality in developed countries and current treatments present certain limitations, particularly for heart tissue repair. After MI, a necrotic and poorly vascularized zone appears, damaged cardiomyocytes are removed by macrophages and gradually replaced by scar tissue due to the limited intrinsic regenerative capacity of the heart. Hence, in recent years, cell-based therapies have attracted considerable interest as an alternative way of achieving cardiac repair. In this regard, it is essential to find a cell source that may give rise not only to cardiomyocytes, but also to endothelial cells, which are necessary to restore cardiac function. Potential cells for transplantation include induced pluripotent stem cells (iPSs) or embryonic stem cells (ESCs), but their inherent risk of tumor formation, the difficulties of easy large-scale cell preparations, and ethical concerns regarding ESCs must still be overcome. From this point of view, adult stem cells have been considered as interesting candidates for cell therapy. Among them, adipose derived-stromal cells (ADSCs) possess a large differentiation potential and can be easily harvested from adipose tissue and amplified in culture (Gimble, Katz et al. 2007; Meliga, Strem et al. 2007). Moreover, they can be used as autologous cells for transplantation and overcome the immunological, ethical as well as availability concerns.

ADSCs secrete many growth factors and cytokines implicated in tissue repair (Bai, Yan et al. 2010), have immunoregulatory properties (Puissant, Barreau et al. 2005; Yañez, Lamana et al. 2006; Bai, Yan et al. 2010; Technau, Froelich et al. 2011), an angiogenic potential (Wosnitza, Hemmrich et al. 2007; Madonna, Geng et al. 2009; Hong, Traktuev et al. 2010), may differentiate into endothelial cells and, particularly, adipose derived mice stromal cells, are also able to differentiate spontaneously into cardiomyocytes (Planat-Benard, Menard et al. 2004; Song, Gehmert et al. 2007; Jumabay, Zhang et al. 2009; Choi, Distingu et al. 2010;

Metzele, Alt et al. 2011). Furthermore, under specific *in vitro* conditions, different subpopulations human ADSC differentiation into cardiomyocytes has been reported using demethylating agent (Choi, Dusting et al. 2010), growth factors (GFs) (Planat-Benard, Menard et al. 2004; Song, Gehmert et al. 2007; Gwak, Bhang et al. 2009), extracellular matrix molecules (Dijk, Niessen et al. 2008) or co-culture with cardiomyocytes (Metzele, Alt et al. 2011). Transplantation of ADSCs after MI has shown benefits on cardiac function through vessel formations, improvement in left ventricular ejection fraction (LVEF) and diminution of scar area (reversed wall thinning in the scar area) (Leobon, Roncalli et al. 2009; Schenke-Layland, Strem et al. 2009; Bayes-Genis, Soler-Botija et al. 2010; Bagno, Werneck-de-Castr et al. 2012). However, the low percentage of living cells remaining in the tissue (Schenke-Layland, Strem et al. 2009) and the limited cardiac differentiation after transplantation (Leobon, Roncalli et al. 2009; Wang, Deng et al. 2009), are problems that need to be taken into consideration for future studies. The use of committed cells can be an alternative to overcome the limited differentiation of grafted cells and to favor their integration within the tissue. In this way, it appears necessary to improve the cardiomyogenic differentiation potential of ADSCs in order to optimize their future use in cell therapy studies.

It is known that the microenvironment plays an important role to determine cell fate and the molecular signals, through cytokines and GFs, are one of the principal actors (Anitua, Sánchez et al. 2008; Discher, Mooney et al. 2009; Srinivas, Anversa et al. 2009). The few studies performed with GFs have described ADSCs cardiac commitment *in vitro* especially in the presence of transforming growth factor beta 1 (TGF β 1) (Gwak, Bhang et al. 2009). ADSC differentiation into cardiomyocytes also seems to be dependent on the autocrine/paracrine action of vascular endothelial growth factor (VEGF) (Song, Gehmert et al. 2007). Recent studies using bone marrow multipotent stromal cells (MSCs) and a GF cocktail have shown that the combination of GFs permits to obtain a more mature cardiac phenotype (Behfar, Faustino et al. 2008; Hahn, Cho et al. 2008; Behfar, Yamada et al. 2010). These factors represent a cocktail of secreted proteins that recapitulate the components of the endodermal secretome critical for cardiogenic induction of the embryonic mesoderm (Behfar and Terzic 2006). Despite the fact that they are very similar, ADSCs seem to be more capable to differentiate into cardiomyocytes than MSCs (de la Garza-Rodea, van der Velde-van Dijke et al. 2012) and we can then hypothesize that ADSCs may display an enhanced differentiation profile in response to such growth factor stimuli. The role of extracellular matrix (ECM) molecules needs also to be taken into consideration. Early studies have shown a role of

fibronectin (FN) and collagen in heart development (Pankov 2002; Dijk, Niessen et al. 2008; Martino, Mochizuki et al. 2009) and collagen and laminin (LM) for myocyte survival and proliferation (Malan, Reppel et al. 2009). Among ECM molecules, LM and FN are the most extensively studied for their importance in cell adhesion, survival, and differentiation (Kuppuswamy 2002). They are expressed in the normal heart and increase after MI (Villarreal and Dillmann 1992; Frøen JF 1995; Willems, Arends et al. 1996; Ulrich, Janssen et al. 1997). FN levels increase from 12 h onwards after MI in the human heart (Choi, Dusting et al. 2010) and LM is described to increase from 3 days post-MI (Morishita, Kusachi et al. 1996).

The combination of both GFs and ECM molecules supplied through the use of biomaterials to improve cell behavior within the infarcted area is an interesting strategy in regenerative medicine. Within this line, we have developed an innovative vector for tissue engineering named pharmacologically active microcarriers (PAMs) (Tatard, Venier-Julienne et al. 2005). These PAMs are biodegradable and biocompatible PLGA microspheres covered with ECM molecules, thus conveying cells on their biomimetic surface and providing a 3-dimensionnal (3D) microenvironment for the transplanted cells in vitro and in vivo which stimulate cell survival (Delcroix, Garbayo et al. 2011; Garbayo, Raval et al. 2011). Moreover, PAMs have a mean size of 60 μm and can be easily implanted through a needle or catheter. They can also contain GFs encapsulated under a nanosolid state to preserve its structure and integrity within the polymer and can deliver it in a sustained and controlled manner for a prolonged period (Giteau A. 2008, Bouffi, Thomas et al. 2010; Delcroix, Garbayo et al. 2011). The delivered factor in combination with the 3D biomimetic surface of the PAMs can then act synergistically to stimulate the survival and/or differentiation of the transplanted cells toward a specific phenotype, therefore enhancing their engraftment but also affecting the host microenvironment allowing a better integration of the grafted cells and/or the stimulation of the injured tissue ((Tatard, Venier-Julienne et al. 2004; Tatard, Sindji et al. 2007; Bouffi, Thomas et al. 2010; Delcroix, Garbayo et al. 2011).

In this study, we first sought to assess the potential benefits of a GF cocktail treatment adapted from the literature (Behfar, Yamada et al. 2010) to further improve ADSCs cardiomyogenic differentiation in vitro. In addition, we compared the effects of the GFs treatment in combination with a co-substrate composed of ECM molecules, FN and LM, which we suggest could be the appropriate adhesion signals to direct ADSCs differentiation. We then hypothesized that the ECM biomimetic coating provided as a 3D support by the

PAMs combined to the addition of a cocktail of GFs in the media could favor ADSCs commitment into the cardiomyogenic lineage. If this is confirmed it may be a reasonable approach to deliver committed cells in tissue-like structures like the heart and preserve cellular integrity.

Material and Methods

Adipose tissue donors

Two primary cultures of ADSCs have been used. The first one was purchased from Lonza (Lonza France, Levallois Perret, France) and the second one obtained from patient lipoaspirates. Briefly, human subcutaneous adipose tissue samples were obtained as waste material after elective surgery and donated upon written informed consent of the patients from Sant' Orsola Hospital of Bologna, Italy. Adipose tissue was harvested from the abdomen by using liposuction. Since similar results were obtained with both cell cultures, no further distinction between these two sources of ADSCs will be made throughout the manuscript.

Isolation of stromal vascular fraction from adipose tissue

Adipose tissue was stored in sterile phosphate-buffered saline (PBS) at 4°C and processed directly after surgery. Briefly, the lipoaspirates were extensively washed with PBS to remove debris and blood cells. The washed aspirates were treated with 0.1% collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO) in PBS for 30 min at 37°C. The collagenase was inactivated with an equal volume of complete medium, consisting of DMEM supplemented with 10% fetal buffer solution (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Lonza, Biowhittaker), and the infranant centrifuged at 800 x g for 10 min. The remaining erythrocytes were swelled in lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 5 min at room temperature. Swelling was blocked by adding the complete medium and then the sample was centrifuged at 800 x g for 10 min. The pellet was washed with PBS, centrifuged at 800 x g for 5 min, resuspended in complete medium, and filtered through a 100-µm filter. The obtained stromal vascular fraction cells were counted using a Bürker's chamber, excluding unviable cells according to the trypan blue staining.

ADSC culture

Freshly isolated stromal vascular cells are a heterogeneous cell population that enriches for ADSCs during adherent culture condition on plastic. Thus, to increase the relative density of

ADSCs, 1×10^3 cells/cm² were seeded on 75 cm² flasks containing DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen, Calif., USA; normal culture medium), in a humidified atmosphere of 5% CO₂ at 37°C, and allowed to reach 80%–90% confluency. Then, the cells were detached with 0.5 mM EDTA/0.05% trypsin (Gibco, Invitrogen) for 5 min at 37°C and replated on plastic dishes till the first or second confluency, changing the medium twice a week.

ADSCs cardiac muscle commitment

To assess ADSC differentiation towards cardiomyocytes, culture-expanded cells were plated into Petri culture dishes at a density of 1×10^3 cells/cm². Twenty-four h later we added to cell culture a cocktail of several cytokines containing 2.5 ng/ml TGF-β1 (Peprotech, Neuilly sur Seine, France), 50 ng/ml IGF-1 (Peprotech, France), 5 ng/ml BMP-2 (Peprotech, France), 10 ng/ml FGF-4 (Peprotech, France), 100 ng/ml IL-6 (Peprotech, France), 1000 U/ml LIF (Sigma, St Quentin Fallavier, France), 40 nM α-thrombin, 10 ng/ml VEGF-A (Peprotech, France), 10 nM retinoic acid (Sigma), supplemented with 3% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were harvested after 1 and 2 weeks and ADSCs cardiac commitment was assessed by qRT-PCR and immuno-cytochemistry.

An enriched treatment by increasing TGFβ-1 concentration in the GF cocktail was also used to investigate beneficial effects of its enrichment on ADSCs differentiation. Briefly, 10 ng/ml TGF-β1 (Peprotech, France) was used in the enriched GF cocktail instead of the 2.5 ng/ml cited previously.

Effect of ECM molecules on ADSCs cardiac muscle commitment

To assess the ECM molecule effects on ADSC differentiation towards cardiomyocytes, culture-expanded cells were plated into Petri culture dishes at a density of 1×10^3 cells/cm², each dish having been coated with 2.5 µg/cm² FN (Sigma), 1 µg/cm² LM (Sigma) or left uncoated, in normal culture medium. The cocktail of GFs was added to the cell culture 24h later. Cells were harvested after 1 and 2 weeks and ADSC commitment was assessed by evaluating the expression of early and late cardiac markers by qRT-PCR and immunocytochemistry.

The enriched treatment with increased concentration of TGF β -1 (10 ng/ml) in the GF cocktail was also used here.

Formulation of PAMs

The organic solution (667 μ l; 3:1 methylene chloride: acetone) containing 50 mg polymer was emulsified in a poly (vinyl alcohol) aqueous solution (90 ml, 4% w/v) maintained at 1°C and mechanically stirred for 1min (Heidolph RZR2041, Merck Eurolab, Paris, France). After addition of 33 ml of deionized water and stirring for 10 min, the resulting o/w emulsion was added to deionized water (167 ml) and stirred at 550 rpm further for 20 min to extract the organic solvent. Finally, the formed microparticles were filtered on a 5 μ m filter (HVLP type, Millipore SA, Guyancourt, France), washed with 500 ml of deionized water and freeze-dried. In order to obtain PAMs presenting the biomimetic surface, the microspheres were coated with 12 μ g/mL poly-D-lysine and 18 μ g/ml FN or LM, stirred for 90 min at 15 rpm in an incubator, freeze-dried, and stored at 4 °C for the in vitro experiments.

Carrier effect of PAMs on ADSCs cardiac muscle commitment

Culture-expanded ADSCs were harvested and cultured under differentiating conditions with PAMs presenting either a FN (FN-PAMs) or a LM (LM-PAMs) biomimetic surface. Briefly, 0.5 mg of FN-PAMs or LM-PAMs were incubated with 2×10^5 ADSCs without any agitation in a 24-well ultra low attachment plate (Costar), in DMEM supplemented with the cocktail of GFs described above. Cardiomyogenic commitment was assessed by qRT-PCR and immunocytochemistry.

Primers design and validation

A panel of cardiac genes, the transcription factors GATA binding protein 4 (GATA4), myocyte enhancer factor 2C (MEF2C), NK2 homeobox 5 (Nkx2.5), the sarcomeric ATPase, Ca⁺⁺ transporting, cardiac muscle, slow twitch 2, variant a (SERCA2a), the cardiac myosin light chain 2 (MLC-2V), the cardiac troponin I (cTnI), and the gap junction protein alpha 1, connexin 43 (CX43/GJA1) have been used to study ADSCs mRNA levels during the commitment (Table 1, Primers ordered from Eurogentec, Angers, France). The following

experimental details were performed following the guidelines of the SCCAN core facility (“Service Commun de Cytométrie et d’Analyse Nucléotidique”, Angers, France). Human sequences were determined using PubMed nucleotide search (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) websites. The online freeware Primer blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was used for primer modeling, clustalw (www.ebi.ac.uk) to align nucleotidic sequences, and nucleotide blast (www.ncbi.nlm.nih.gov) to confirm the specificity of the defined primer sequences. When possible, pairs of primers were designed across intron-spanning regions to avoid genomic DNA contamination. Sense and antisense desalted primer pairs (Eurogentec, Angers, France) were mixed in RNase free water at a final concentration of 5 mM and validated using cDNA from human adult or foetal heart and commercial qPCR Human Reference cDNA (Clontech, Takarabio, Saint-Germain-en-Laye, France). The melting peak of the amplicon had to be narrow and unique, and its size and specificity were confirmed by electrophoresis. Finally, a serial dilution of the PCR product was reamplified to draw a linear curve $C_t = f(\text{Quantity})$. The efficiency of the primer was calculated from the slope of the linear curve: $E = [10^{(-1/\text{slope})} - 1] \times 100$. Only primer pairs with an efficiency greater than 80% were validated for use (Table 1).

Real-time Quantitative PCR

ADSCs were detached using trypsin-EDTA (Sigma) and washed in DPBS. Following the manufacturer’s guidelines, cells were lysed in a 1% β -mercaptoethanol containing buffer and RNA extracted following a treatment by DNase to remove any traces of genomic DNA (Total RNA isolation Nucleospins® RNA II, Macherey Nagel, Hoerd, France). First strand cDNA synthesis was performed with a Ready-To-Go You-Prime First-Strand Beads® kit in combination with random hexamers (Amersham Biosciences, Orsay, France) using 1 μ g RNA according to the manufacturer’s guidelines. Following first-strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France) and eluted in 50 μ L RNase free water (Gibco). Five μ L of cDNA (1:20) was mixed with iQ SYBR Green Supermix (Biorad) and primer mix (0.2 mM) in a final volume of 15 μ L. Amplification was carried out on a Chromo4 thermocycler (Biorad) with a first denaturation step at 95°C for 3 min and 40 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 15 s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. A mean cycle threshold value (C_t) was obtained from 2 measurements for each cDNA. Several housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH,

NM_002046), hypoxanthine phosphoribosyltransferase 1 (HPRT1, NM_000194), b-actin (Actb, NM_001101), 30S ribosomal protein S18 (Rps18, NM_001093779) and heat shock 90 kDa protein 1b (Hspcb, NM_007355) were tested and the three best ones chosen for normalization. The relative transcript quantity (Q) was determined by the D cT method $Q = E^{(Ct_{\text{min}} - Ct_{\text{sample}})}$, where E is related to the primer efficiency (E=2 if the primer efficiency=100%). Relative quantities (Q) were normalized using the multiple normalization method described in Vandesompele et al. (2002).

Immunofluorescence

Uninduced and induced cells were used for GATA4, Nkx2.5, MEF2C, cTnI and CX43 immunocytofluorescence. After washing the slides three times with DPBS, cells were fixed with 4% paraformaldehyde at 4°C for 15 min and then permeabilized with 0.2% TritonX 100 (Sigma) for 5 min. Slides were blocked with DPBS, 10% normal goat serum (Sigma), 4% bovine serum albumin (BSA) (Sigma) at room temperature for 45 min. After washing, slides were incubated overnight at 4°C with goat anti GATA4 (1:100, Abcam, Paris France), mouse anti-Nkx2.5 (1:200, Abcam), mouse anti-cTnI (1:100, Abcam), rabbit anti-MEF2C (1:100, Abcam) and rabbit anti-CX43/GJA1 (1:100) in DPBS, 4% BSA, 0.2% TritonX-100. Isotypic controls were made with IgG1k (cloneMOPC-31C, #557273, BD Biosciences) and IgG2bk (clone27 35, #555740, BD Biosciences). After rinsing, the cells were incubated with the corresponding secondary biotinylated anti-mouse or anti-goat or anti rabbit antibody (1:200, Abcys) in DPBS, 4% BSA, 0.2% TritonX-100 for 1 h. Finally, after rinsing again and following incubation with streptavidin-FITC (1:500, Dako) or streptavidin-rhodamin (1:500, Dako) in DPBS for 40 min, the slides were mounted (Mounting Media, Dako) and observed with a fluorescence microscope (Axioscop, Carl Zeiss, LePecq, France).

Table 1: Human specific cardiac primers

Gene	Full Name	NM accession number	Sequences
GATA4	GATA binding protein 4	NM_002052.3	Fwd: AGATGCGTCCCATCAAGACG Rev: GGAGCTGGTCTGTGGAGACT
MEF2C	Myocyte enhancer factor 2C	NM_001131005.2	Fwd: CTAATCTGATCGGGTCTTCCTTCAT Rev: TTTTCTCCCATAGTCCCG
Nkx2.5	NK2 homeobox 5	NM_004387.3	Fwd: CTATCCACGTGCCTACAGCG Rev: GCCGCTCCAGTTCATAGACC
SERCA2a	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2 (ATP2A2) variant a	NM_001681.3	Fwd: ACCTGGAACCTGCAATACTGG Rev: TGCACAGGGTTGGTAGATGTG
MLC-2V	Myosin light chain 2, cardiac, slow	NM_000432.3	Fwd: GATGGAGCCAATTCCAACGTG Rev : ACGTTCACACGCCCAAGAG
cTnI	Troponin I type 3 (cardiac)	NM_000363.4	Fwd CCTGCGGAGAGTGAGGATCT Rev: CAGTGCATCGATGTTCTTGCG
CX43	Gap junction protein alpha 1, 43kDa	NM_000165.3	Fwd: TCTGAGTGCCTGAACCTTGCC Rev: CACCTTCCCTCCAGCAGTTG

Results

ADSC commitment with the cocktail of cytokines

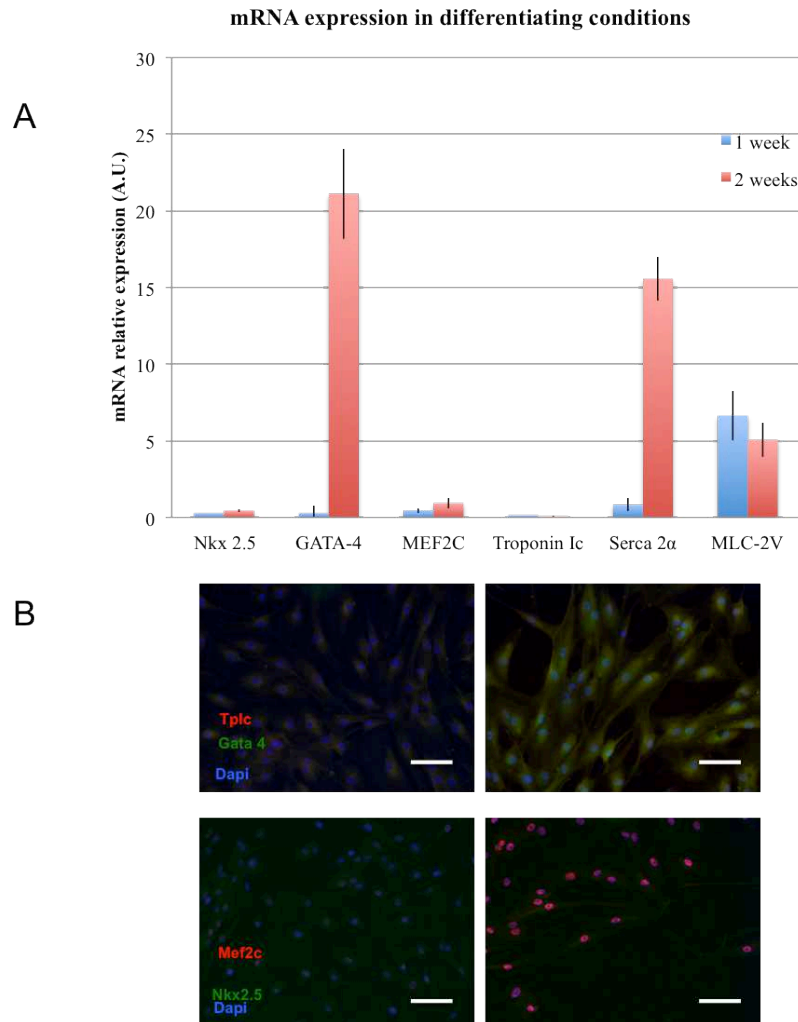


Figure 1: GF cocktail enhanced cardiac transcription factors expression of ADSC. Using real time quantitative PCR (RT-qPCR), we observed that the mRNA of the sarcomeric protein MLC-2V was expressed in ADSCs cultured on uncoated dishes with the cocktail of growth factors after 1 and 2 weeks. The mRNA expression of the transcription factors gata-4 and the other sarcomeric protein Serca2a was also observed at 2 weeks. The expression of the transcription factor gata-4 (FITC) and mef2c (rhodamin) was also observed by immunofluorescence.

Immunocytofluorescence for cardiac troponin-I (rhodamin) and gata-4 (FITC) in ADSCs cultured without GF cocktail for 2 weeks (B), with GF cocktail for 2 weeks (C). Immunocytofluorescence for Nkx2.5 (FITC) and Mef2c (rhodamin) in ADSCs cultured without GF cocktail for 2 week (D), with GF cocktail for 2 weeks (E). All cell nuclei were counterstained with DAPI (blue). Scale bar represent 100μm

Results

ADSC cardiac commitment with the cocktail of GFs

We first studied the transcription factors GATA4 and Nkx2.5 that initiate the differentiation pathway toward the cardiomyocyte lineage (Naito, Tominaga et al. 2003; Yamada, Sakurada et al. 2007; Armiñán, Gandía et al. 2009). After the pre-treatment of ADSCs with the GFs cocktail, Nkx2.5 was not expressed at the two time-points examined (1 week and 2 weeks), while GATA4 was only highly expressed at 2 weeks (21 fold increase compared to the first week) (fig 1A). Although we did not observe the expression of MEF2C, which may be induced by these factors, the mRNA expression of the contractile proteins MLC-2V and SERCA2a was observed, the latter being particularly expressed at 2 weeks (15 fold) (fig 1A). Interestingly, the double immunofluorescence analysis showed that GATA4 protein was expressed after 2 weeks. Moreover, MEF2C protein was observed within the cell nucleus even though no mRNA was found, probably due to a transitory expression of its mRNA (Fig 1B).

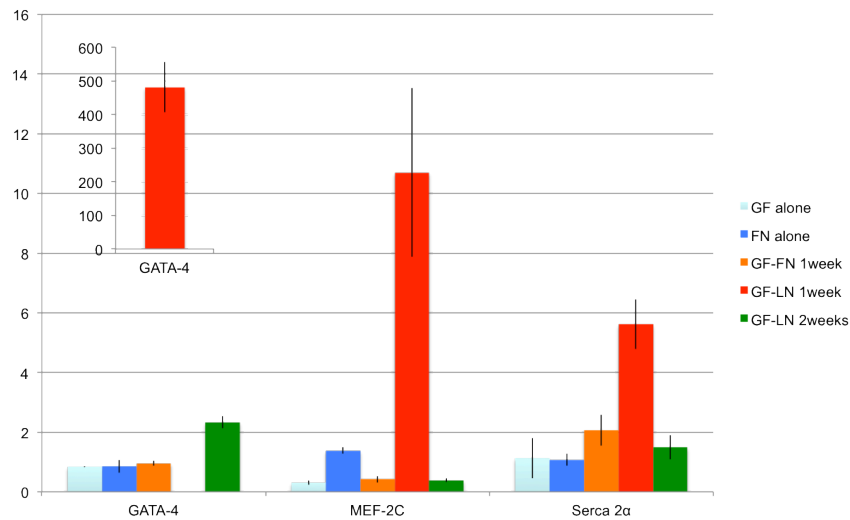


Figure 2: Laminin (LM) enhanced the extent of ADSC cardiac markers expression in combination with GF cocktail. Using real time quantitative PCR (RT-qPCR), we observed that the mRNA of the sarcomeric protein serca2a was expressed in ADSCs cultured on GF-FN and GF-LM conditions after 1 week. The transcription factors gata-4 and Mef2c were both expressed in GF-LM conditions the first week while only gata-4 was expressed at 2 weeks.

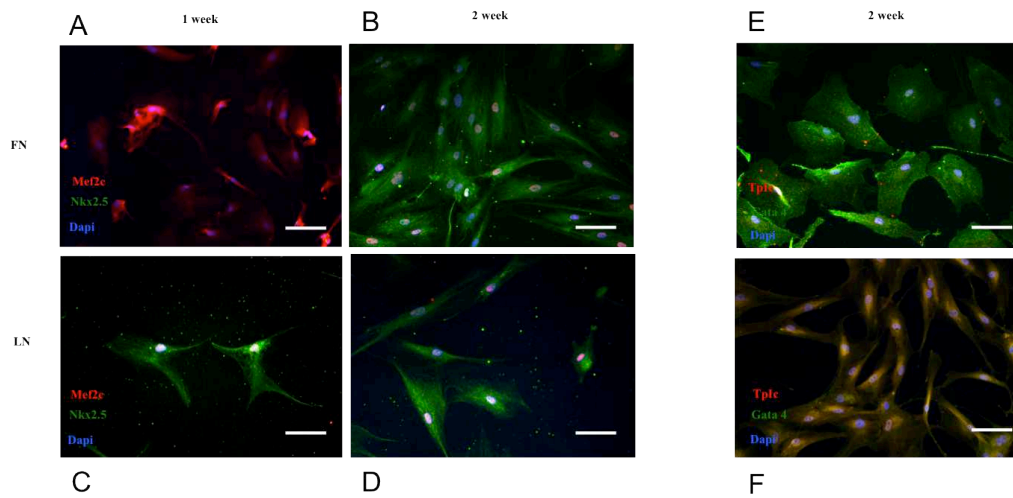


Figure 3: LM enhanced better than FN the extent of ADSC cardiac markers expression in combination with GF cocktail. Using immunocytofluorescence, we observed that ADSCs cultured for 1 week on FN expressed cytoplasmic MEF2C (rhodamin) (A) while its expression was nuclear with laminin (C), the transcription factor was translocated to the nucleus the second weeks with the FN and is maintained in the nucleus with LM (respectively B, D). The transcription factor NKX2.5 (FITC) was only expressed at 2 weeks with FN (B) while it was already expressed with LM the first week (C). GATA4 (FITC) was expressed on GF-LM and GF-FN cells at 2 weeks as observed (E, F) and the cTnI (rhodamin) was only expressed in GF-LM (F). The blue colour indicates cell nuclei. Scale bar represents 100µm

ECM molecules further induce ADSC cardiac commitment

To assess the influence of LM and FN in combination with the GF cocktail on the cardiac commitment of ADSCs, the mRNA expression of several cardiac markers was studied after 1 and 2 weeks of treatment. At 1 week we did not observe any Nkx2.5 expression (data not shown), nor significant changes in GATA4 mRNA levels with FN, LM or GFs alone or with the combination of GF-FN. Importantly, GATA4 was very highly expressed the first week in the GF-LM condition (450 fold compared to the other conditions at 1 week), suggesting an early engagement towards a cardio-muscular lineage. Furthermore, at the same time-point, the mRNA of MEF2C (10 fold) and SERCA2a (6 fold) were expressed in the same conditions. At two weeks only a weak presence of GATA4 mRNA was still observed, suggesting a transitory expression of these latter factors (Fig 2).

The double immunostaining revealed that after 1 week in differentiating conditions, only the transcription factor MEF2C was expressed and was located in the cytoplasm when cells are cultivated on a FN substrate supplemented with the GF cocktail (Fig 3A). Interestingly, at 2 weeks in the same conditions Nkx2.5 was found in the cytoplasm, GATA4 was highly expressed and a nuclear translocation of MEF2C was observed (Fig 3B, 3E). At this time-point however, cTnI, which normally appears later in the differentiation process of cardiomyocytes, was not found (Fig 3E). On the contrary, when cells were cultured on a LM coating, GATA4 together with Nkx2.5 were found at both time-points in the cytoplasm (3C, 3D). In addition, MEF2C was already observed in the nucleus of some cells the first week and remained in the same compartment at 2 weeks in the majority of the cells (Fig 3C, 3D). Moreover, using the LM substrate, cTnI was observed at 2 weeks (Fig 3F), suggesting that this ECM molecule better induces the cardiogenic differentiation of ADSCs. Finally, on the FN substrate a higher number of cells was observed at 2 weeks when compared to cells on the LM substrate.

TGF- β 1 increases cardiac markers expression in ADSCs

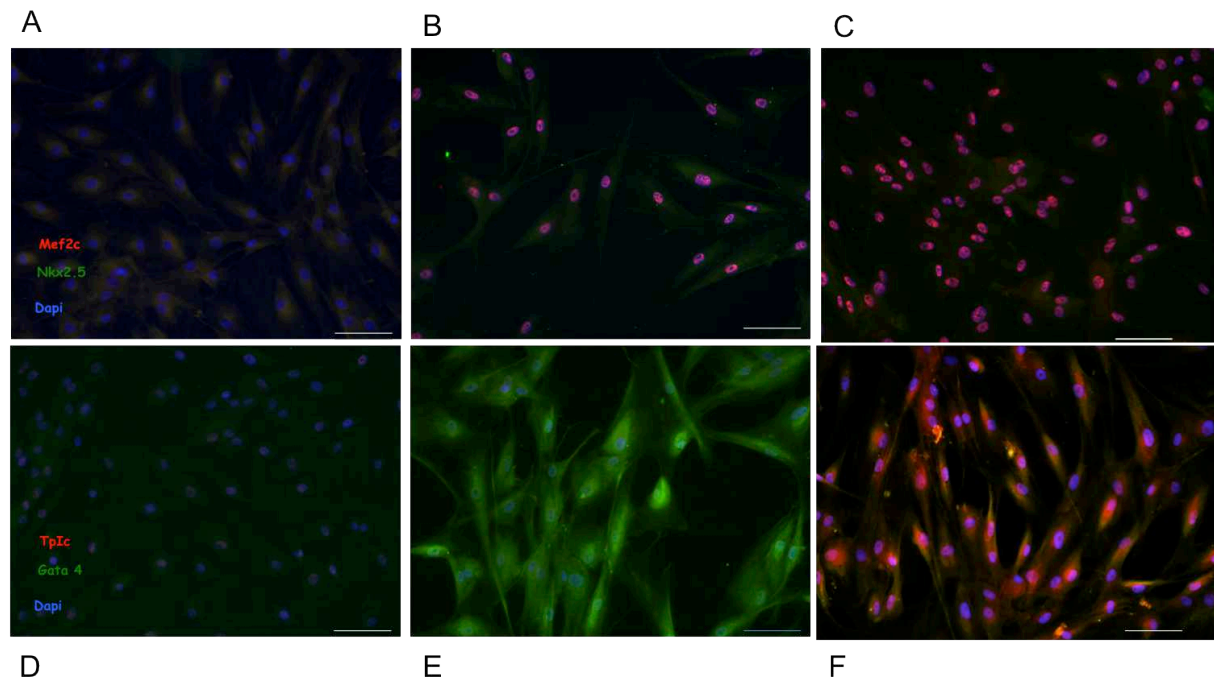


Figure 4: TGF- β 1 potentiates ADSC cardiac markers expression. Using immunofluorescence we observed that without cocktail, no protein expression was observed (A, D). We observed that GATA4 (FITC) and MEF2C (rhodamin) were expressed after two weeks with the TGF- β 1 cocktail. MEF2C is localized in the nucleus and some GATA4 positive cells expressed it within the nucleus (B, E). GATA4 immunostaining is higher with TGF- β 1 cocktail than GF cocktail (compared to Fig 3E and 3F). Combining TGF- β 1 cocktail to LM, cTnI (rhodamin) was expressed and MEF2C expression was increased as observed after 2 weeks (C, F). The blue color indicates cell nuclei. Scale bar represent 100 μ m

TGF β -1 induces ADSC differentiation into immature cardiomyocytes

Since the TGF family is strongly involved in cardiomyogenesis, we hypothesized that TGF β 1 would have improved the cardiac commitment of our cells as suggested by the literature (Lim JY, Kim WH et al. 2007; Li TS, Komota T et al. 2008 ; Gwak SJ, Bhang SH et al. 2009; Kawaguchi N. 2011.). To assess the influence of the increase in TGF β 1 concentration on ADSC differentiation, the protein expression of several cardiac markers was studied after 1 and 2 weeks of treatment with the GF cocktail enriched in TGF β 1 (GF-TGF β 1). As illustrated in Fig 4, at two weeks we observed a high expression of GATA4 and MEF2C, while their cofactor Nkx2.5 was slightly expressed when cells were cultured only with the cocktail supplemented with TGF β 1 (Fig 4B and Fig 4E compared to 4A and 4D controls). Interestingly, some cells expressed GATA4 within their nucleus as well as MEF2C indicating a nuclear translocation of GATA4 from the cytoplasm to the nucleus (Fig 4E), but we did not find cTnI, the marker of a more mature differentiating state, suggesting that TGF β 1 would increase the expression of the transcription factors and participate to the commitment through the induction of their nuclear translocation (Fig 4E). When we combined TGF β 1 with LM, which seems to be more appropriate than FN, MEF2C and cTnI were highly expressed, whilst GATA4 was very slightly expressed and Nkx2.5 not expressed at all (Fig 4C and 4F). Interestingly, a morphological change was observed when ADSCs were cultured with the TGF β 1 cocktail in combination with LM, passing from a broader cell body to a more elongated shape, suggesting that LM better induces the cardiogenic differentiation of ADSCs in combination to the TGF β 1 cocktail (Fig 4F).

PAMs potentiate ADSC cardiac muscle commitment

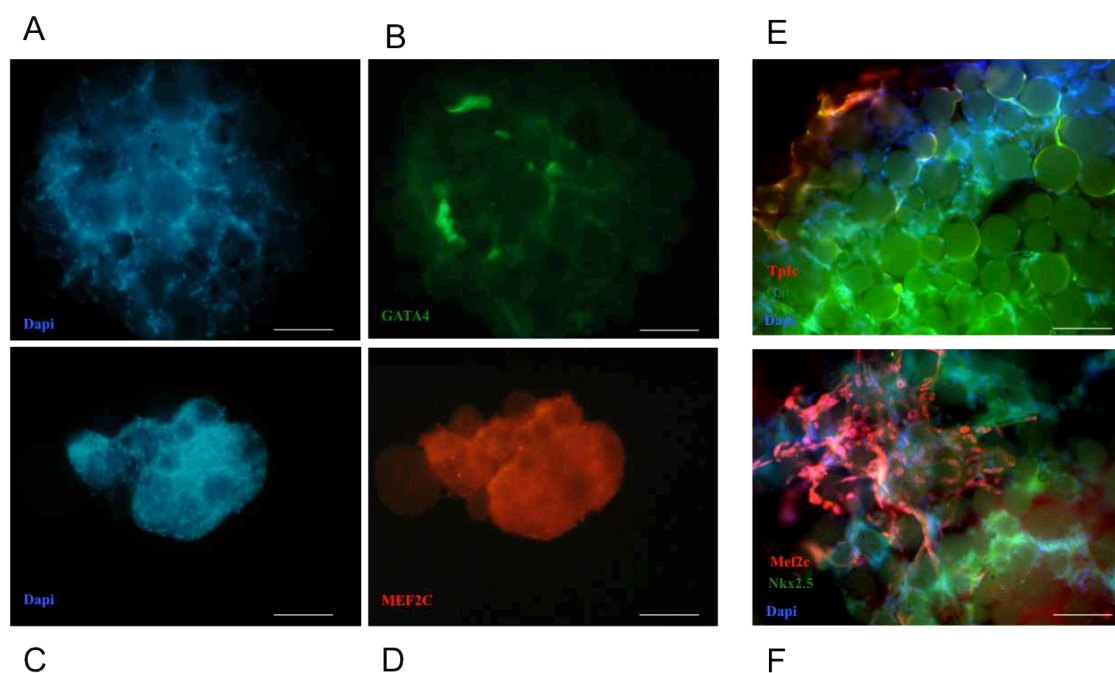


Figure 5: The expression of the transcription factor GATA4 (FITC) and MEF2C (rhodamin) was also observed by immunofluorescence after 1 week on FN-PAM (C, D) and LM-PAM (E, F). Troponin Ic and nkx2.5 were also found on ADSCs cultured on LM-PAM after 1 week with GF as observed. Scale bar represent 100µm.

	Condition	Nkx2.5	Gata 4	Mef2C	TpIc
2D	Cells alone	-	-	-	-
	FN 2.5µg/cm ² coating	-	-	-	-
	LN 1µg/cm ² coating	-	-	-	-
	Cocktail GF +FN 2. FN 2.5µg/cm ² coating	-	+-	+	-
	Cocktail GF +LN 1µg/cm ² coating	+	+-	+	-
3D	Cocktail GF + PAM with a FN biomimetic coating	ND	+	++	ND
	Cocktail GF + PAM with a LN biomimetic coating	+-	++	++	+-

Table 2: The table shows a comparative protein expression between the 2D and 3D culture condition. We observed that in both 3D culture conditions, proteins expression of the transcription markers GATA4 and MEF2C were significantly higher than 2D culture condition and also for cTnI. The blue staining indicates cell nuclei. Abbreviation, ND: Not done

PAMs potentiate ADSC cardiac muscle commitment

To further provide a 3D structure to the cells we cultured ADSCs on PAMs with a LM or FN biomimetic surface for 1 week in combination with the cocktail of GFs or with that enriched in TGF β 1. First, the protein expression profiles of cardiac markers by ADSCs on FN-PAMs and LM-PAMs in GF cocktail only were compared (Fig 5). We verified that on 3D support, FN did not have a better effect than LM (Fig 5E and 5F compared to 5B and 5D). Table 2 summarizes ADSC protein expression and offers a comparative protein profile between the 2D and 3D culture conditions after 1 week. We observed that in both 3D culture conditions, protein expression was significantly higher than in 2D. For Nkx2.5 expression, no differences were observed between the 2D and 3D culture conditions. Moreover, Nkx2.5 was only observed in cells on a LM substrate. Interestingly GATA4 and MEF2C were more expressed in 3D than in 2D culture conditions. When the 3D conditions were compared between them, we observed that LM-PAMs better induced cardiac differentiation of ADSCs than FN-PAMs as illustrated by the expression of cTnI.

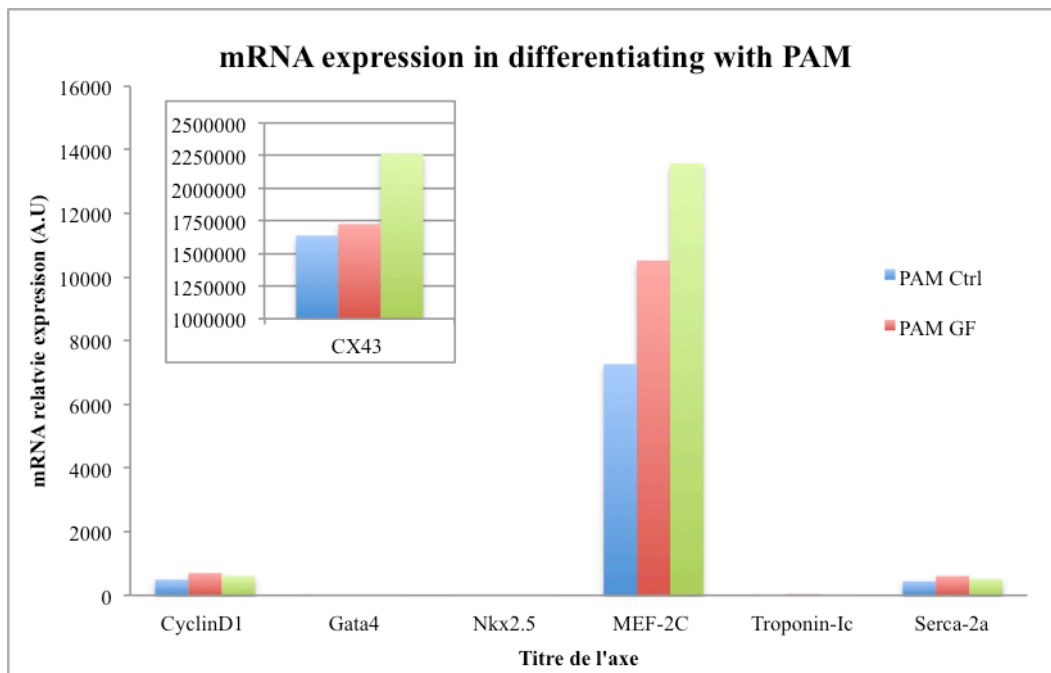


Figure 6: Relative mRNA expression of cardiac markers in ADSCs cultured on laminin coated PAM with the cocktail of Growth factors after 1 week. No significant expression was observed with SERCAa and cyclin D1. mRNA levels of the transcription factor MEF2C were increased with TGFb1 cocktail as well as the mRNA levels of the gap-junction protein CX43.

After having confirmed that a LM biomimetic surface is more appropriate for the ADSC commitment on PAMs, we then studied the effect of the GF-TGF β 1 cocktail in combination with LM-PAMs. The expression of Cyclin D1 was also studied to evaluate whether the cells progress in cell cycle and proliferate rather than differentiate. We obtained similar mRNA expression of Cyclin D1 and SERCA2a but no significant difference in both control (PAMs only) and differentiating conditions (PAMs with GF or PAMs with GF-TGF β 1). The mRNAs of GATA4, Nkx2.5 and cTnI were not detected after 1 week in all conditions. On the contrary, at the same time-point MEF2C expression was significantly higher with PAMs in GF-TGF β 1 conditions when compared to PAMs with the GF cocktail only (1,400 compared to 1,000). Additionally, the mRNA of CX43 was also highly expressed with the GF-TGF β 1 cocktail compared to the other conditions (2,250,000 compared to 1,750,000 for GF-PAMs).

Concerning protein expression, interestingly, GATA4, MEF2C, Nkx2.5, cTnI and CX43 were all expressed when ADSCs were cultured on LM-PAMs with the GF-TGF β 1 cocktail at 1 week (Fig 7A-F). Surprisingly, cell proliferation was observed at the centre of the PAM-ADSCs complexes where KI67 -positive cells were found (Fig 7G). Due to its 3D conformation, a confocal microscopy observation was performed to know how the late cardiac differentiation marker-expressing cells were distributed and organized within the PAM-ADSCs. Interestingly, the results revealed that cTnI was only expressed at the surface of the PAM-ADSCs complexes while CX43 was also found within the complexes (Fig.8).

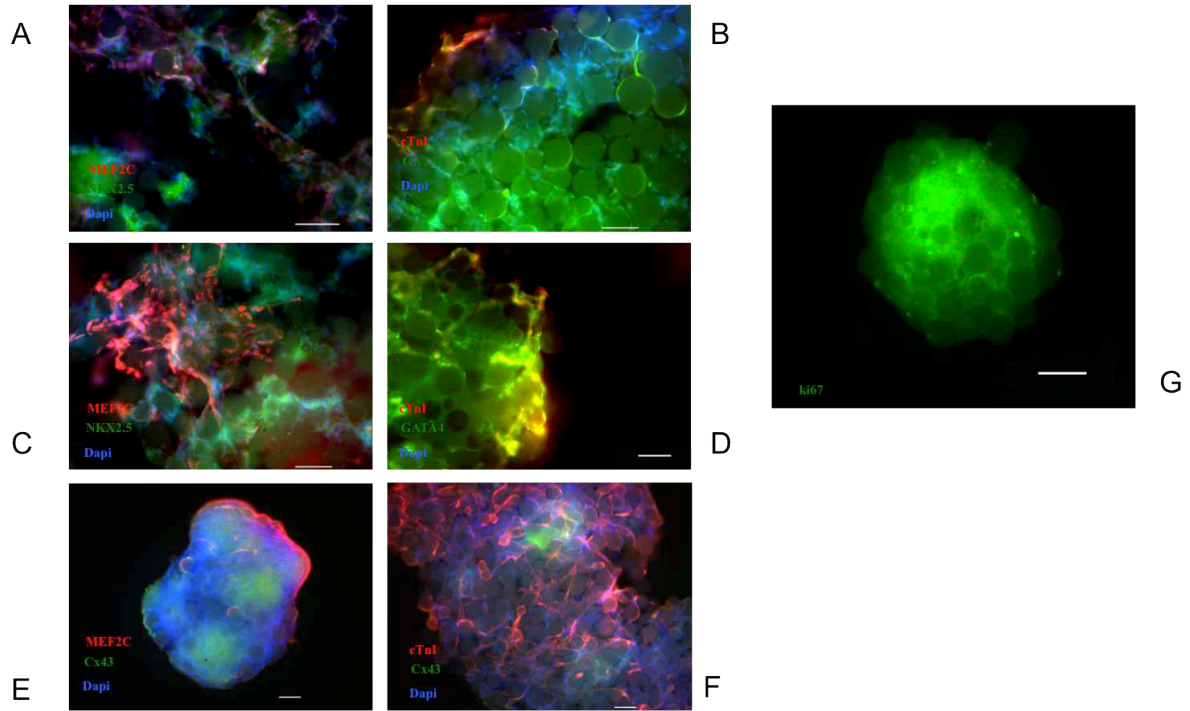


Fig 7: Using immunofluorescence, we observed that the transcription factors NKX2.5 (FITC), GATA4 (FITC) and MEF2C (rhodamin) were expressed in GF-LM-PAM and TGFb1-LM-PAM (respectively A, C, B, D). Although cTnI (rhodamin) is expressed on both ECM-coated -PAM, its expression is higher with LM PAM (D) than FN-PAM (B). The gap-junction protein cx43 (FITC) and the contractile protein TpIc (rhodamin) were also co-expressed (E, F) on LM PAM. The blue staining indicates cell nuclei. Scale bar represent 100µm

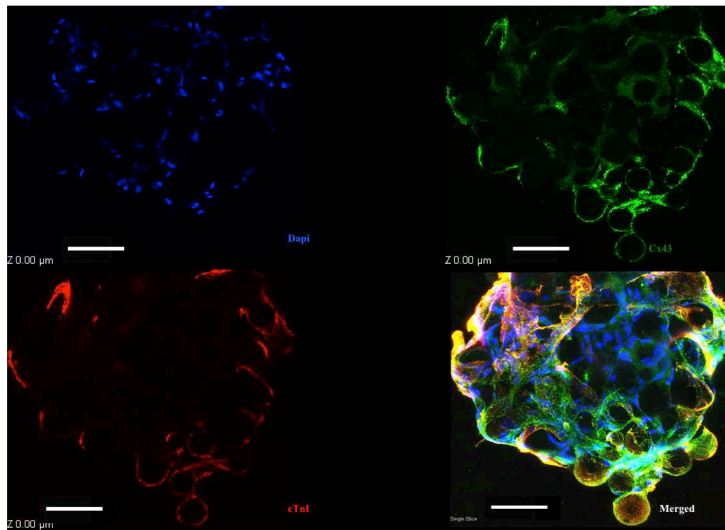


Fig 8 : Confocal observation of ADSC-PAM complexes after culture with the growth factor cocktail supplemented with TGF-β1 for 1 week. CX43 positive cells (FITC) and cTnI-positive cells (rhodamin) were localized at the surface of the PAM-ADSCs complexes as observed. The blue staining indicates cell nuclei. Scale bar represents 100µm.

Discussion

The use of adult stem cells for cardiac cell therapy provides exciting perspectives to overcome the poor regenerative capacity of the damaged heart. In this regard, beneficial effects were found *in vivo* using freshly isolated ADSCs from the stromal vascular fraction and immature cultured ADSCs. However, the use of undifferentiated stem cells and the possible undesirable differentiation phenotypes that may arise after transplantation requires being cautious with this approach. Most studies have reported their differentiation into beating cardiomyocytes through the use of the DNA demethylating agent 5-azacytidine, which is not likely clinically applicable (Choi, Dusting et al. 2010). This phenomenon was also found co-culturing them with cardiomyocytes, and recently also with cardiomyocyte extracts without cell contact, suggesting that connection with myocytes and probably with ECM molecules and/or secreted factors is important to develop cardiomyocytes from adult stem cells (Metzele, Alt et al. 2011).

In this study, a set of recombinant GFs was first applied to induce ADSC differentiation into immature cardiomyocytes. We observed that the pre-treatment of ADSCs for 2 weeks with the GF cocktail permitted the translocation within the nucleus of MEF2C and enhanced the mRNA level of SERCA2a and MLC-2V, indicating that MEF2C could induce the transcription of SERCA2a and MLC-2V as already reported (Moriscot, Sayen et al. 1997; Leszczynski and Esser 2003). Similar results have been described using MSCs and a cocktail of GFs, as documented by the expression in the cytoplasm of the main transcription factors of the master cardiac genes after 5 days of treatment and their nuclear translocation after 20 days (Behfar, Yamada et al. 2010). In other studies the effect of GFs on MSCs and ADSCs subpopulation (Planat-Benard, Menard et al. 2004; Jumabay, Zhang et al. 2009) differentiation was more pronounced because some end-stage proteins were also expressed and beating cardiomyocytes were even obtained. By contrast, the ADSCs exposed to the GF cocktail in our study showed a less mature cardiac phenotype. It should however be taken into account that in these studies very high percentages of serum were used (20% and 15%, respectively) which probably further enhanced the actual GF cocktail differentiating effect.

ADSC differentiation towards the cardiac lineage was also demonstrated to improve in vitro in the presence of LM (Dijk, Niessen et al. 2008). However, their differentiation was achieved through the administration of 5-azacytidin, which cannot be used in the clinical setting. Furthermore, LM has been described to increase from the third day post-MI (Morishita, Kusachi et al. 1996) but no further studies were performed in vivo to investigate its possible role on cardiac repair. We here showed that the use of a LM substrate combined to the GF cocktail is important for the engagement of ADSCs into the cardiac lineage, as a cascade of events occurred starting from the expression of the earlier cardiac transcription factors Nkx2.5, GATA4 and MEF2C and followed by the expression of one of the targets of MEF2C, the sarcomeric protein cTnI (Kirby and Waldo 2002; Dunwoodie 2007; Chien, Domian et al. 2008; Taubenschmid and Weitzer 2012). Indeed, GATA4 and Nkx2.5 are mutual cofactors considered as the key regulators of cardiomyogenesis (Daniel Durocher 1997), which interact with a third transcription factor MEF2C. These cofactors are necessary to induce the differentiation pathway into cardiomyocytes and the expression of late-stage differentiating proteins such as cTnI. By contrast, we did not observe these steps with FN likely because of the lack of Nkx2.5 and the permanent cytoplasmic localization of MEF2C that did not allow reaching this degree of differentiation.

As TGF β 1, is known to induce the cardiomyogenic differentiation of hMSC and rat ADSCs (Gwak, Bhang et al. 2009), we investigated the effect of higher TGF β 1 concentrations in our GFs cocktail to know whether it could potentiate differentiation. In our study, TGF β 1 was always present in the GF cocktail, but by increasing its concentrations we could potentiate the expression of GATA4 and MEF2C. Interestingly, some cells showed GATA4 within the nucleus indicating its translocation from the cytoplasm. Nevertheless, we did not find either Nkx2.5 or cTnI, suggesting that TGF β 1 could increase the expression of only some transcription factors and participate to cardiac commitment through GATA4 nuclear translocation. Another study, applying a GF cocktail on MSCs in vitro, showed the role of TGF β 1 in increasing Nkx2.5 and MEF2C cytosolic expression but without affecting their nuclear translocation. This work mentioned the implication of IGF-1, which is one of the components of our GF cocktail, in the nuclear translocation of the transcription factors, but the concomitant role of TGF β 1 and IGF-1 in cardiac differentiation needs to be explored. The combination of LM and GFs cocktail supplemented with higher TGF β 1 concentrations, induced the nuclear translocation of GATA4 and MEF2C, as well as the increase in cTnI

expression, but there was no production of Nkx2.5. However, Nkx2.5 and GATA4 can independently participate to cardiomyocytes differentiation, and have the ability to induce cardiac commitment even alone. This could explain why we obtained cTnI expression in the absence of Nkx2.5.

ADSC differentiation before transplantation might be beneficial if a homogenous population of cells can be obtained. However, in cardiac cell therapy the fibrotic scar, the lack of adhesion signals, and the loss of contact between the cells and the ECM molecules do not allow good cell engraftment within the damaged heart. The use of 3D supports covered by ECM molecules appears to be a reliable strategy to deliver cells and tissue-like structures. This approach might increase cell delivery efficiency and reduce cell death, optimizing engraftment efficacy and transplanted cell function. In order to implement the PAMs developed in our laboratory for cardiac cell therapy, we investigated whether these 3D supports could influence this differentiating process in vitro. Others authors demonstrated the role of LM in the cardiac lineage engagement of ESCs in 3D structure. They investigated the effect of 3D constructs and matrix composition on control and guidance of ESCs growth and differentiation in vitro. It was observed that the presence of FN in 3D collagen constructs strongly stimulated endothelial cell differentiation and vascularization, while LM increased the ability of ESCs to give rise to beating cardiomyocytes. Similarly, our results suggested that the 3D condition established by LM-PAMs was better than the 2D standard culture condition to induce the cardiac commitment in ADSCs. This was attested by the expression of GATA4, MEF2C, Nkx2.5 and cTnI with LM-PAMs compared to all the 2D conditions after only 1 week of treatment. Moreover, LM-PAMs were better inducers of cardiac differentiation than FN-PAMs.

By combining the enriched TGF β 1 GF cocktail with LM-PAMs, we found that the mRNA levels of MEF2C and CX43 were increased compared to the GF cocktail LM-PAM conditions. Furthermore, we observed the presence of all the studied transcription factor proteins but also that of more mature cardiac proteins, such as SERCA2a, cTnI and CX43, demonstrating the progression of ADSCs cardiac differentiation. Some ADSCs stained positive for Ki67 indicating that they continued to proliferate, which was confirmed by the presence of cyclin D1. Moreover, the Ki67-positive cells were localized within the PAM-

ADSCs complexes and not at the periphery, suggesting that the pool of potentially growing undifferentiated cells remained at the center of the complexes where there is a poor GF diffusion gradient and also less oxygenation which maintains ADSC “stemness” (Smith, Barile et al. 2007; Leri, Davis et al. 2009). However, this hypothesis needs to be further confirmed. Interestingly, cTnI-positive cells were localized at the surface of the PAM-ADSCs complexes, while an expression gradient was observed for CX43, the cells with the higher expression levels situated also at the periphery as shown by confocal microscopy analysis.

Gap junctional coupling is essential in establishing electrochemical communication between cardiomyocytes. These structures consist of two hexameric assemblies of connexin proteins embedded in the plasma membranes of neighboring cells, thereby forming the so-called connexon hemi-channels. CX43, the major gap junction protein of the working myocardium is involved in cardiac development by transferring cardiomyogenic signals between cardiomyocytes (Saffitz JE, Yamada KA. 1998; Wang Y, Cheng Y. 2009.). Lack of such a protein results in serious cardiac malformation during development and is correlated to the arrhythmic phenomenon encountered following cell transplantation (Danik SB, Liu F et al. 2004 ; Wang Y, Cheng Y. 2009 ; Maass K, Chase SE et al. 2009). Our results suggest that TGFβ1 increased CX43 expression in ADSC-LM-PAMs complexes. The role of TGFβ1 on CX43 expression of smooth muscle cells has been already investigated (Neuhaus, Heinrich et al. 2009). It was demonstrated that TGFβ1 up-regulated CX43 expression and intercellular communication, in concert with the increased expression of α -actin, calponin and smooth muscle heavy chain isoform 1, thus indicating the role of this growth factor in improving CX43-mediated gap-junction communication and enhancing contractile activity (Neuhaus, Heinrich et al. 2009). The presence of CX43-expressing cells in ADSC/PAM complexes represents a further advantage for cell therapy, as it should allow a better ADSCs engraftment and communication with host cells.

In conclusion, these results suggest that ADSCs are able to express both early and late cardiac muscle markers when treated with a cocktail of GFs and LM under 2D conditions. The 3D support offered by PAMs in combination with the LM biomimetic coating and the GFs cocktail improve the biosynthesis of cardiac markers and the supplementation of TGFβ1 potentiates their expression. The presence of CX43 in treated ADSCs also suggests the possibility of communication between these cells. Taken together, these findings let us to

hypothesize that PAMs conveying cardiac committed ADSCs and releasing TGF β 1 may increase, in the post-MI region, their survival and differentiation and favor cell communication with hosted cells, allowing a better process of myocardial regeneration and recovery of cardiac function.

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Conclusion

In cardiac cell therapy studies using ADSCs it is commonly acknowledged that it is important to control as much as possible their behavior after transplantation into the ischemic heart in order to avoid undesirable effects. The transplantation of ADSCs adhered to PAMs capable of inducing or maintaining ADSC commitment towards the cardiomyogenic lineage is therefore a potential interesting strategy.

After having investigated the effect of a GF cocktail on ADSCs commitment, we have demonstrated that the ECM molecule LM further improves GF-induced cell differentiation towards the cardiac lineage through the expression of transcription factors GATA4, NKX2.5 and MEF2C, but also through the expression of a late differentiation stage protein, the sarcomeric protein cTnI. Although the LM effect on ADSCs differentiation into cardiomyocytes has already been evaluated (van Dijk A, Niessen HW et al. 2008), this study is one of the first combining ECM molecules and GFs to improve ADSC commitment *in vitro* with a low serum percentage.

Interestingly, independently of the surrounding biomimetic ECM coating, the 3D support offered by PAMs further increases and accelerates the differentiation. Additionally, LM-PAMs are better than FN-PAMs to induce ADSCs differentiation through cardiomyogenic lineage as demonstrated by the expression of the contractile sarcomeric cTnI protein.

Moreover, the addition to the GF cocktail of TGF β 1, which is strongly implied in cardiomyogenesis, potentiates the differentiation in 2D as well as on PAM- 3D complexes. Finally ADSCs adhered on PAMs adopt a similar organization as that of cardiospheres, with a pool of poorly differentiated cells and proliferating cells expressing ki67 at the center of the complexes and more mature differentiated cells expressing the contractile protein cTnI and connexin 43 at the surface (Smith, R. R., L. Barile, et al. 2007; Davis DR, Kizana E et al 2010, Davis DR, Zhang Y et al. 2009).

These results suggest that the use of PAMs offering a LM biomimetic surface may represent an efficient tool for applications of committed adult stem cells in tissue-engineering strategies of damaged organs and may be investigated as an alternative approach to the use of cardiospheres constituted by CPCs. Growth factors released *in situ* from PAMs could further potentiate or maintain this differentiation state *in vivo*.

Chapter 3

Pharmacologically active microcarriers as
growth factor releasing biomimetic 3D
scaffold for cardiac tissue engineering

Scientific context

Combining ADSCs with PAMs releasing a GF for a prolonged period is of real interest for tissue engineering. Concerning cardiac repair, the PAMs can constitute an injectable scaffold that offer a 3D biomimetic support for ADSCs adhesion. Furthermore, they could continuously release a GF that could induce or maintain ADSCs differentiation into cardiac lineage over time while modulating the ischemic environment through angiogenesis or endogenous cell recruitment or proliferation. Moreover, we have shown that PAMs coated with LM further induce ADSCs commitment into cardiomyogenic lineage compared to the 2D conditions in combination with a cocktail of GFs. Although the choice of the LM as the biomimetic coating has been performed, the choice of the GF to encapsulate within the PAMs still remains to be defined. We have thus chosen HGF a pro angiogenic (Ruvinov, Leor et al. 2010) and anti fibrotic factor involved in CPCs recruitment (Urbanek 2005) and MSCs differentiation into cardiomyocytes (Forte, Minieri et al. 2006; Deuse, Peter et al. 2009). Another GF widely used for cardiac repair due to its anti-apoptotic and pro-survival as well as its proliferative properties, in particular on endogenous CPCs, is IGF-1 (Urbanek 2005; Davis 2006; Suleiman, Singh et al. 2007; Hahn, Cho et al. 2008; Padin-Iruegas, Misao et al. 2009). Finally VEGF, a potent angiogenic factor also presenting proliferative and anti-apoptotic properties (Hughes, Biswas et al. 2004; Sadat, Gehmert et al. 2007; Song, Gehmert et al. 2007; Pons, Huang et al. 2008; Zisa, Shabbir et al. 2009; Zisa, Shabbir et al. 2009; Silva and Mooney 2010; Miyagi, Chiu et al. 2011), was also chosen for PAM formulation.

The destabilization of the protein within the microsphere or during their release limits their exploitation (Giteau, Venier-Julienne et al. 2008; Paillard-Giteau, Tran et al. 2010). Modification of bioactivity could be due to undesirable effects, i.e. protein aggregation, not enabling protein recognition by the organism. We and others, have developed strategies to overcome these problems and protect the protein, such as protein nanoprecipitation (Giteau, Venierjulienne et al. 2008). For protein encapsulation, we here use the s/o/w formulation technique developed in our laboratory, based on the suspension of nanosolid proteins in an organic solvent. Additionally, an additive (poloxamer 188) is co-precipitated with the protein and these nanoparticles are then encapsulated. In this way, the protein structure is preserved by limiting its adsorption onto the organic phase, and the amount of active protein released is increased (Giteau, Venierjulienne et al. 2008). In this study, several parameters such as

concentration, ionic strength, pH or protein/additive ratio, have first been investigated using model proteins to develop the precipitation conditions of IGF-1, VEGF and HGF. After formulating the PAMs with the GF and functionalizing the surface of PAMs, the protein release profiles from PAM were studied.

To favor PAM retention within the beating infarcted heart, combining them with an injectable in situ thermosensitive HG is a valuable approach. Moreover, the HG could provide appropriate mechanosensitive cues by changing the reticulation state and thus the stiffness of the material to modulate the differentiation profile of the grafted cells as already observed (Young and Engler 2011). Therefore the PAMs were combined to a thermosensitive HG of poloxamer 407 (P407, Pluronic® F127). The release profile of a model protein from the PAMs within the HG and the behavior of the cells have also been studied. Finally, the cardiomyogenic commitment of hADSCs adhered on PAMs releasing IGF-1 and VEGF with and without the HG was also assessed.

Abstract

One challenge of tissue engineering of the infarcted heart is how to improve stem cell engraftment, survival and homing for myocardial repair. We propose to integrate pharmacologically active microcarriers (PAMs), a 3 dimensional (3D)-carrier of cells and growth factors (GF), into a viscoelastic thermosensitive injectable hydrogel (HG) of poloxamer 407 (P407) to obtain a system that stimulates the survival and/or differentiation of the grafted cells toward a cardiac phenotype. PAMs are biodegradable and biocompatible poly (lactic-co-glycolic acid) (PLGA) microspheres conveying cells on their 3D biomimetic surface, that deliver continuously and in a controlled manner a growth factor (GF) acting on the transported cells and on the microenvironment to improve engraftment. The choice of the appropriate GF and its protection during the formulation process and delivery is essential. In this study, 3 GFs have been encapsulated under a nanosolid state that limit their interaction with the polymer and conserve their structure and integrity, vascular endothelial growth factor VEGF, hepatocyte growth factor (HGF) and insulin-like growth factor (IGF-1). To formulate PAMs-GF, precipitation conditions of each GF and the release profile have been first investigated. The released IGF-1 and HGF induced human adipose-derived stem cell (ADSC) expression of cardiac differentiation markers GATA4, MEF2C, NEK2.5, cTnI and CX43 after 1 week in vitro. Integrating PAMs with P407 HG decreased the protein release profile, improved ADSC survival and increased the elastic properties of the PAM-HG complexes. Finally, cardiac markers were expressed and the gap junction protein CX43 expression was increased in ADSCs-PAMs-GFs complexes embedded within the HG. These results suggest that the combination of particulate and HG scaffolds may be beneficial for applications combining adult stem cells, GF delivery, ECM molecules and substrate elasticity in tissue-engineering strategies for myocardial infarction.

Key words: Adipose-derived stem cells, growth factors, hydrogel, pharmacologically active microcarriers, myocardial infarction.

Pharmacologically active microcarriers as growth factor releasing biomimetic 3D scaffold for cardiac tissue engineering

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Introduction

Heart failure constitutes one of the first causes of morbidity in developed countries. Cell-based strategies have emerged as an alternative way of achieving cardiac repair, and a great deal of attention has been placed on finding the best cell-type to repair injured tissue (Segers and Lee 2008; Boudoulas and Hatzopoulos 2009; George 2010; Marban and Cheng 2010; Forte, Chimenti et al. 2011; Menasche 2011). Beneficial effects on cardiac remodeling have been obtained with adult stem cells on animal models of MI, leading to clinical studies that have demonstrated their safety and benefits on left ventricular function (Bolli, Chugh et al. 2011; Menasche 2011; Makkar, Smith et al. 2012). Additionally, besides their large differentiating potential and secretion of factors involved in tissue repair, their easy accessibility and immunoregulatory function render these cells as interesting candidates for cardiac cell therapy. However, the efficiency of stem cell transplantation is limited by low cell retention, survival and differentiation, as the number of functional surviving cells following injection is proportional to the functional improvement. Indeed, regardless of the type of cell used, 90% of the cells die within the following 24h after injection in the ischemic myocardium. Several factors play a role in this early cell death after engraftment such as the inflammatory reaction, the absence of survival factors, and the loss of contact between the cells and the extracellular matrix (ECM) molecules.

Growth factors (GF) such as insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) injected alone or together with cell grafts, are able to overcome the inhospitable ischemic environment and induce myocardial regeneration (Post, Laham et al. 2001; Forte, Minieri et al. 2006; Sadat, Gehmert et al. 2007;

Pons, Huang et al. 2008; Deuse, Peter et al. 2009; Padin-Iruegas, Misao et al. 2009; Srinivas, Anversa et al. 2009; Zhu, Zhang et al. 2009; Jay, Shepherd et al. 2010; Silva and Mooney 2010). Depending on the cell type grafted they can stimulate their survival, their differentiation or their proliferation . They also induce endogenous cell protection, migration and angiogenesis (Kitta 2002; Laflamme, Chen et al. 2007; Hahn, Cho et al. 2008; Pons, Huang et al. 2008). Nevertheless, it is essential to find the best delivery strategy of these GF and cells in order to obtain appropriate cell engraftment.

An appealing approach for myocardium repair combines cells and drug-releasing three-dimensional scaffolds capable of localized and sustained release of the GF during the degradation of the polymer. In this way, the GF is protected within the scaffold and exerts its beneficial effect on the transported cells and the surrounding environment. Hydrogels (HG) and particulate systems are two possible candidates. HG may mechanically support the beating myocardium due to their elastic properties but usually a short-term protein release is obtained (Tibbitt and Anseth 2009; Wang, Li et al. 2010; Tous, Purcell et al. 2011; Young and Engler 2011). With microspheres a prolonged release of several weeks may be obtained (Sy and Davis 2010) and they may advantageously increase cell–material interactions due to a large specific surface (Yu, Gu et al. 2009; Yu, Du et al. 2010). Furthermore, microspheres covered with a biomimetic surface provide additional cues stimulating the transported cell survival and differentiation (Tatard, Venier-Julienne et al. 2005; Bouffi, Thomas et al. 2010; Delcroix, Garbayo et al. 2011; Musilli, Karam et al. 2012). Indeed, extracellular matrix (ECM) molecules like laminin or fibronectin induce adult stem cell cardiac differentiation and survival, respectively (Pankov 2002; Dijk, Niessen et al. 2008; Dijk, Niessen et al. 2008; Malan, Reppel et al. 2009). Within this line, we have developed pharmacologically active microcarriers (PAMs), which combine in an adaptable and simple device, an implantable 3-dimensional (3D) biomimetic biomaterial-based scaffolds transporting cells and in situ controlled drug delivery. These PAMs are biodegradable and biocompatible, Poly(lactic-co-glycolic) acid (PLGA) microspheres that have been covered with ECM molecules while delivering GFs in a sustained and controlled manner for a prolonged period (Tatard, Venier-Julienne et al. 2005). We have shown that these combined parameters promote or maintain transported cell survival, differentiation and integration in the host tissue after complete degradation of the carrier (Tatard, Sindji et al. 2007; Bouffi, Thomas et al. 2010; Delcroix, Garbayo et al. 2011; Garbayo, Raval et al. 2011).

It has been demonstrated that a sustained release of VEGF and of IGF-1 better induced muscle regeneration, revascularization and gain of function than a single high dose (Borselli, Storrie et al. 2009). In addition, it has been recently reported that a combined delivery of GFs better stimulate neovascularization of ischemic tissues than a single GF therapy (Saif, Schwarz et al. 2010; Banquet, Gomez et al. 2011). However, the prolonged, sustained and complete delivery of proteins remains a technological challenge, which is necessary to overcome for future effective clinical applications. We have shown that proteins encapsulated under a nanosolid state within the PLGA polymer preserve their structure and integrity allowing the release of a bioactive factor (Giteau, Venierjulienne et al. 2008; Tran, Karam et al. 2012). In addition, the co-precipitation of an amphiphilic polymer (poloxamer P188) with the protein further protects it from the hydrophobic environment.

We thus developed PAMs with a biomimetic surface of laminin and engineered to release HGF, IGF-1 and VEGF in order to implement them for cardiac tissue engineering studies. The optimum conditions to obtain reversible particles of these proteins were prior determined with model proteins currently used in the laboratory. After encapsulation, the protein release profile and bioactivity were studied. The differentiation potential of ADSCs into the cardiomyogenic lineage, forming complexes with PAMs, has been evaluated *in vitro*. As we envisage to combine the PAMs within a thermosensitive injectable HG of poloxamer 407 (P407, Pluronic® F127), that may also induce stem cell lineage specification depending of its rheological characteristics, these properties and the protein release profile from PAMs was evaluated. Finally, the viability of MSCs and ADSCs on PAMs with and without the HG was studied.

Material and Methods

Precipitation of the protein

Protein, glycofurol and sodium chloride were used without further purification. Experiments were carried out at 4°C. The overall mixture was prepared directly inside a centrifugation tube as follows. The protein powder was first dissolved in a non-buffered aqueous solution of sodium chloride at given pH, this solution was then introduced into glycofurol. Thirty minutes later, the protein particles were recovered by centrifugation (10,000g, 30 min, 4°C). Mixing and centrifugation times of 30 minutes were selected in order to optimize the quantity of precipitated protein.

Screening of protein precipitation

To define the optimum conditions of precipitation, an experimental design was used. Three parameters influencing protein precipitation were modified: the ionic strength, the pH, and the protein concentration. The precipitation efficiency was considered as the percentage of active protein recovered after precipitation and rehydration. The reference was the initial activity, i.e. the activity measured for the initial protein mass whose activity was 100%. The protein suspensions in glycofurol were centrifuged, the supernatant eliminated and the pellet of protein particles rehydrated.

The measured responses were the precipitation efficiencies of three model proteins, lysozyme, peroxidase and bovine serum albumin (BSA). Lysozyme was rehydrated in TRIS-HCl 0.01M buffer, pH 7.4, peroxidase in potassium phosphate buffer, pH 6.0 and BSA in PBS, pH 7.4. The biological activity of lysozyme was determined by measuring the turbidity change in a *Micrococcus lysodeikticus* bacterial cell suspension as reported by Aubert-Pouëssel et al. (Aubert-Pouëssel, Bibby et al. 2002). The enzymatic activity of peroxidase was determined with pyrogallol as substrate while BSA was measure using micro-BCA dosage method (Pierce, Bezon, France).

Protein encapsulation

Poly (lactic-co-glycolic acid) (PLGA)-microspheres of an average diameter of 60 μm were prepared using a previously described emulsion solvent evaporation- extraction process (Aubert-Pouessel A, Venier-Julienne MC et al. 2004). The total protein loading was 0.6% w/w of the amount of polymer, i.e. 0.1% HGF, IGF-1 or VEGF₁₆₅ (VEGF-A) (Peprotech, France) and 0.5% HSA.

The proteins were co-precipitated with poloxamer 188 as additive in a ratio additive/protein 20:1. After 30 min (at 4°C) the protein particles were recovered by centrifugation (10,000g, 4°C, 30 min), carefully dispersed in the organic solution containing 50 mg of polymer. This organic suspension was emulsified in a poly (vinyl alcohol) aqueous solution (30 mL, 4% w/v) at 1°C and mechanically stirred at 550 rpm for 1 min. Then, deionised water was added to the resulting o/w emulsion to extract the organic solvent. The microsphere suspension was sieved, washed with deionised water and then freeze-dried before storage at -20°C as described by Giteau et al. (Paillard-Giteau, Tran et al. 2010).

Unloaded microparticles were prepared in the same manner without adding the protein. PLGA microspheres were either coated with fibronectin-like sequence (Sigma, Saint-Quentin Fallavier, France) or with LM combined with the highly charged poly-D-lysine molecule (Sigma, Saint-Quentin Fallavier, France) to obtain LM-PAMs and FN-PAMs, as previously described (Delcroix, Garbayo et al. 2011) to favor cell attachment to the PAM surface. Briefly, microspheres were suspended in Dulbecco's phosphate buffered saline (DPBS), sonicated until full dispersion and were then incubated with a mixture of both molecules (9 $\mu\text{g.mL}^{-1}$ LM and 6 $\mu\text{g.mL}^{-1}$ PDL for a final concentration of the coating molecules of 15 $\mu\text{g.mL}^{-1}$) by continuous stirring at 15 rpm for 90 min (Giteau A, Venier-Julienne MC et al. 2008). PAMs were then freeze-dried and stored at 4° C for the experiments or immediately used for release kinetics studies.

Protein encapsulation efficiency

VEGF-A, HGF and IGF-1 encapsulation yield were determined by measuring the entrapped proteins using ELISA kits (Peprotech, France for VEGF and R&D systems, France for HGF and IGF-1). The biological activity of encapsulated lysozyme was determined by measuring

the turbidity change in a *Micrococcus lysodeikticus* bacterial cell suspension as reported above (Aubert-Pouëssel, Bibby et al. 2002)). Briefly, protein PLGA microspheres (5 mg) were dissolved in 1 mL acetone in silanized glass tube. After 1h, the entrapped protein was separated from the dissolved polymer by centrifugation (15-min, 14,000 rpm) and the acetone was removed. To ensure that the entire polymer was dissolved, the step was repeated twice and the pellet dissolved in phosphate buffered saline (PBS), pH 7.4 with 0.1% BSA to determine the protein activity.

Release kinetics, in vitro study

The in vitro release profile of protein from PAMs was determined by adding 250 μ L of PBS buffer, pH 7.4, containing 1% w/v BSA to 2.5 mg of microspheres into eppendorf tubes. The tubes were closed and incubated in a shaking water bath (37°C, 125 rpm). At different times, the tubes were centrifuged for 5 min at 3,000 rpm and 250 μ L of the supernatant were collected for analysis and replaced by fresh buffer. The percentage of released GF was evaluated by ELISA (Peprotech, France and R&D systems, France).

To confirm the biological activity of the released VEGF and HGF, a bioassay was performed using a human umbilical vein endothelial cells (HUVECs) proliferation assay; HUVECs were cultured and passaged in standard endothelial cell medium conditions containing the growth supplement. The HUVECs cells were then plated (5×10^3 cells) onto 24 well plates, with filtered supplement-free medium conditioned with recombinant rVEGF or rHGF at different concentrations and the effect on HUVEC proliferation was investigated at several time points 24h, 48h, 5 days and 1 week by the Alamar Blue assay (Invitrogen). The best effect was observed with 4ng/mL of rVEGF and 2ng/mL of HGF for five days. The effect of bioactive VEGF and HGF released from the microspheres on HUVEC proliferation was performed with the different aliquots collected throughout time and diluted to 4 ng/mL and 2 ng/mL respectively, according to the ELISA results and compared to the supplement-free medium alone or supplement-free medium containing 4 ng/ml VEGF and 2ng/mL HGF.

Hydrogel preparation and rheology

Poloxamer 407 (P407, pluronic F127) HG was prepared by dissolving P407 (Sigma Aldrich, St. Louis, MO) in sterile PBS (pH 7.4) at 4°C. Once completely dissolved at low temperature, P407 formed a clear solution which turned into a transparent gel when brought up to room temperature or 37°C. The rheological properties of 3 hydrogel concentrations (18%, 20% and 22% w/v) were investigated with a Kinexus® rheometer (Malvern S.A.), used with plate-plate geometry (20 mm-diameter) with a 800 µm gap.

The HG properties were evaluated as followed in triplicate:

Strain sweep experiments: Frequency was fixed at 1 Hz and elastic (G') and viscous (G'') moduli were measured vs. strain variation from 0.01 to 100 % to determine the base regime. Experiments were performed at 37°C.

Frequency sweep experiments: The base regime (Strain fixed at 0.1 %), elastic (G') and viscous (G'') moduli were determined vs. frequency variation from 0.01 to 50 Hz. Experiments were performed at 37°C.

Temperature ramp experiments: Elastic (G') and viscous (G'') moduli were determined vs. temperature cycles from 37 to 4°C and from 4°C to 37°C at 5°C/min. Strain and frequency were fixed at 0.1 % and 1 Hz base regime respectively.

PAMs were added within the 18%, 20% and 22% hydrogels at 1% w/v concentration and in vivo injection concentration, corresponding 10% w/v. The rheological properties were also evaluated.

Protein release profile from PAMs within the hydrogels

PAMs releasing lysozyme were formulated as described by Giteau A. et al. (Paillard-Giteau, Tran et al. 2010) and were then coated with ECM. 1% w/v of PAMs were then added within the 3 HGs. The tubes were closed and incubated in a shaking water bath (37°C, 125 rpm) and at interval times were centrifuged for 5 min at 3,000 rpm. The supernatant was collected for analysis and replaced by fresh buffer. The biological activity of released lysozyme was determined by measuring the turbidity change in a *Micrococcus lysodeikticus* bacterial cell suspension as reported above.

Cell Culture

ADSCs have been purchased from the American type culture collection (ATTCs). 1×10^3 cells/cm² were seeded on 75 cm² flasks containing DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen, Calif., USA; normal culture medium), in a humidified atmosphere of 5% CO₂ at 37°C, and allowed to reach 80%–90% confluence. The cells were then detached with 0.5 mM EDTA/0.05% trypsin (Gibco, Invitrogen) for 5 min at 37°C and replated on plastic dishes till the first or second confluence, changing the medium twice a week.

Cell viability and cardiac differentiation in 3D culture conditions

For 3D cell studies, culture-expanded ADSCs were harvested and cultured with FN coated PAMs for cell viability studies and LM coated PAMs for cell differentiation.

ADSC/PAM complex formation: Culture-expanded ADSCs were harvested and cultured under differentiating conditions with PAMs presenting either a FN (FN-PAMs) or a LM (LM-PAMs) biomimetic surface. Briefly, 0.5 mg of FN-PAMs or LM-PAMs were incubated with 2×10^5 ADSCs without any agitation in a 24-well ultra low attachment plate (Costar), in DMEM for 4h.

HG containing ADSC and ADSC/PAM complexes: HG was synthesized in the characterized stiffness range of 18% – 22% total polymer content. 18%, 20% and 22% P407 HG were prepared by dissolving sterile poloxamer 407 (Sigma Aldrich, St. Louis, MO) in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen, Calif., USA; normal culture medium), at 4°C. PAMs-cells complexes incorporation within the HGs, an adhesion step incubating cells with PAMs for 4h was necessary.

Viability studies: For viability studies, hMSCs and hADSCs were suspended within the HGs with and without PAMs at a concentration of 0.5×10^6 cells/cm³ into an ULA microplate. Prior these cell-loaded hydrogels were then incubated at 37°C in 5% CO₂ for 5 days and hydrogels removed at 1, 2, 3 and 5 days. Hydrogel samples were then assayed for cell number and viability using the Cyquant GR assay (Molecular Probes, Eugene, OR) against generated cell number standards.

Cardiac Differentiation studies: With ADSC/PAM complexes alone or ADSC/PAM

complexes within a HG. For cardiac differentiation studies, with ADSC/PAM complexes within the gel only the 22% P407 HG was prepared by dissolving sterile poloxamer 407 (Sigma Aldrich, St. Louis, MO) in DMEM supplemented with 3% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen, Calif., USA; normal culture medium), at 4°C. ADSCs were then cultured with LM-PAMs releasing HGF and LM-PAMs releasing IGF-1 with and without the HG. Briefly, 0.25 mg of LM-PAM-HGF and 0.25mg LM-PAM-IGF-1 were first incubated for 4h with 2×10^5 ADSCs without any agitation in a 24-well ultra low attachment plate (Costar), in DMEM supplemented 3% FBS. Once adhered, PAMs-cell complexes were added within the 22% P407 HG. One week after, cardiomyogenic commitment of ADSCs adhered to PAMs-GFs without HG was assessed by qRT-PCR and immunocytochemistry while for PAMs with HG it was done by qRT-PCR.

Cardiac primer design and validation

A panel of cardiac genes, the transcription factors GATA binding protein 4 (GATA4), myocyte enhancer factor 2C (MEF2C), NK2 homeobox 5 (Nkx2.5), the sarcomeric ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2, variant a (SERCA2a), the cardiac myosin light chain 2 (MLC-2V), the cardiac troponin I (cTnI), and the gap junction protein alpha 1, connexin 43 (CX43/GJA1) have been used to study ADSCs mRNA levels during commitment (Table 1). The following experimental details were performed following the guidelines of the SCCAN core facility (“Service Commun de Cytométrie et d’Analyse Nucléotidique”, Angers, France). Human sequences were determined using PubMed nucleotide search (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) websites. The online freeware Primer blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was used for primer modelling, clustalw (www.ebi.ac.uk) to align nucleotidic sequences, and nucleotide blast (www.ncbi.nlm.nih.gov) to confirm the specificity of the defined primer sequences. When possible, pairs of primers were designed across intron-spanning regions to avoid genomic DNA contamination. Sense and antisense desalted primer pairs (Eurogentec, Angers, France) were mixed in RNase free water at a final concentration of 5 mM and validated using cDNA from human adult or foetal heart and commercial qPCR Human Reference cDNA (Clontech, Takarabio, Saint-Germain-en-Laye, France). The melting peak of the amplicon had to be narrow and unique, and its size and specificity were confirmed by electrophoresis. Finally, a serial dilution of the PCR product was reamplified to draw a linear curve $\text{Ct} = f(\text{Quantity})$. The efficiency of the primer was calculated from the slope of the linear curve:

$E = [10^{(-1/\text{slope})} - 1] \times 100$. Only primer pairs with an efficiency greater than 80% were validated for use (Table 1).

Real-time Quantitative PCR

ADSCs were washed in DPBS. Following the manufacturer's guidelines, cells were lysed in a 1% β -mercaptoethanol containing buffer and RNA extracted following a treatment by DNase to remove any traces of genomic DNA (Total RNA isolation Nucleospins® RNA II, Macherey Nagel, Hoerd, France). First strand cDNA synthesis was performed with a Ready-To-Go You-Prime First-Strand Beads® kit in combination with random hexamers (Amersham Biosciences, Orsay, France) using 1 μ g RNA according to the manufacturer's guidelines. Following first-strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France) and eluted in 50 μ L RNase free water (Gibco). Five μ L of cDNA (1:20) was mixed with iQ SYBR Green Supermix (Biorad) and primer mix (0.2 mM) in a final volume of 15 μ L. Amplification was carried out on a Chromo4 thermocycler (Biorad) with a first denaturation step at 95°C for 3 min and 40 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 15 s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. A mean cycle threshold value (Ct) was obtained from 2 measurements for each cDNA. Several housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_002046), hypoxanthine phosphoribosyltransferase 1 (HPRT1, NM_000194), b-actin (Actb, NM_001101), 30S ribosomal protein S18 (Rps18, NM_001093779) and heat shock 90 kDa protein 1b (Hspcb, NM_007355) were tested and the three best ones chosen for normalization. The relative transcript quantity (Q) was determined by the $\Delta\Delta C_T$ method $Q = E^{(C_{T \text{ min}} - C_{T \text{ sample}})}$, where E is related to the primer efficiency (E=2 if the primer efficiency=100%). Relative quantities (Q) were normalized using the multiple normalization method described in Vandesompele et al. (2002).

Immunofluorescence

ADSCs adhered on empty PAMs or LM-PAMs releasing GFs were used for GATA4, Nkx2.5, MEF2C, cTnI and CX43 immunocytofluorescence. Briefly, PAM/cell complexes were transferred into an 1.5mL and washed three times with DPBS, the PAM/cell complexes were

fixed with 4% paraformaldehyde at 4°C for 15 min and then permeabilized with 0.2% TritonX 100 (Sigma) for 5 min. They were then blocked with DPBS, 10% normal goat serum (Sigma) and 4% bovine serum albumin (BSA) (Sigma) at room temperature for 45 min. After washing, PAM/cell complexes were incubated overnight at 4°C with goat anti GATA4 (1:100, Abcam, Paris France), mouse anti-Nkx2.5 (1:200, Abcam), mouse anti-cTnI (1:100, Abcam), rabbit anti-MEF2C (1:100, Abcam) and rabbit anti-CX43/GJA1 (1:100) in DPBS, 4% BSA, 0.2% TritonX-100. Isotypic controls were made with IgG1k (BD Biosciences) and IgG2bk (BD Biosciences). After rinsing, the PAM/cell complexes were incubated with the corresponding secondary biotinylated anti-mouse or anti-goat or anti rabbit antibody (1:200, Abcys) in DPBS, 4% BSA, 0.2% TritonX-100 for 1 h. Finally, after rinsing again and following incubation with streptavidin-FITC (1:500, Dako) or streptavidin-rhodamin (1:500, Dako) in DPBS for 40 min, a 2 min centrifugation at 1000rpm was done to remove the supernatant. PAM/cell complexes were mounted on slides (Mounting Media, Dako) and observed with a fluorescence microscope (Axioscop, Carl Zeiss, LePecq, France).

Table 1: Human specific cardiac primers

Gene	Full Name	NM accession number	Sequences
GATA4	GATA binding protein 4	NM_002052.3	Fwd: AGATGCGTCCCATCAAGACG Rev: GGAGCTGGTCTGTGGAGACT
MEF2C	Myocyte enhancer factor 2C	NM_001131005.2	Fwd: CTAATCTGATCGGGTCTTCCTTCAT Rev: TTTTCTCCCCATAGTCCCCG
Nkx2.5	NK2 homeobox 5	NM_004387.3	Fwd: CTATCCACGTGCCTACAGCG Rev: GCCGCTCCAGTTCATAGACC
SERCA2a	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2 (ATP2A2) variant a	NM_001681.3	Fwd: ACCTGGAACCTGCAATACTGG Rev: TGCACAGGGTTGGTAGATGTG
MLC-2V	Myosin light chain 2, cardiac, slow	NM_000432.3	Fwd: GATGGAGCCAATTCCAACGTG Rev : ACGTTCACACGCCCAAGAG
cTnI	Troponin I type 3 (cardiac)	NM_000363.4	Fwd CCTGCGGAGAGTGAGGATCT Rev: CAGTGCATCGATGTTCTTGCG
CX43	Gap junction protein alpha 1, 43kDa	NM_000165.3	Fwd: TCTGAGTGCCTGAACTTGCC Rev: CACCTTCCTCCAGCAGTTG

Results:

Protein precipitation conditions

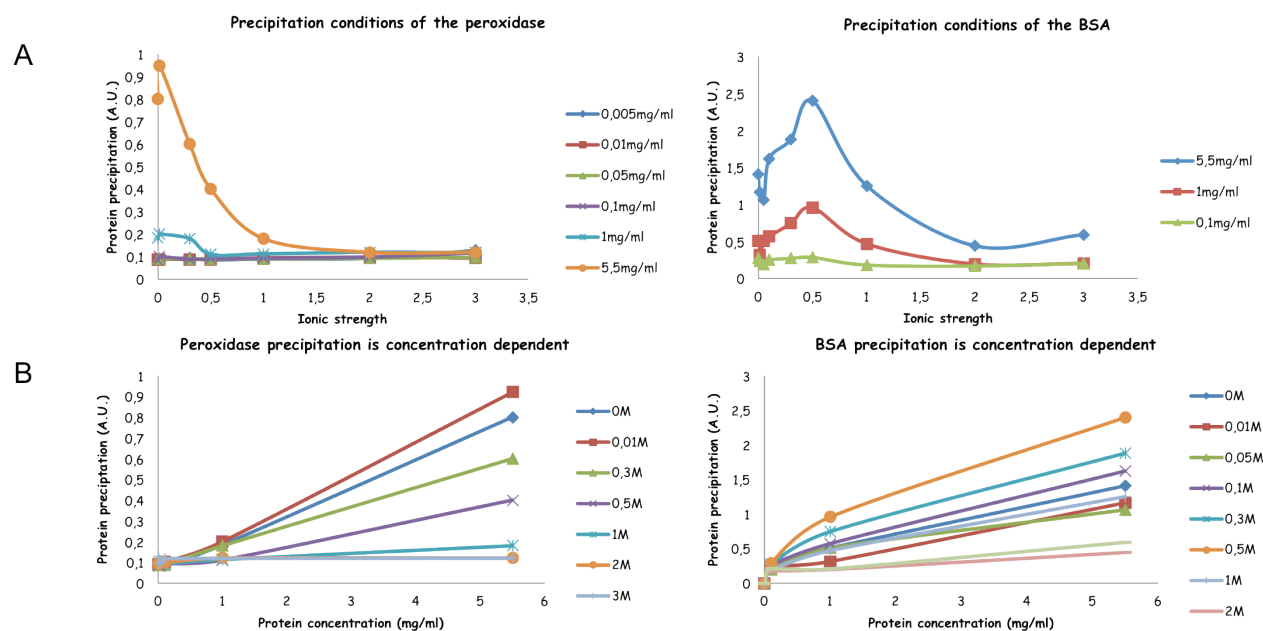


Figure 1: Precipitation conditions of the model proteins peroxidase and BSA to predict VEGF and HGF precipitation respectively. Precipitation of peroxidase occurred at 0,01M for a concentration of 5,5mg/ml while BSA precipitation occurred at 0,5M for a concentration of 5,5mg/ml (A). Interestingly, to precipitate the protein, a 5,5mg/ml concentration was necessary.

Protein	Mw (kDa)	Isoelectric point	Ionic strength (NaCl)	Protein concentration (mg/mL)	Precipitation efficiency (%)
Lysozyme	14	11	0,3M	5,5	83 %
Peroxidase	40	7,2	0,01M	5,5	100 %
BSA	66,7	4,7	0,05M	5,5	100%
VEGF	38,2	7,3	0,01M	5,5	80 ± 20%
HGF	80	7,8	0,5M	5,5	90 ± 10%
IGF-1	7,6	7,8	2M	5,5	80 ± 20%

Table 2: Recapulative table of proteins characteristics and precipitation conditions.

Protein precipitation conditions

An excess of water-miscible organic solvent, glycofurol, and sodium chloride were conjunctively added to protein solutions to induce the formation of fine protein particles. As the efficiency of precipitation vary with the process conditions, the composition of the medium was varied in a defined experimental domain at 4°C. Peroxidase and bovine serum albumin were used as model proteins for VEGF and HGF respectively based on their molecular weight and isoelectric points. These physicochemical parameters have been shown to influence protein precipitation as described by Giteau et al (Giteau A, Venier-Julienne MC et al. 2008). Precipitation optima corresponded to the recovery of more than 95% of the initial protein mass in its biologically active state. For BSA, the precipitation efficiency was above 90% for all the experiments while for peroxidase it was 100%. We found that the protein concentration also greatly influence protein precipitation and should be also taken into consideration. Combined analysis of these results underline that protein precipitation optima correspond to higher protein concentrations (>5.5 mg/mL).

For peroxidase, we can precipitate 50µg of the protein in a solution at 5.5 mg /mL in NaCl 0.01M with 1.04g of glycofurol (Table 2). These conditions were thus applied to VEGF and the output of precipitation of VEGF was determined by ELISA. 80± 20% of VEGF could thus be precipitated by simply applying the conditions of precipitation of peroxidase.

In a similar manner, the defined conditions for BSA precipitation were applied to HGF. We can precipitate 50µg of the protein in a solution at 5.5mg/mL in NaCl 0.5M with 1.04g of glycofurol (Table 2). In these conditions 90±10% of HGF could thus be precipitated as determined by ELISA.

The physicochemical properties of IGF-1 are different to the other model proteins. Applying a screening method placing pH equal to the isoelectric point and varying the protein concentration and ionic strength, we found that we could precipitate 10µg of the protein in a solution at 5.5mg/mL in NaCl 2M with 1.04g of glycofurol (Table 2). With these conditions, 80± 20% of IGF-1 could be precipitated. Lysozyme precipitation was obtained using 7µL of an NaCl 0.3 M solution containing 50 µg of protein and 1 mg of Poloxamer 188 was added to 1.04 g glycofurol to form a 1 mL suspension. After 30 min (at 4°C), the protein particles were recovered by centrifugation (10,000g, 4°C, 30 min).

PAM characterization and GF release profile

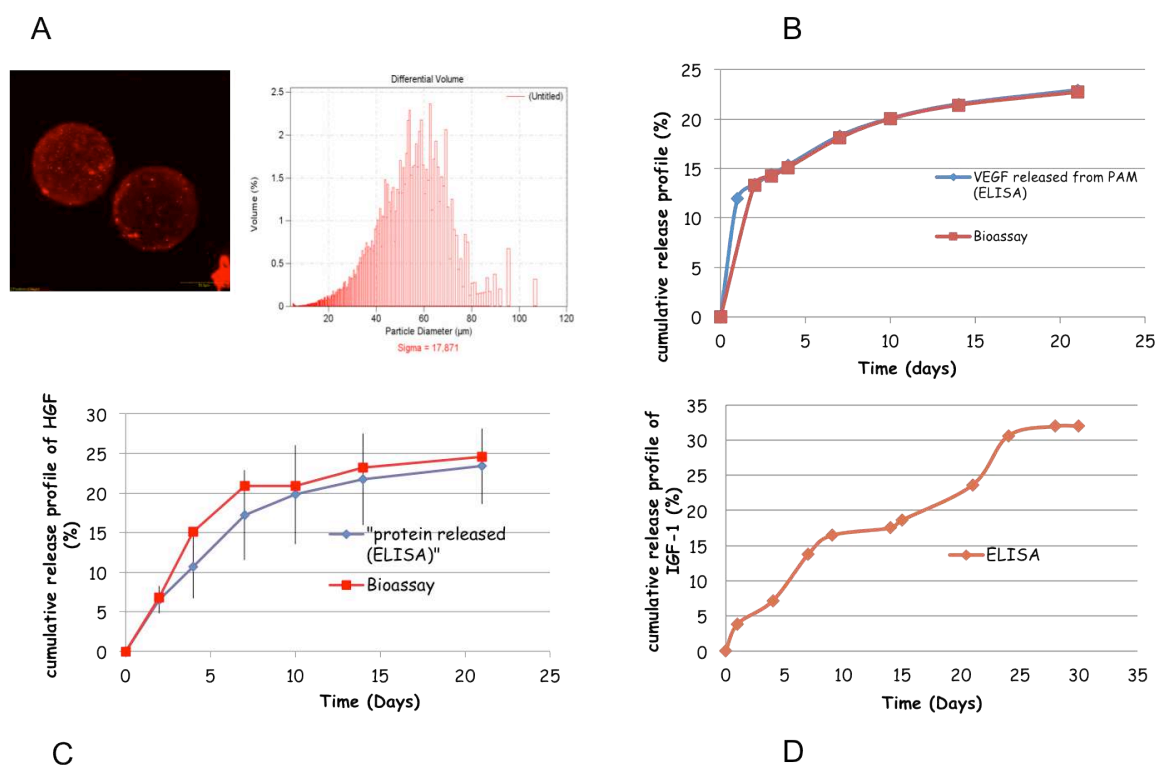


Figure 2: The PAMs size distribution was homogenous and the surrounding biomimetic coating was also homogeneously distributed at their surface (A). 23% of the encapsulated VEGF (B), 24% of the entrapped HGF (C) and 32% of IGF-1 (D) were released after 3 weeks. The proteins were released under their bioactive form (B, C).

PAM characterization and GF release profile

$62 \pm 5\mu\text{m}$ microspheres with a homogenous size distribution were obtained for all the formulations performed (for illustration, Fig 2A). After coating the microspheres, an immunofluorescence study showed a homogeneous distribution of the ECM molecules on the surface of the PAMs (Fig 2A). The encapsulation yield of biologically active VEGF within PAMs was $85 \pm 23\%$, of biologically active HGF was $95 \pm 13\%$ and IGF was $99 \pm 4\%$.

VEGF release profile: The release of VEGF from PAMs overtime was quantified in vitro by ELISA. The cumulative release profile showed a small burst of 12% of released VEGF after 24 h and 23% of the encapsulated VEGF was released after 3 weeks (Fig. 2B). For the protein loading of $1\mu\text{g}$ VEGF/mg of microspheres the release of VEGF was estimated to be 133ng/mL for 1 mg of PAMs during the first 2 days and approximately 100 ng/mL for the 15 next days. In vitro release of bioactive VEGF was then evaluated using a proliferation bioassay. The VEGF collected from each sample of the kinetics release assay (diluted to 4ng/mL) was able to stimulate HUVEC proliferation for a period of 5 days in a similar manner as the recombinant VEGF at 4ng/mL (data not shown). The cumulative release curve of bioactive VEGF was parallel to that measured by ELISA VEGF overtime (Fig.2B) and all the protein released was bioactive.

HGF release profile: A continuous release of HGF from PAMs was observed for at least 3 weeks consisting in a cumulative release of 1204 ng/mL and representing 24% of the entrapped protein (Fig. 2C). The HGF collected from each sample of the kinetics release assay (diluted to 2ng/mL) was able to stimulate HUVEC proliferation for a period of 5 days in a similar manner as the recombinant HGF at 2ng/mL.

IGF-1 release profile: The IGF-1 released from PAMs overtime was quantified in vitro by ELISA. The cumulative release profile showed a small burst of 5% of IGF-1 released after 24 h and 32% IGF-1 was released after one month representing 1600ng/mL (Fig.2D).

In vitro cardiomyogenesis of ADSCs adhered on HGF and IGF-1-PAMs

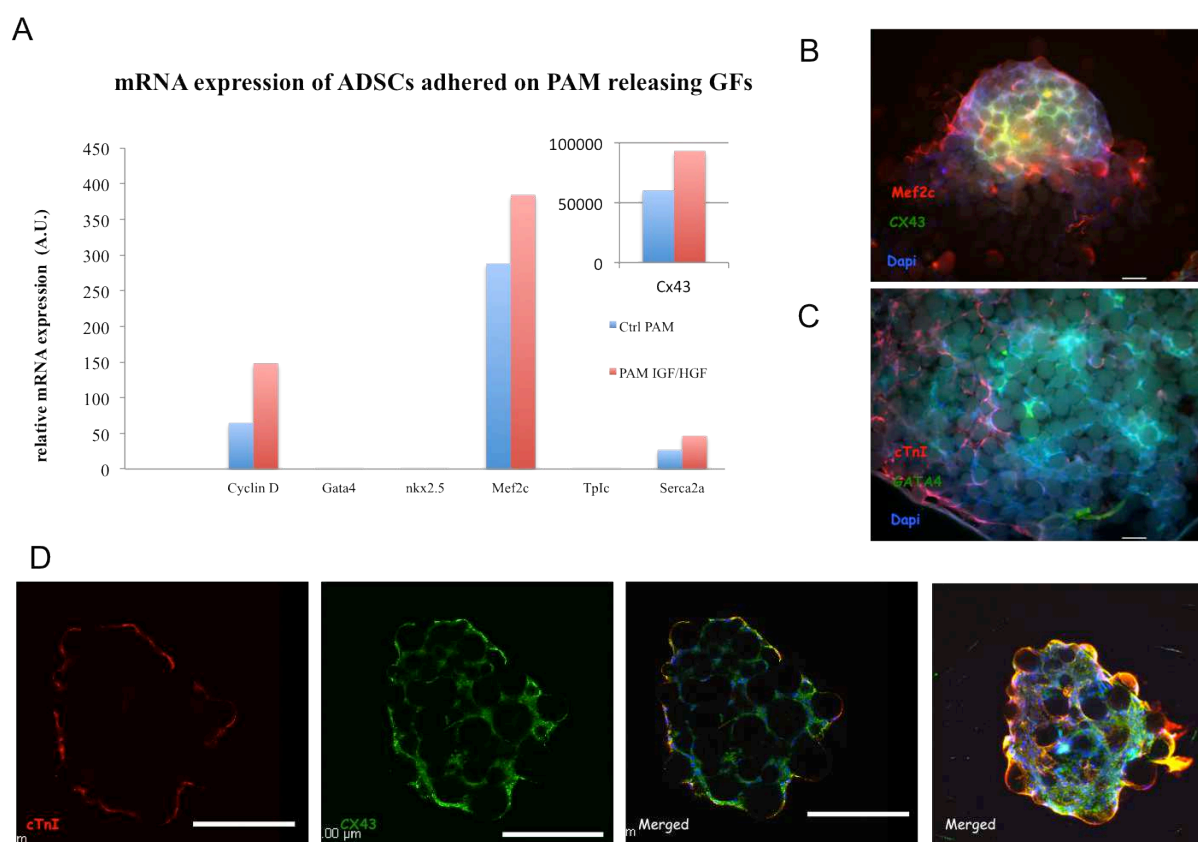


Fig 3:

Relative mRNA expression of cardiac markers in ADSCs cultured on laminin coated PAM releasing IGF-1 and HGF, after 1 week. No significant expression was observed with SERCAa. mRNA levels of cyclin D1 and the transcription factor MEF2C were increased as well as the mRNA levels of the gap-junction protein CX43.

Using immunofluorescence, we observed that the transcription factors GATA4 (FITC) and MEF2C (rhodamin) were expressed in LM-PAM-GF (C). The gap-junction protein cx43 (FITC) and the contractile protein Tplc (rhodamin) were also co-expressed (C) on LM PAM-GF. The blue staining indicates cell nuclei. Scale bar represent 100µm

Confocal observation of ADSC-PAM-GF complexes after culture for 1 week. CX43 positive cells (FITC) and cTnI-positive cells (rhodamin) were localized at the surface of the PAM-ADSCs complexes as observed. CX43 was found throughout. The blue staining indicates cell nuclei. Scale bar represents 100µm

In vitro cardiomyogenesis of ADSCs adhered on HGF and IGF-1-PAMs

To provide a 3D structure to the cells, we cultured ADSCs on PAMs releasing or not GFs (IGF-1 and HGF) with a LM biomimetic surface for 1 week. Incubation of 4×10^5 ADSCs with 0.5 mg PAMs allowed the rapid adhesion of the cells onto the surface of the microspheres. Adhesion was similar whether PAMs released or not GFs. Indeed, as soon as 2h after incubation, most of the cells adhered on the PAMs with almost no cells in suspension (not shown). ADSCs adhered to surrounding cells formed PAM/cell complexes that became bigger with time, no cells in suspension could be observed at any time after day 2.

The expression of several cardiac markers and Cyclin D1 was studied, to evaluate whether the cells progress in cell cycle and proliferate rather than differentiate. We first tried to measure the transcription factors GATA4 and Nkx2.5 that initiate the differentiation pathway toward the cardiomyocyte lineage (Sepulveda JL, Belaguli et al. 1998) but we did not detect them. Among the cardiomyogenic markers tested, MEF2C which is specifically expressed during cardiomyocytes specification and CX43 were highly expressed and significantly induced after ADSC culture on LM-PAMs releasing GFs compared to ADSCs cultured on LM-PAMs only (Fig.3A). Interestingly, the sarcomeric protein SERCA2-A and the late-stage cardiac marker cardiac troponin I (cTnI) were also expressed. The cell-cycle cyclin D1 marker was expressed as well, suggesting a proliferation of some of the cells.

After immunostaining, we observed that in 3D ADSC culture conditions with PAMs releasing GFs, cardiac protein expression was significantly higher than with LM-PAMs. With LM-PAM-GF and LM-PAMs the early transcription factor Nkx2.5 was not observed (not shown). However, GATA4, MEF2C, CX43 and cTnI were expressed by ADSCs in LM-PAM-GF complexes while we did not find it with LM-PAMs alone (Fig 4B and 4C). Interestingly, KI67-positive cells were observed at the center of both LM-PAMs and GFs-LM-PAM-GF/ADSCs complexes confirming cell proliferation in this area (not shown).

Due to its 3D conformation, a confocal microscopy observation was performed to evaluate the distribution of the cells expressing the late cardiac differentiation within the ADSC/ PAM-GF complexes. Interestingly, the results revealed that cTnI was expressed at the periphery of the ADSCs-PAMs-GF complexes while the CX43 were expressed throughout all the complexes (Fig.4D).

Pluronic F127/poloxamer 407 hydrogel characterization

A

	0% microsphere		1% microsphere		10% microsphere	
	T _{G-S} (°C)	T _{S-G} (°C)	T _{G-S} (°C)	T _{S-G} (°C)	T _{G-S} (°C)	T _{S-G} (°C)
Gel 18%	15.5 ± 1.4	29.9 ± 1.8	16.6 ± 2.2	23.8 ± 2.1	20.5	27
Gel 20%	18.8 ± 0.9	25.0 ± 0.6	14.4 ± 1.6	24.4 ± 1.2	---	---
Gel 22%	20.1 ± 0.6	22.5 ± 0.6	15.5 ± 0.8	21.4 ± 0.5	19.5	20

T_{G-S} and T_{S-G} – gel-solution and solution-gel transition temperature at 0.1 %-constant strain and 1 Hz-constant frequency;

B

	0% microsphere		1% microsphere		10% microsphere	
	G' (10 ³ Pa)	G'' (10 ³ Pa)	G' (10 ³ Pa)	G'' (10 ³ Pa)	G' (10 ³ Pa)	G'' (10 ³ Pa)
Gel 18%	1.9 ± 0.7	0.6 ± 0.2	8.7 ± 0.3	0.5 ± 0.02	3.8 ± 1	0.8 ± 0.1
Gel 20%	8.1 ± 0.5	2.1 ± 0.1	9.8 ± 0.6	0.7 ± 0.003	---	---
Gel 22%	10.2 ± 1.2	2.7 ± 0.4	12.3 ± 0.5	0.5 ± 0.03	15.3 ± 1.3	1.13 ± 0.03

G' and G'' – elastic and viscous modulus at 0.1 %-constant strain and 1 Hz-constant frequency;
f_c – critical frequency where gel properties are lost at 0.1 %-constant strain.

Table 2: Elastic moduli and transition temperatures of different P407 HG (18%, 20% and 22% w/v). Addition of microspheres into the gels modified the rheological properties (B) and modify the elasticity of the gel (B) but do not modify the temperatures of transition (A)

PAM protein release profile decrease within the HGs

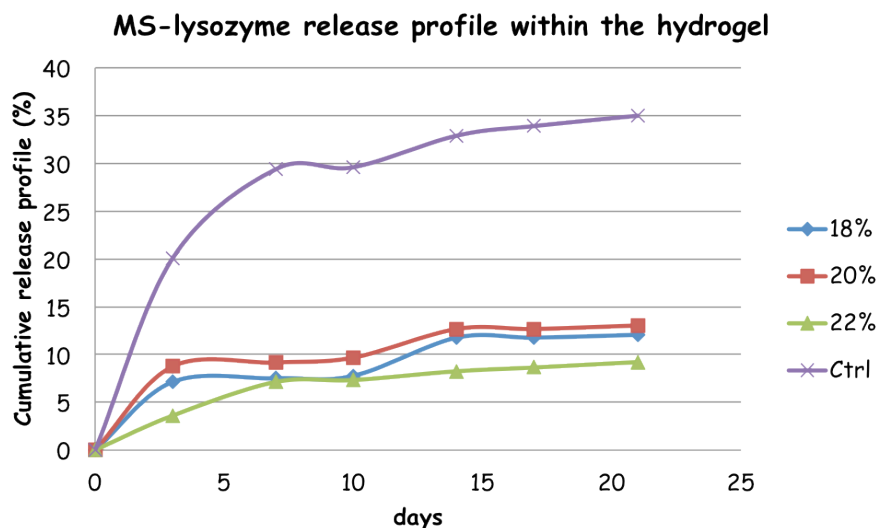


Fig 4: The effect of P407 concentration (18%, 20% and 22%) on lysozyme released from PAM. Increasing the concentration of PF-127 from 18% (w/v) to 20% (w/v) decreased the cumulative percent drug released, 14% and 9% of lysozyme were released respectively within the 18% and 22% HG after 3 weeks compared to the 35% of lysozyme released with control PAM in release buffer.

Pluronic F127/poloxamer 407 hydrogel characterization

The elastic modulus increases with the concentration (percentage w/v) of poloxamer 407.

The elastic modulus found here for each gel in the graph was 1.9 kPa, 8 kPa, and 10.2 kPa for 18%, 20% and 22% P407 gels, respectively (table 2). Interestingly, addition of microspheres into the gels modified the rheological properties when compared to the gel alone (G' and G'' , T_{G-S} and T_{S-G}) of native gels. These properties were strongly modified for the gel at 18% and were less modified for the gels at 20% and 22%. Indeed, the elastic moduli were 9.8 kPa, and 12.3 kPa for 20% and 22% P407 gels with the addition of 1% microsphere w/v, respectively. Addition of 10% microspheres w/v into the gels did not modify the rheological properties (G' and G'' , T_{G-S} and T_{S-G}) of the 22% gel, (a slight increase of the slow modulus to 15.3 kPa) (table 2). On the other hand, the rheological properties of the gel at 18% were modified by the addition of microsphere as addition of 1% w/v of microspheres within the gel improved the gel properties (elastic modulus = 8,7kPa), while addition of 10% of microsphere seemed to destabilize the gel network (a decrease of the elastic modulus to 3.8 kPa) (table 2A). Moreover we can observe that addition of PAMs do not modify a lot the temperatures of transition for the 20% and 22% HG (variability < 5°C). Additionally, the elastics moduli do not change on the contrary to the elasticity which is increase as an increase in the ratio G'/G'' is observed when PAMs are added to the HGs (14 compared to 3,9 for 20% HG and 24,6 compared to 3,8.for HG 22% when 1% PAMs are added to HGs and 13,5 compared to 3,8 for HG 22% when 10% PAMs are added to HG) (Table 2B).

PAM protein release profile decrease within the HGs

The effect of P407 concentration (18%, 20% and 22%) on lysozyme released from PAM at 37°C is shown in Figure 5. It is apparent that the protein release rate decreases as the P407 concentration increases. Increasing the concentration of PF-127 from 18% (w/v) to 20% (w/v) decreased the cumulative percent drug released, 14% and 9% of lysozyme were released respectively within the 18% and 22% HG after 3 weeks compared to the 35% of lysozyme released with control PAM in release buffer. This phenomenon is due to an increase in chain entanglement and gel formation that favors hydrophobic interactions. This entanglement is also more marked at higher concentrations of P407, yielding an increase in gel strength and consequently a decrease of the drug release rate.

PAMs further improve hADSCs viability within P407

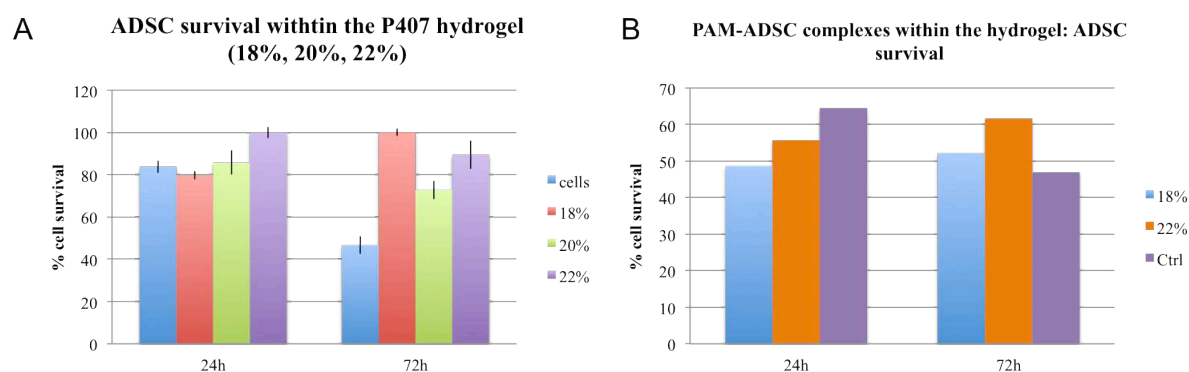


Fig 5: ADSCs survival within the P407 HG, adhered or not on PAMs. When ADSCs adhered onto PAMs were mixed within the gels both at 18% and 22%, survival was improved compared to PAMs alone. The 22% HG-PAM conditions exhibited better cell survival compared to control.

ADSC/PAM-GF complexes integrated within the P407 HG increased cardiac mRNA levels

mRNA expression of ADSCs PAMs-GFs complexes within the 22% P407 HG

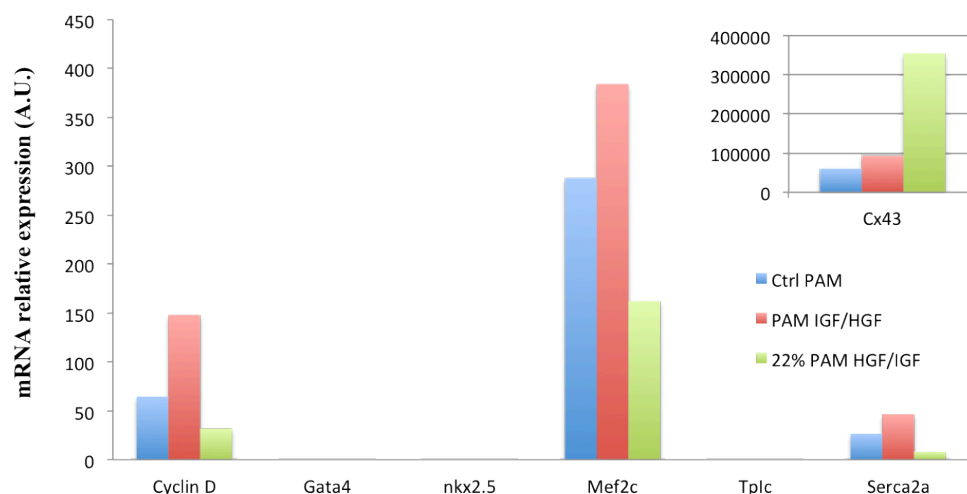


Fig 6: mRNA expression of ADSCs-PAM-GF within the 22% P407 HG. The transcription factors GATA4, NKX2.5 were not expressed as well as the contractile protein, cTnI. Interestingly, the gap junction CX43 was strongly increased with PAMs-GFs within the HG compared to control PAM-GF without gel and PAM in standard culture medium.

PAMs further improve hADSCs viability within P407

We first tested whether the P407 hydrogel could be cytotoxic for ADSCs. Culture medium incubated for 24, 48 or 72 h with P407 hydrogels showed a significant cytotoxicity when mixed with ADSCs for 72 h, as assessed by Cyquant proliferation assay (Fig. 5). However, in contrast with cells seeded on polystyrene dishes, the cells did not spread, but retained a round shape. We then analyzed the interactions of ADSCs adhered onto PAMs within the 18% and 22% HGs. When ADSCs adhered onto PAMs were mixed within the gels both at 18% and 22%, survival was improved compared to PAMs alone. Interestingly, in a 22% HG the number of living cells was slightly increased after 72h compared to 18% HG, where it was maintained and to the control of PAMs in culture conditions (Fig 5).

ADSC/PAM-GF complexes integrated within the P407 HG increased cardiac mRNA levels

We checked whether the P407 hydrogel could influence ADSC commitment toward the cardiomyogenic lineage when adhered onto PAMs for 1 week. A 5 fold-decrease of the mRNA levels of cell-cycle molecule, Cyclin D1, was observed in ADSCs within the HG alone compared to ADSC/PAM complexes, probably suggesting that fewer cells were proliferating. The early cardiac transcription factors NKX2.5 and GATA4 were not expressed in all conditions, while a 2.5 fold-decrease of the third transcription factor, MEF-2c, a cofactor of the two first cited was observed as well as the decrease of the sarcomeric contractile SERCA2-A mRNA levels compared to ADSC/PAM-GF conditions. Interestingly, while cTnI was not found in ADSCs within the HG alone, a 6 fold increase in mRNA levels was observed in ADSC/PAM complexes and a 4 fold increase in CX43, suggesting gap-junction communications between the cells (Fig 6).

Discussion

The use of adult stem cells for cardiac cell therapy combined to biodegradable injectable 3D-biomimetic scaffolds releasing growth factors in a prolonged manner provides exciting perspectives to overcome the poor regenerative capacity of the damaged heart. In this study, we developed PAMs with a biomimetic surface composed mainly of laminin as bioadhesive, differentiation-inducing component for cells, including MSCs and ADSCs, and delivering three growth factors IGF-1, VEGF and HGF, validated for cardiac tissue engineering as angiogenic, differentiation and pro-survival factors (Opgaard and Wang 2005; Davis 2006; Forte, Minieri et al. 2006; Chen, Minatoguchi et al. 2007; Sadat, Gehmert et al. 2007; Haider, Jiang et al. 2008; Pons, Huang et al. 2008; Deuse, Peter et al. 2009; Penn and Agarwal 2010). We obtained the proof of principle that these PAMs combined or not to a thermosensitive HG induced the differentiation of adult stem cells toward the cardiomyogenic lineage.

Together with hydrogels, biodegradable and biocompatible microparticles may be powerful tools for adult stem cell implantation and heart regeneration. Indeed, although encouraging results have been reported in cardiac cell therapy studies, only a few of the transplanted cells survive and integrate the host myocardium. Recent studies with HG, microparticles and other scaffolds conveying cells and releasing GF have shown interesting results on cardiac function *in vivo*. Although interesting results are obtained, the amount of GF released and the release profile are not well documented. We here offer a 3D scaffold with an instructive biomimetic coating of LM and releasing continuously a known concentration of a defined GF overtime. We have recently shown that a 3D-laminine biomimetic support provided by PAMs stimulate cardiomyogenic commitment when the appropriate GFs are added to the medium. We have tried to encapsulate 3 GFs released from LM-PAM at similar concentrations than those used in the GF cocktail for 1 week.

In order to appropriately implement these approaches for cardiac tissue engineering, it is essential to obtain a sustained GF release from such scaffolds, at physiological and controlled doses. In this way, the GF and cells may not only initiate tissue repair, but also exert their trophic effects during the entire process. The first step to obtain a complete and sustained release of an active protein implies its protection during the encapsulation and release process. To preserve protein integrity during the PLGA microspheres preparation, we have developed

a technique to obtain a protein in a solid state, where it is less fragile and exhibits restricted conformational flexibility. Due to the expensive cost of therapeutic proteins, model proteins with similar physic-chemical properties were used to predict their precipitation environment as proposed by Giteau et al. (Giteau, Venierjulienne et al. 2008). Thus, the precipitation conditions of VEGF and HGF were obtained with peroxidase and BSA, respectively. We found that by placing the protein at 4°C in a solution at a pH equal to its isoelectric point and at a particular range of salt-induced ionic strength, the glycofurol induced the protein precipitation. Moreover, this precipitation could be visualized at a minimum protein concentration of 5.5 mg/mL for these GF, particularly for the small polypeptide, IGF-1. In these conditions we obtained a good precipitation efficiency of 100% for VEGF and HGF and 80% for IGF-1.

PAMs with a homogenous biomimetic coating of LM released continuously the three GFs for up to 3 weeks. An initial burst followed by a release of around 25 % of the encapsulated protein was observed for the 3 proteins. This incomplete release usually may be due to protein aggregation and non-specific adsorption by electrostatic interactions to the PLGA matrix (Giteau, Venier-Julienne et al. 2008; Paillard-Giteau, Tran et al. 2010). However, the release profile of bioactive HGF and VEGF were similar to those detected by ELISA, indicating that the released protein was active. For 1mg PAMs, which constitutes an easily implantable quantity, we obtained around 233 ng/mL release of VEGF, 344 ng/mL HF, 320 ng/mL IGF-1 corresponding to physiological doses of the GFs. Additionally, when ADSCs were cultured for 1 week on PAMs releasing HGF and IGF-1 in vitro, the expression of cardiac transcription factors GATA4 and MEF2C was induced as well as the expression of the contractile sarcomeric protein cTnI and the gap junction protein GJA1/CX43, indicating a late-stage cardiac differentiation of the adhered cells. Moreover, CX43 was highly expressed in these conditions and the cells expressing this gap-junction protein were found homogeneously distributed within and all around the complexes.

HGF has been described as an inductor of embryonic stem cells (ESCs) and MSCs differentiation into cardiomyocytes. Recently, it has been demonstrated that in vitro injection of 20ng/mL HGF induced mouse MSCs commitment into cardiac lineage. Another recent study has described the role of injection of HGF and IGF-1 in endogenous CPCs recruitment and proliferation within the infarcted area. PAMs conveying ADSCs which have better differentiative potential than MSCs and releasing HGF and IGF-1, could induce ADSCs

commitment into cardiac lineage while inducing CPCs recruitment and proliferation. Additionally, FN-PAMs releasing VEGF could be used in combination to these formulation for in vivo application to improve cell survival but also angiogenesis as they have shown improvement in adhesion and survival of early endothelial progenitor cells (eEPCs) but also adult endothelial cells migration in vitro (Musilli, Karam et al. 2012).

The use of an injectable thermosensitive HG is an interesting approach because it could mechanically support the beating myocardium without interfering with the electric signal conduction. Integrating PAMs releasing a protein in a thermosensitive injectable hydrogel of poloxamer 405 (P407 or Pluronic®F127) resulted in a slower release profile. This phenomenon is due to an increase in chain entanglement and gel formation that favors hydrophobic interactions. This entanglement is also more marked at higher concentrations of P407, yielding an increase in gel strength and consequently a decrease of the drug release rate. Moreover, the rheological properties are increased when 1% w/v of PAMs is added within the gel. When the amount of PAMs within the HG were increased to reach the injection concentrations of PAMs generally used for in vivo experiments, a slight increase in the gel modulus was observed with the gel at the higher concentration (22%). On the contrary, the elastic modulus of the 18% HG decreased, indicating the loss of the viscoelastic behavior of the HG probably due to a loss of structure. Interestingly, P407 viscoelastic properties obtained with the 22% HG are close to those used in the literature to demonstrate the effect of matrix stiffness on cell fate (Engler, Sen et al. 2006; Engler, Rehfeldt et al. 2007; Reilly and Engler 2010; Young and Engler 2011). Indeed, it has been described that playing on matrix elasticity properties, stem cells can be induced into a defined lineage in a viscoelastic-dependent manner. Interestingly, although the normal modulus of the heart is approximately from 10-20kPa, gels with a 10-13kPa induce MSC's toward a muscular phenotype without the need of additional molecular signal (Tse JR 2011), reinforcing the importance of the substrate stiffness. As the 22% HG with the PAMs has an elastic modulus within this range from 12-15 kPa, the cells adhered onto PAMs contained inside the gel could be induced toward a myogenic lineage and furthermore into a cardiomyogenic commitment with the appropriate differentiating signals. Additionally, although the addition of PAMs into the gel does not modify the elastic modulus, its elasticity is increased as measured by the increase of the ratio G'/G'' suggesting that PAMs integrated within the HG would have sufficient elasticity to support the beating myocardium and appropriate stiffness to induce ADSCs commitment toward the cardiomyogenic lineage.

Thus, we further evaluated whether the 22% P407 hydrogel could influence the commitment of ADSCs adhered on PAMs and releasing GFs, into the cardiomyogenic lineage after 1 week. In this condition, the mRNA levels of the cell-cycle molecule, cyclin D1 markedly decrease compared to ADSC/PAM complexes alone, suggesting fewer proliferating cells. The mRNA of the cardiac transcription factors, NKX2.5 and GATA4, were not expressed and a decrease in the expression of their cofactor, MEF2C was observed. Furthermore, a slight expression of the sarcomeric contractile molecule SERCA2-A was observed, and the mRNA of the gap junction protein GJA1/CX43 was strongly increased. The absence of the mRNA of GATA4, NKX2.5 and cTnI could indicate a transitory expression, as they were also not expressed by ADSCs cultured on PAMs releasing GFs for 1 week although the immunostaining revealed that the proteins GATA4, MEF2C and cTnI were expressed together with CX43. Additionally, cell survival is maintained when cells are adhered to PAMs within the P407 at high gel concentration (Fig). As no cell death was observed with PAMs complexes embedded within the 22% HG, the decrease of cyclin D probably comes from an arrest of cell proliferation to favor the differentiation. This assumption is favored by the high expression of the late stage-differentiation marker, gap junction molecule CX43.

Gap junctional coupling is essential in establishing electrochemical communications between cardiomyocytes. CX43, the major gap junction protein of the working myocardium is implied in cardiac development, i.e. by transferring cardiomyogenic signals within cardiomyocytes (Saez JC, Nairn AC et al. 1997; W. Lo C. 2000; Hervé JC, Derangeon M. et al. 2008). Lack of such a protein results in serious cardiac malformation during development and is correlated to the arrhythmic phenomenon encountered following cell transplantation (NS 1996; Duffy 2008; Fernandes, Rijen et al. 2009). The presence of CX43-expressing cells in PAMs complexes represents a further advantage for cell therapy, as it should allow a better ADSCs engraftment and communication with host cells. Additionally, as its expression is increased when PAMs-ADSCs complexes are integrated within an injectable thermosensitive hydrogel in which no cell death was observed after 72h, we can hypothesize that the transplanted cells would better engraft and home within the infarcted area. However, the protein delivery is lower so the amount of protein released needs to be adapted to cell demand.

In conclusion, these results suggest that PAMs with a biomimetic coating of LM, releasing bioactive VEGF, HGF and IGF-1 can be used as a tool to convey cells and repair the infarcted

heart. PAMs-HGF and PAMs-IGF-1 are able to induce the expression of both early and late cardiac muscle markers when ADSCs are adhered on their surface. While a slower protein release profile is observed, an increase in CX43 expression is also observed when ADSC/PAM-GF complexes are integrated within a P407 hydrogel. Moreover, no cell death was observed when ADSCs-PAMs are integrated within the 22% HG. The presence of CX43 in these conditions also suggests the possibility of communication between these cells. Finally, this communication between cells may affect both mechanical and electrical function, influencing the recovery of cardiac function and proneness to arrhythmias in a manner that needs to be tested.

Taken together, these findings let us to hypothesize that PAMs conveying ADSCs and releasing IGF-1 and HGF within a thermosensitive hydrogel may increase, in the post-MI region, cell survival and differentiation and favor cell communication with hosted cells, allowing a better process of myocardial regeneration and recovery of cardiac function.

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Conclusion

In this article, VEGF, HGF and IGF-1 have been encapsulated within the microspheres after having investigated their precipitation conditions using model proteins (lysozyme, peroxidase, bovine serum albumin). Their release profile corresponds to those described by the literature, with a burst for the first days followed by a slow release of the proteins for up to 3 weeks (Bouffi, C., O. Thomas, et al. 2010, Delcroix G, Garbay E. et al. 2011). The released GFs were bioactive and induced cell proliferation as shown by the NIH3T3 bioassay for IGF-1 and HUVEC cell proliferation bioassay for HGF and VEGF.

The two GFs, HGF and IGF-1 released from LM-PAMs induced the ADSC expression of cardiac transcription factors GATA4 and MEF2C as well as the expression of contractile sarcomeric protein cTnI and the gap junction protein GJA1/CX43, indicating a late-stage differentiation step of the adhered cells. Interestingly, the CX43 was co-localized with the cTnI and MEF2C at the periphery of the complexes suggesting the presence of more differentiated cells. These results are similar to those obtained with ADSCs adhered on LM-PAMs in combination with a cocktail of GFs, indicating that both HGF and IGF-1 could together induce the differentiation of ADSCs into cardiomyogenic lineage.

Moreover, the elastic modulus is increased when 1% w/v of PAMs is added to the gel. Increasing the amount of PAMs within the HG to reach the injection concentrations generally used for in vivo transplantation (10%), a slight increase in the young modulus was observed with the HG with higher P407 concentrations. It could thus be adapted to induce ADSCs within the myogenic lineage as described for MSCs and smooth muscle cells (SMCs) and additionally synchronically respond to the electromechanical properties of the heart. The combination of a thermosensitive viscoelastic hydrogel improved hADSCs survival in vitro but decreased the release profile of the model protein lysozyme. Interestingly, after 96h hADSCs and hMSCs survival remain stable when they are adhered to empty FN-coated PAMs suggesting an anti-apoptotic/survival effect of PAMs. In this way, the combined thermosensitive HG/PAM/cell complex could allow injection through a needle or a catheter, thus reducing heavy surgery while increasing their retention in the host tissue (cartilaginous

tissue or myocardium) by in situ gelling process. This study suggests that the use of PAMs in combination with a defined P407 HG holds great promise as a drug releasing system enhancing adult stem cell retention, commitment and survival as an efficient regenerative medicine approach.

Chapter 4

Protein-loaded PLGA–PEG–PLGA microspheres: A tool for cell therapy

Scientific context

In parallel to the previous work, a strategy to further ameliorate the release profile of GFs from the PAMs was elaborated. Indeed, the continuous and complete release of a bioactive factor from PLGA microspheres remains a technological challenge. It is now well established that low and incomplete protein release occurs from PLGA microspheres, due to protein-polymer interaction during the release period. Protein nano-coprecipitation with poloxamer 188 permits to overcome its adsorption onto the hydrophobic surface of the polymer, considered as one of the major problems during the first week of the release (Giteau A. et al. 2008). Indeed, Poloxamer188 is an amphiphilic tensio-active containing a hydrophobic polypropylene oxide (PPO) at the center and two hydrophilic extremities of polyethylene oxide (PEO), allowing both interaction of the protein with the polymer and the protection from aggregation and adsorption to the surface of the polymer (Lee R., Despa F. et al. 2006). However, with this co-precipitation approach an incomplete release is still obtained.

Association of this co-precipitation technique and the use of a more hydrophilic copolymer could constitute an ideal strategy to formulate microspheres showing a complete protein release profile. It has been shown that the presence of hydrophilic polyethylene glycol (PEG) segments provides protein stability by limiting protein –polymer interaction and also permits a better release profile (Kissel T., Li YX et al. 2002; Paillard-Giteau A., Tran VT et al. 2010). Therefore, in this work a tri-block copolymer of PLGA-PEG-PLGA (ABA) was used to formulate the microspheres. The impact of different combinations of (B) and (A) segments on protein encapsulation and delivery has been investigated with a model protein (lysozyme) currently used in our laboratory (Aubert-Pouessel A. et al. 2002). The mechanisms of lysozyme instability during the protein release were also studied. Furthermore, as PAMs are covered with a protein biomimetic surface, the adsorption of FN to the microspheres was studied. Finally, hMSCs adhesion and viability on these PAMs have been assessed to determine the effectiveness of this strategy for tissue engineering.



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Protein-loaded PLGA–PEG–PLGA microspheres: A tool for cell therapy

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ABSTRACT

A promising strategy to repair injured organs is possible by delivering a growth factor *via* poly-(D,L lactide-co-glycolide) (PLGA) microspheres; the latter are coated with adhesion molecules that serve as a support for cell delivery. At present, PLGA is not the optimal choice of polymer because of poor or incomplete protein release. The use of a more hydrophilic PLGA–PEG–PLGA (A–B–A) copolymer increases the degree of protein release. In this work, the impact of different combinations of (B) and (A) segments on the protein-release profile has been investigated. Continuous-release profiles, with no lag phases, were observed. The triblock ABA with a low molecular weight of PEG and a high molecular weight of PLGA showed an interesting release pattern with a small burst (<10% in 48 h) followed by sustained, protein release over 36 days. Incomplete protein release was found to be due to various causes: protein adsorption, protein aggregation and protein denaturation under acidic conditions. Interestingly, cell viability and cell adhesion on microspheres coated with fibronectin highlight the interest of these polymers for tissue engineering applications.

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1. Introduction

Cell therapy carried out by grafting autologous or non-autologous cells is a promising strategy to repair injured organs (Delcroix et al., 2009; Dennis et al., 2007). However, the survival and functional state of the cells after transplantation still need to be improved (Delcroix et al., 2009; Tabata, 2000). Growth and differentiation factors may improve cell survival, cell differentiation and affect the immediate environment, thus enhancing graft integration. Nevertheless, the delivery of these factors still remains a technological challenge due to their fragile structure and their short half-life after administration. To overcome these difficulties, the growth factors can be protected in biodegradable microparticles which offer controlled and sustained release after administration (Aubert-Pouëssel et al., 2004; D'Aurizio et al., 2011).

Our group has demonstrated the interest of a tissue engineering system named Pharmacologically Active Microcarriers (PAMs) to improve grafting in the host tissue. These biodegradable particles made with poly-(D,L-lactic-co-glycolic acid) (PLGA) with an adapted size (60 µm), presenting a biomimetic surface of cell adhe-

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sion/extracellular matrix molecules, served as a support for cell administration and the programmed delivery of an appropriate protein. The combined effect of the 3D, biomimetic surface and the delivered growth factor increased cell survival and differentiation of the transported cells and also enhanced the regenerative potential of stem cells (Bouffi et al., 2010; Delcroix et al., 2011; Tatard et al., 2004, 2005a). A PLGA polymer was first chosen because of its biodegradable and non-toxic nature (Fournier et al., 2003). However, low and incomplete protein release from PLGA microspheres are related to protein instability during the release period (Determan et al., 2006; Fu et al., 2000). Our group and others have shown that by introducing hydrophilic segments poly(ethylene glycol) (PEG) into hydrophobic polyesters, PLGA, protein release from PLGA–PEG–PLGA (ABA) triblock copolymer microspheres was enhanced (Kissel et al., 2002; Paillard-Giteau et al., 2010). Due to the presence of PEG segments, cross-linked biodegradable hydrogel formed upon contact with water thus favouring protein release (Li and Kissel, 1993). The PEG segment itself also promotes the stability of proteins (Kissel et al., 1996). In this regard, Kissel et al. (2002) demonstrate a complete release of lysozyme from PLGA–PEG–PLGA with a high protein loading of 5%.

Tissue engineering approaches combining 3D biomimetic systems and the sustained release of therapeutic factors represent a technological improvement for cell therapy studies. Accurate delivery of therapeutic proteins at physiological levels requires low encapsulation loading of these otherwise expensive proteins

(VEGF, CNTF, GDNF) (Aubert-Pouëssel et al., 2004; Bertram et al., 2009; Boerckel et al., 2011), which accentuates protein destabilisation following polymer–protein interaction. However, most of research in the literature was focused at high protein loading (more than 3%) (Kissel et al., 2002) or focused on the *in vivo* bio-application where the reason of incomplete protein release was not clarified (Chen and Hu, 2011). It is therefore essential to investigate protein release from ABA copolymers with low loading (<1%) in order to elucidate protein behaviour during a long release period. Furthermore, although the co-precipitation of the stabilizer agent with the protein presents particular interest to stabilize the protein without affecting the burst effect (Paillard-Giteau et al., 2010), the association of the co-precipitation with the systematic study of the polymers was not yet evaluated. In the present study, the impact of ABA copolymer composition and molecular weight on the release profile and protein stability were studied. Lysozyme was used as a model protein because it is representative of the physical and chemical properties (isoelectric point and molecular weight) and the adsorption behaviour of therapeutic growth factors such as NGF, TGF- β 3 and NT3 (Aubert-Pouëssel et al., 2002; Paillard-Giteau et al., 2010).

The adsorption of fibronectin on ABA copolymers was also evaluated to assess the capability of creating an appropriate 3D biomimetic surface with these copolymers. Fibronectin was used as a bioadhesive substance for this biomimetic surface because it enhances the attachment of various stem cells *in vitro* and affects their behaviour such as survival, migration and proliferation (Delcroix et al., 2009). Cell adhesion and cell survival on the microspheres with a fibronectin biomimetic surface were studied. The goal of this study is to follow the stability of the protein released from different PLGA–PEG–PLGA triblock copolymers and to create a biomimetic 3D surface by fibronectin adsorption in order to improve a tissue-engineering approach.

2. Materials and methods

2.1. Materials

Lysozyme (chicken egg white) and its substrate *Micrococcus lysodeikticus*, glycofurol (tetraglycol or α -[(tetrahydro-2-furanyl)methyl]- ω -hydroxy-poly(oxy-1,2-ethanediyl)), fibronectin, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), BSA-FITC and dextran-FITC were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Polyvinyl alcohol (Mowiol® 4-88) was obtained from Kuraray Specialities Europe (Frankfurt, Germany). Pluronic F68 was kindly supplied by BASF (Levallois-Perret, France). Culture mediums, penicillin, streptomycin and trypsin were obtained from Invitrogen (Cergy Pontoise, France). Uncapped (free carboxylic acid group at the terminal end) PLGA37.5/25 (Mn 14,000 Da) was provided by Phusis (Saint-Ismier, France). DL-Lactide and glycolide were obtained from Purac (Gorinchem, The Netherlands) and poly(ethylene oxide) from Fluka.

2.2. Methods

2.2.1. Polymer synthesis and characterisation

A series of nine PLGA–PEG–PLGA copolymers were synthesised from the combination of various molecular weights of PLGA segments (20, 30 and 40 kDa) and PEG segments (4, 12 and 20 kDa). The polymers are indicated as PLGxPEGy, in which “x” and “y” are the PLGA and PEG segment molecular weights in kDa, respectively.

The triblock copolymer PLGA–PEG–PLGA (ABA copolymer) was prepared by ring-opening polymerisation of DL-lactide and glycolide using PEG as an initiator, and stannous octoate [Sn(Oct)₂] as a catalyst (Garric et al., 2008). Briefly, precise amounts of various

PEG, DL-lactide and glycolide were mixed and introduced into 100 mL round-bottom flasks with the catalyst. The mixtures were heated to 140 °C and degassed by vacuum-nitrogen purge cycles in order to remove the moisture and the oxygen, inhibitors of this polymerisation. Following this, the flasks were sealed under dynamic vacuum at 10^{−3} mbar and the polymerisation was allowed to proceed at 140 °C under constant agitation. After 5 days, the products were recovered by dissolution in methylene chloride and then precipitated by adding the same volume of ethanol. Finally, the polymer was filtered and dried overnight under reduced pressure, up to constant weight.

The ABA copolymer was characterised by ¹H NMR spectra and size exclusion chromatography (SEC). The molecular weight of the PLGA block was determined by using the integration ratio of resonance of PEG blocks at 3.6 ppm and PLGA blocks at 4.76 ppm in the ¹H NMR spectra. The molecular weights of the copolymers were determined by SEC using Waters Inc. equipment fitted with a Plgel 5 μ m mixed-C (60 cm) column as the stationary phase and a Waters 410 refractometric detector, eluted with DMF at 1 mL min^{−1}. Typically, samples were dissolved in DMF at 10 mg/mL and filtered on PTFE filter Millex®-FH (pore size 0.45 μ m) from Millipore Corporation, prior to 20 μ L of the solution of polymer being injected. The Mn and Mw were expressed according to calibration against poly(styrene) standards.

Differential scanning calorimetry (DSC) was performed with a Mettler Toledo Star System (Mettler-Toledo, Viroflay, France). Samples (10 mg) were placed in a sealed aluminium crucible; they were first heated from 25 to 80 °C, then thermograms covering a range from −50 to 100 °C were recorded at a heating rate of 10 °C min^{−1}. The T_g of the polymer were determined thanks to the DSC technique. The PLGA 37.5/25 with the known T_g (42.5 °C) was used as control.

For analysis of the swelling properties of polymers, the relative water-uptake of polymer film was measured: polymer film was prepared by solvent casting; 10 mg of polymer were dissolved in DMSO, poured onto a glass dish and subsequently air-dried. Polymer films were immersed in distilled water at room temperature to obtain the wet weight. After 2 days of incubation, the relative water-uptake was calculated as a percentage of water absorbed into the polymer films against initial dry polymer film weight. The relative water-uptake of polymer films was repeated three times and the values were presented as mean \pm SD.

2.2.2. Microsphere preparation and characterisation

Microspheres were prepared using an s/o/w emulsion solvent evaporation–extraction process adapted from Giteau et al. (2008): 45 μ L of an NaCl 0.3 M solution containing 900 μ g of protein and 9 mg of Poloxamer 188 was added to 1.04 g glycofurol to form a 1 mL suspension. After 30 min (at 5 °C), the protein particles were recovered by centrifugation (10,000g, 5 °C, 30 min). They were then carefully dispersed in the organic solution (2 mL; 3:1 methylene chloride:acetone) containing 150 mg of polymer. This organic suspension was emulsified in a poly(vinyl alcohol) aqueous solution (90 mL, 4%w/v) maintained at room temperature and mechanically stirred at 550 rpm for 1 min (Heidolph RZR 2041, Merck Eurolab, Paris, France). After the addition of 100 mL of deionised water and stirring for 10 min, 500 mL of deionised water was added to the resulting o/w emulsion and stirred at 300 rpm for 20 min to extract the organic solvent. The suspension was sieved through a 125 μ m stainless mesh and then recovered by sieving through a polypropylene 37 μ m filter. Microspheres were washed with 500 mL of deionised water and then freeze-dried before storage at −20 °C.

To track the protein during the *in vitro* release, lysozyme-FITC was encapsulated into PLG40PEG4 microspheres with the same process. Lysozyme-FITC was prepared as defined by Bezemer et al. (2000).

To assess the pH level in the microspheres during the *in vitro* release, 1.5 mg of dextran-FITC was added to the aqueous protein solution before the precipitation step.

The surface morphology of the microparticles was investigated by scanning electron microscopy (SEM, JSM 6310F, JEOL, Paris, France). Freeze-dried microparticles were mounted onto metal stubs using double-sided adhesive tape, vacuum-coated with a film of carbon using a MED 020 (Bal-Tec, Balzers, Lichtenstein).

The average particle size and size distribution were determined using a Coulter Multisizer (Coultronics, Margency, France). The microparticles were suspended in isotonic saline solution and sonicated for a few minutes prior to analysis. The mean particle sizes are expressed as volume distributions.

The protein encapsulation yield was determined considering the biologically active entrapped protein as previously reported (Aubert-Pouëssel et al., 2002; Giteau et al., 2008). Lysozyme-loaded microspheres (10 mg, 3 batches) were dissolved in 0.9 mL DMSO in a 5 mL PTFE tube. After 1 h, 3 mL of 0.01 M HCl were added. The solution was left to stand for one more hour and was then incubated with a *M. lysodeikticus* suspension for lysozyme activity determination. The amount of active protein was calculated using a standard curve.

2.2.3. *In vitro* release study

The *in vitro* release profile of lysozyme from the microspheres was determined by adding 500 μ L of 0.05 M TRIS–HCl buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl to 10 mg of microspheres, into the centrifugation tubes. The tubes were closed and incubated in a shaken water bath (37 °C, 125 rpm). At determined intervals, the tubes were centrifuged for 5 min at 2800g to collect the supernatant and to analyse the pH, lysozyme and poloxamer release. The supernatant was then replaced by fresh buffer.

2.2.3.1. Assessment of protein integrity. To follow the behaviour of unreleased protein during the *in vitro* study, lysozyme-FITC encapsulated into microspheres was observed by fluorescent microscopy. At determined time-points, microspheres were collected by centrifugation (5 min, 2800g) to assess the bioactivity of remaining lysozyme in the matrix.

The aggregation of remaining lysozyme in the microspheres was investigated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The microspheres were dissolved in DMSO (500 μ L). After 1 h, 1 mL of 0.01 N HCl was added and the tubes were slowly vortexed for one more hour. The tubes were centrifuged at 13,400g for 5 min and 1 mL of the supernatant was saved for freeze-drying. The freeze-dried protein was then dissolved in 50 μ L of 0.05 M Tris–HCl buffer. Fifteen microlitre of extracted lysozyme were diluted with SDS buffer (1/1 w/w) and subjected to electrophoresis on a 10% SDS–polyacrylamide gel under reducing (with β -mercaptoethanol) and non-reducing conditions. Electrophoresis was performed using a Bio-Rad Mini-Protein electrophoresis system at a constant voltage (80 V). Gels were stained with 0.1% Coomassie blue solution and then processed with an aqueous solution of 10% methanol and 10% acetic acid. The molecular weights of the detected bands were compared to standard molecular weights. Standard lysozyme treated under the same condition was used as a control.

2.2.3.2. Poloxamer release. The amount of encapsulated poloxamer was determined by a colorimetric method based on the formation of a coloured complex between polyethylene oxide segments of poloxamer and cobalt(II) thiocyanate (Paillard-Giteau et al., 2010). Briefly, 10 mg of microspheres were digested with 400 μ L of NaOH 0.2 N for 8 h (until the solution became limpid) and neutralised with 400 μ L of HCl 0.2 N. This solution was lyophilised; the freeze-dried poloxamer was dissolved in 40 μ L of Tris HCl 0.05 M.

Six hundred microlitre of cobalt thiocyanate reagent, 100 μ L of ethyl acetate and 200 μ L of absolute ethanol were then added and the mixture was centrifuged (1 h, 13,360g). The sediment was washed with ethanol and subsequently dissolved in 1 mL of acetone. The absorbance was measured at 624 nm. The quantity of poloxamer was compared to a standard curve. A control (microspheres without poloxamer) was carried out to consider the polyethylene oxide segment of the ABA copolymer.

To quantify the released poloxamer, release buffers were collected and lyophilised; the freeze-dried poloxamer was quantified as described above.

2.2.3.3. Polymer degradation. During an *in vitro* release study, the microspheres were collected, washed and freeze-dried at determined time intervals.

DSC measurements were carried out to determine the variations of polymer T_g .

The molecular weights of the polymers were determined by SEC. To avoid interference with BSA and lysozyme, the microspheres were dissolved in 4 mL of dichloromethane; 4 mL of distilled water was then added to extracted water-soluble substances under shaking for 8 h. The dichloromethane phase was then recovered and the solvent was removed under vacuum. The polymer was then dissolved in DMF at 5 mg/mL, which was analysed by SEC as described in polymer characterisation.

2.2.3.4. pH measurement inside and outside the microspheres. To assess the buffer effect of the release medium, a release study of four types of ABA copolymers was achieved in 1 mL of the same buffer solution and replaced with fresh buffer at 2-day intervals. The micro-pH in the microspheres was assessed as described by Shenderova et al. (1999). Briefly, 1% w/w of dextran-FITC loaded microspheres was incubated under *in vitro* release conditions, typically 1 mL of releasing buffer containing 100 μ g dextran-FITC at 37 °C under shaking. The release buffer was totally replaced every 2 days to stabilise the pH of the release medium. The microparticles were observed by confocal microscopy (Olympus Light Microscope Fluoview FU300, Paris, France). The excitation wavelength was 488 nm and the emission filter was 515 nm. To investigate the pH-dependence of fluorescence intensity, images of probe solutions at 0.1 mg/mL at pH 2, 5 and 7.4 were taken. The black and white images were transformed into colour with Adobe PhotoShop software (Adobe Systems Incorporated, CA). The indexed colour mode was selected and the level of grey from black and white was transformed into colours from violet to red with a spectrum colour table.

2.2.3.5. Lysozyme stability study. To assess the biological stability of lysozyme, a full factorial experimental design (2^3) was used to study the effect of three additives on lysozyme stability at pH 2, 3, 4 and 5 over a period of 8 days. The concentration was determined by considering the *in vitro* conditions: lysozyme 0.12 mg/mL, BSA 1 mg/mL, Poloxamer 2 mg/mL, NaCl 0.88 mg/mL.

2.2.4. Biomimetic surface, cell adhesion and viability

2.2.4.1. Biomimetic coating. Films of polymer were incubated in a 10 μ g/mL solution of fibronectin for 1.5 h at 37 °C. The coated polymer films were washed three times with PBS. The fibronectin coating was determined by immunofluorescence. Briefly, a saturation step with a PBS solution containing 4% BSA for 60 min at room temperature was performed. Polymer films were washed three times with PBS, followed by incubation with a monoclonal mouse, anti-human, fibronectin antibody (1:100) overnight at 4 °C. The sections were then washed with PBS and incubated with a biotinylated horse, anti-mouse, IgG antibody (1:200) for 60 min, washed in PBS and incubated with Streptavidin-fluorophore 547 (1:500) for 40 min. Isotype control was also performed.

Fibronectin adsorption was also evaluated on ABA microspheres to ensure the similarity of the protein coating between the 2D and 3D adsorption model. Fibronectin-coated microspheres were prepared in the same conditions as polymer films.

Streptavidin-fluoprobe was visualised by using a Axioskop 2 Zeiss microscope and was analysed using METAMORPH software.

2.2.4.2. Cell adhesion. Human marrow stromal cells (MSC) were isolated and cultured as previously described (D'Ippolito et al., 2004). Briefly, cells from the stromal vascular fraction of bone marrow were seeded on 1.25 ng/cm² of a fibronectin-like coated flask at 100 cells/cm² and cultured for several passages in DMEM supplemented with 3% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, in a humidified atmosphere of 3% O₂, 5% CO₂ at 37 °C. The media were changed twice a week. When reaching 50–60% confluency, the cells were detached with 0.5 mM EDTA/0.05% trypsin for 5 min at 37 °C.

Microparticles (0.5 mg) were coated at 37 °C in a 5% CO₂ incubator with 18 µg/cm² of fibronectin-like material in a serum-free medium under 15 rpm continuous rotation for 1.5 h. The coated microparticles (PAM) were immediately washed three times with double-distilled water supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin after the centrifugation steps, freeze-dried and then stored at 4 °C before use. They were then incubated with 1.2×10^5 cells in DMEM with 3% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin for 4 h without any agitation in 24 Ultra low attachment plates (Costar). After centrifugation (2 min, 1000 rpm) to remove non-adherent cells, the percentage of microcarriers conveying MSC was evaluated by CyQuant® cell proliferation kit (Invitrogen) according to the manufacturer's indications. After subtracting the absorbance of the microcarriers, the number of adherent cells on the ABA microspheres was calculated using a standard curve.

3. Results

3.1. Polymer characterisation

Characteristics of the nine synthesised ABA copolymers are reported in Table 1. The molecular weights of the copolymers were measured by ¹H NMR and were found to be close to the theoretical molecular weights. SEC data show that the polydispersity index for most of the copolymers are between 1.5 and 2. These values are commonly obtained with this polymerisation method compared to controlled radical polymerisation.

As expected, polymer *T_g* values increased with the molecular weight of PLGA segments and decreased with the molecular weight of PEG segments, PEG segments plasticise the material (Table 1). The *T_g* of the nine studied polymers were all under the *in vitro* temperature (37 °C); the rubbery form of these copolymer matrices could thus contribute to favour protein diffusion through this carrier system.

The percentage of water uptake in the polymer films is shown in Table 1. The hydrophobicity of the PLGA segment limited water-uptake. With the small hydrophilic PEG segment (4 kDa), water-uptake is low (<60%) regardless of the size of PLGA segments. By increasing the PEG segment to 20 kDa, the water-uptake increases up to 254%. The “anti-water-uptake” effect of PLGA was more pronounced with long PEG segments. This will significantly influence the characteristics of lysozyme-loaded microspheres.

3.2. ABA microspheres characterisation

Microsphere diameters were in the range of $[61\text{--}89] \pm [19\text{--}24]$ µm. A variation of lysozyme encapsulation efficiency is shown in Table 1 and the microsphere surface observed by scanning

electron microscopy is shown in Fig. 1. Microspheres based on the smallest PEG segments and the longest PLGA segments, copolymer (PLG40PEG4), showed the highest level of encapsulation efficiency (70%) and presented a smooth surface, whereas the microspheres with the longest chains of PEG and shortest chains of PLGA (PLG20PEG20) showed the lowest level of encapsulation efficiency with the highest level of porosity. The PEG segment thus increased the porosity of the microspheres and reduced encapsulation efficiency. In order to comprehend the impact of polymer composition on protein release, taking into account encapsulation efficiency and particle structure, these formulations were used without an optimisation step.

Fig. 2 shows the *in vitro* lysozyme release profiles over 50 days as a function of the molecular weights of PEG. At 48 h, lysozyme release increased dramatically from 8% for PLG40PEG4 to 28% for PLG20PEG20. The molecular weight of PEG was the most influent factor on protein release compared to the PLGA segment. Increasing the molecular weight of the PEG segment increased the quantity of protein released at the initial stage and thus achieved high protein release levels at the plateau. After a burst, PLG20PEG20 had released 52% of lysozyme by Day 8 and reached 61% at the plateau. In contrast, PLG40PEG4 presents an interesting release profile with a small burst; only 8% was initially released within 48 h, followed by a continuous, sustained release to achieve 31% by Day 45.

3.2.1. Bioactivity of lysozyme in the microspheres

After the screening step, PLG40PEG4 appears to be a good candidate with a high encapsulation efficiency, a low burst effect and a good continuous-release profile over 36 days. However, there is still 70% of unreleased protein, so the lysozyme state and environment within the microspheres were investigated in the PLG40PEG4 microspheres. Toward this aim, the *in vitro* release of lysozyme-FITC was studied (Fig. 3A). Although the microspheres were degraded into large blocks of polymer by Day 43, the protein still remained in the polymer matrix. The quantification of active lysozyme remaining in the PLG40PEG4 matrix was achieved. At Day 8 and Day 22, 4 and 48% of lysozyme was denaturated respectively and remaining lysozyme was totally inactive by Day 43. Lysozyme gradually lost its biological activity within the matrix during the *in vitro* incubation period (Fig. 3B).

Poloxamer has the capacity to protect lysozyme from adsorption onto hydrophobic PLGA segments (Paillard-Giteau et al., 2010). Poloxamer encapsulated into PLG40PEG4 was quantified at 0.3% w/w (30 µg poloxamer/10 mg microspheres). Due to its high hydrophilicity, more than 50% of poloxamer leaked out into the release buffer after 48 h of incubation and 91% after 1 week. The anti-adsorption effect of poloxamer was thus limited to 1 week.

The protein conformation was studied by SDS-PAGE (Fig. 4). After 2 days under *in vitro* release conditions, only a single band of lysozyme was observed. By Day 8, a slightly stained, second band of protein between the 25 and 37 kDa reference bands was observed. This band matched dimer lysozyme aggregation, which was dissociated under reducing conditions (Fig. 4.2). On Day 22, a similar amount of lysozyme aggregations, resistant to denaturing conditions, was observed.

3.2.2. Polymer degradation and protein denaturation under acidic conditions

The *in vitro* degradation study of the PLG40PEG4 microspheres showed that the molecular weight of the copolymer decreased dramatically from 89 to 62 kDa by Day 2, to 17 kDa by Day 8 and finally to 5 kDa by Day 29 (Fig. 5A). On the contrary, the *T_g* of PLG40PEG4 microspheres increased from 26 to 35 °C by Day 8 and decreased gradually to 28 °C by Day 29 (Fig. 5B). The *T_g* increase during the first week suggests that the PLGA was no longer

Table 1
Physical characterisation of PLGA–PEG–PLGA copolymers.

Theoretical molecular weight		Code name	Actual molecular weight				T_g (°C)	Relative water-uptake of polymer films (%)***	Lysozyme encapsulation efficiency of microspheres (%)
PEG segment Mn (kDa)	PLGA segment Mn (kDa)		PEG segment Mn (kDa)	PLGA segment Mn (kDa)*	Mn total (kDa)**	Mw total (kDa)**			
4	20	PLG20 PEG4	4	21	60	103	1.7	26.3 ± 1.3	66 ± 4
4	30	PLG30 PEG4	4	30	41	156	3.2	32.7 ± 0.1	50 ± 9
4	40	PLG40 PEG4	4	45	100	159	1.6	32.6 ± 0.8	49 ± 22
12	20	PLG20 PEG12	12	17	57	89	1.6	0.5 ± 0.7	191 ± 47
12	30	PLG30 PEG12	12	27	80	160	2.0	14.1 ± 1.4	119 ± 9
12	40	PLG40 PEG12	12	38	49	86	1.8	19.3 ± 0.9	87 ± 41
20	20	PLG20 PEG20	20	17	53	81	1.5	−10.3 ± 0.8	254 ± 38
20	30	PLG30 PEG20	20	29	41	69	1.7	−4.0 ± 1.3	146 ± 57
20	40	PLG40 PEG20	20	38	39	93	2.4	10.0 ± 1.1	32 ± 20

* Determined by ^1H NMR: molecular weight of PLGA block was determined by using the integration ratio of resonance due to PEG blocks at 3.6 ppm and PLGA blocks at 4.76 ppm in the ^1H NMR spectra.

** Determined by size exclusion chromatography (SEC).

*** Determined after 48 h of incubation in distilled water.

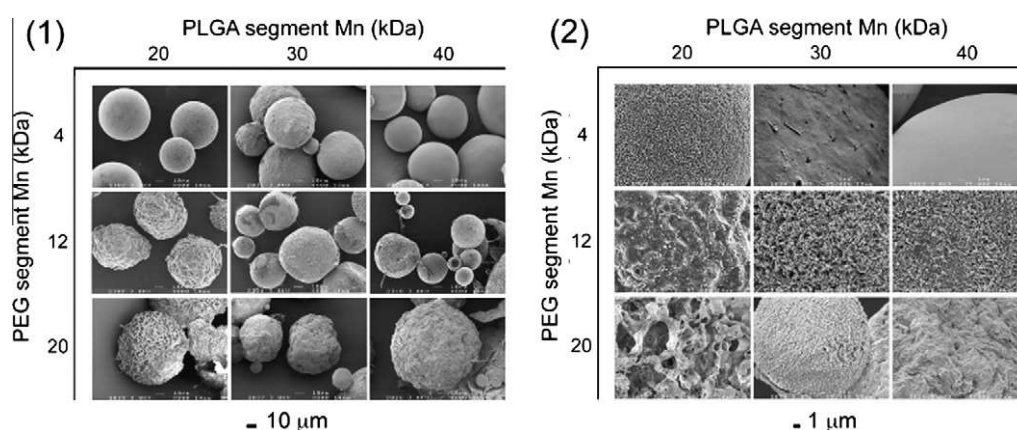


Fig. 1. Scanning electronic microscopy of microspheres at (1) 800× and (2) 5000× magnification.

plasticised by PEG, the PEG segment was partially lost. Relatively short PEG segments diffuse out of the residual specimen from the beginning of degradation was also reported previously (Kissel et al., 2002). After that, the T_g decreased in correlation with polymer degradation.

The acidic polymer degradation products changed the pH of the buffer medium (Fig. 6A). The most acidic pH was obtained with the polymer containing the smallest PEG, for a defined PLGA segment. Increasing the PEG molecular weight (4, 12 and 20 kDa) reduced the onset-time of the decrease in pH (Days 22, 15 and 8, respectively). This result can be attributed to a faster degradation rate and to a higher exchange between the release buffer and the core of the microspheres of high Mw PEG-containing polymers. During dissolution test, to avoid drug accumulation in the release medium, sink conditions were provided. In the case of PLGA-based drug delivery systems these conditions are particularly important to limit the acidic environment (Klose et al., 2011).

By increasing the buffer volume and changing it more frequently the pH of the release buffer was maintained constant during the release period (Fig. 6B). Under these conditions, an increase in the amount of lysozyme released from PLG40PEG20 was observed compared to previous conditions, due to higher lysozyme release levels between Day 4 and Day 8 (Fig. 6C). Increasing the release buffer volume may favour protein release via diffusion at the initial stage. However, no beneficial effect on the total protein release profile was observed when the pH was kept constant. A plateau was still reached by Day 30, regardless of polymer type (Fig. 6C).

The pH of the microenvironment within the microspheres was assessed using a pH-sensitive probe, fluorescein. The intensity of

a fluorescein emission decreases when the pH decreases. As described by Shenderova et al. (1999), if the microsphere microenvironment is acidic, no emission from within the microsphere can be observed. Although the pH of the release buffer is kept at about 7.4 (orange-green colour), the pH of the microenvironment of PLG40PEG4 microspheres decreased by Day 8 (blue colour in the core of the microspheres) and became totally acidic by Day 14 (Fig. 6B). On the contrary, the pH of the microenvironment of PLG20PEG20 was acidic on Day 2 and was then neutralised by the release buffer. The higher exchange between the core of PLG20PEG20 microspheres and the release buffer due to their swollen porous structure limited the accumulation of polymer degradation products and consequently the internal acidic microenvironment (Kissel et al., 1996). But, the lag time between the onset of polymer degradation and the leakage of degradation products must be considered. With the highest PEG segment (PLG20PEG20), at least 8 days are necessary to wash out the acidic microclimate.

As pH levels in the microspheres decreased, the bioactivity of lysozyme in aqueous solution at various pH levels, with or without NaCl, BSA or poloxamer, were assessed. At pH 4 and 5, lysozyme activity was protected independently of the composition of the release buffer over 8 days. At pH 2, lysozyme stability is strongly dependent on the composition of the medium (Table 2).

3.3. Biomimetic surface and cell adhesion

Fig. 7A shows the semi-quantitative fluorescence intensity of the fibronectin biomimetic surface on the various polymers (polymeric films and microspheres). High fluorescence intensity

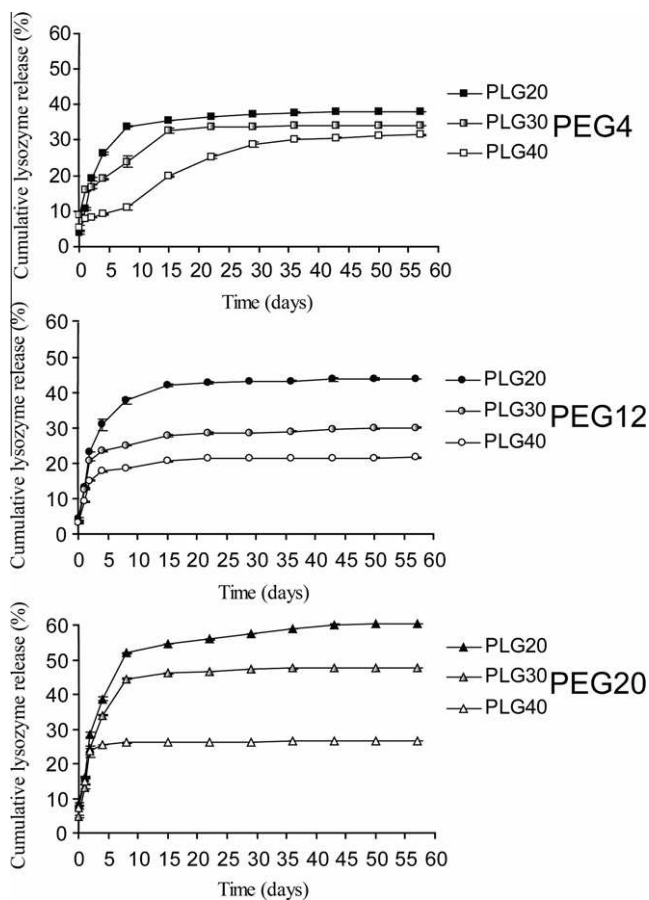


Fig. 2. *In vitro* release profile of lysozyme (mean \pm SD) from ABA microspheres ($n = 3$ for each point) (error bar $<1\%$).

indicates high fibronectin adsorption on the polymer films studied. As PLGA 37.5/25 has been used to formulate the PAMs in previous studies (Bouffi et al., 2010; Tatard et al., 2007b), fluorescence

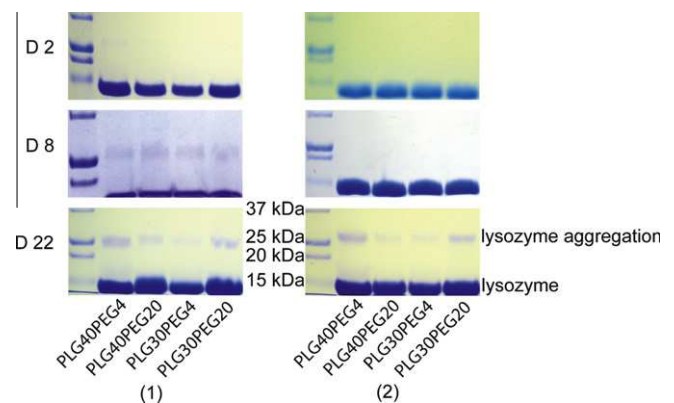


Fig. 4. SDS-PAGE of lysozyme remaining in the PLG40PEG4, PLG40PEG20, PLG30PEG4 and PLG30PEG20 microspheres during *in vitro* release period under non-reducing conditions (1) and reducing conditions (2) on Days 2, 8 and 22.

intensity measured from fibronectin-coated PLGA 37.5/25 was set at 1000 as a reference. For polymers containing 4 and 12 kDa PEG chains, high levels of fibronectin adsorption was found with high molecular weights of PLGA whereas low fibronectin adsorption was found with small PLGAs (PLG20PEG12). Highly hydrophilic polymers (PEG 20 kDa) show weak fibronectin adsorption regardless of the molecular weight of the PLGA segments. Fibronectin adsorbed on microspheres showed the same tendency as on polymeric films. Fibronectin-coated PLG40PEG4 microspheres showed the best fibronectin adsorption (Fig. 7A.2).

PLG40PEG4 and PLG20PEG20 were selected to study the marrow stromal cell (MSC) behaviour on ABA microspheres and compared to PLGA 37.5/25 microspheres used as a reference. After 4 h, the cell adhesion on microspheres with a fibronectin biomimetic surface was determined (Fig. 7B). MSCs showed good adherence (around 90%) onto the fibronectin-coated ABA microspheres regardless of their composition and of the quality of their biomimetic surface. Furthermore, all the polymers (PLG40PEG4, PLG20PEG20 and PLG37.5/25) showed good compatibility with MSC cells. Cell viability after one week of culture with the three different microspheres

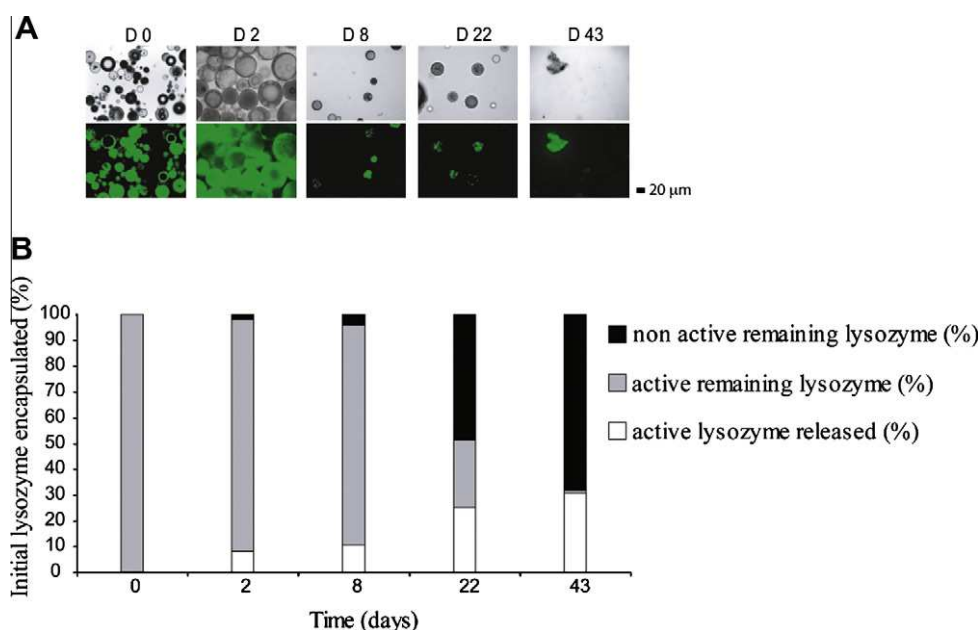


Fig. 3. (A) Optical and fluorescence images of lysozyme-FITC (represented by the bright zone) remaining in PLG40PEG4 microspheres during *in vitro* release on Days 0, 2, 8, 22 and 43 and (B) bioactivity of lysozyme in the PLG40PEG4 microspheres during the *in vitro* release period.

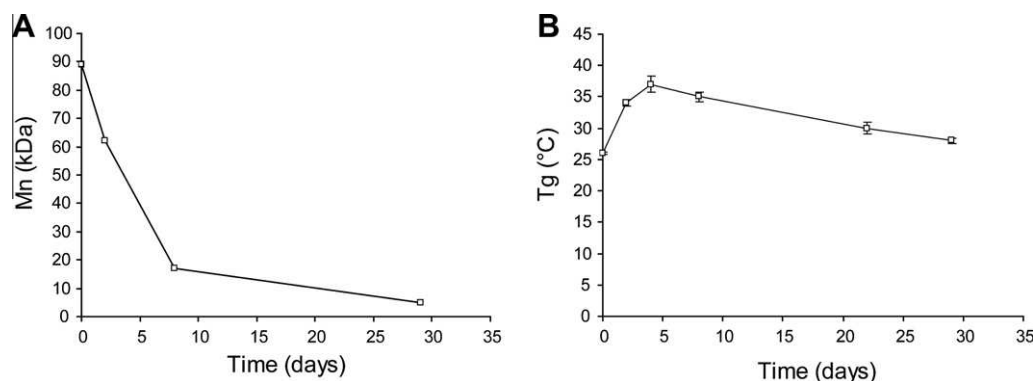


Fig. 5. Molecular weight (A) and T_g changes (B) of PLG40PEG4 during *in vitro* release studies.

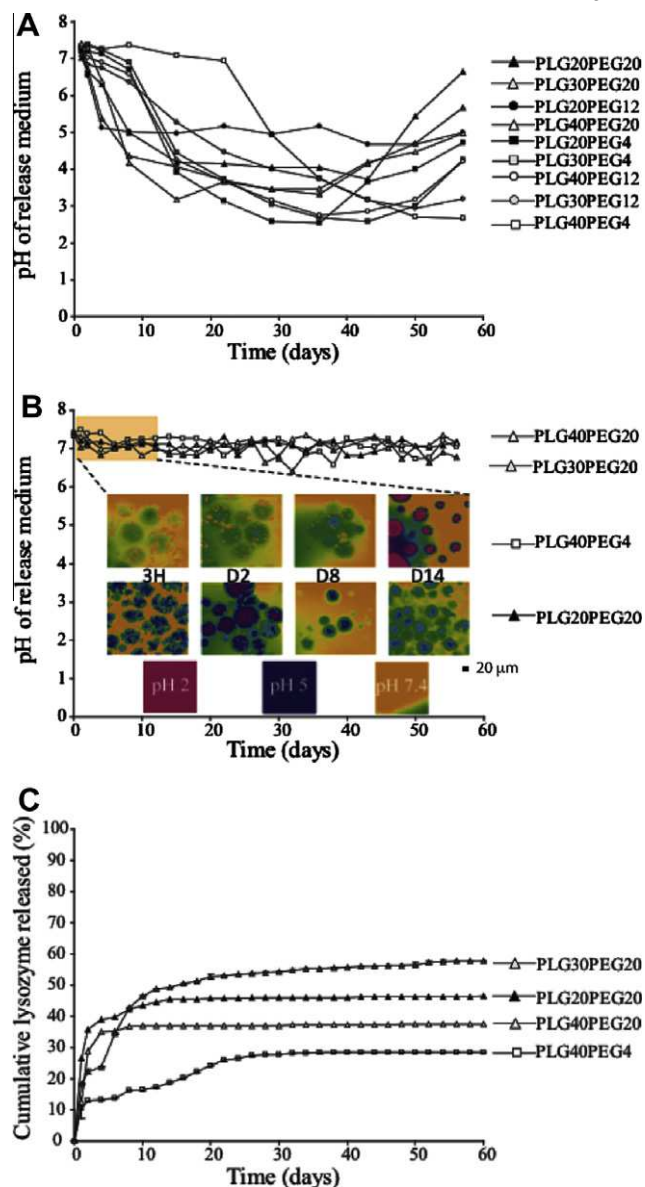


Fig. 6. pH of release buffer: (A) in 500 μ L of Tris–HCl 0.05 M, with 7 day interval removal, (B) in 1000 μ L of Tris–HCl 0.05 M with 2 day interval removal, and (C) the lysozyme release profile under (B) conditions. (B) Confocal microscopy of the PLG40PEG4 and PLG20PEG20 microspheres containing the pH-sensitive fluorescent probe after 3 h, 2 days, 8 days and 14 days during *in vitro* release (1000 μ L Tris–HCl 0.05 M, 37 °C, with 2-day interval replacement conditions). The colour of 0.1 mg/mL fluorescein solution images at pH 2, 5 and 7.4 are shown for comparison.

Table 2

Biostability of lysozyme in acidic medium (pH 2) with or without NaCl, BSA, Poloxamer 188 after 8 days of incubation.

Experiment	BSA (mg/mL)	Poloxamer 188 (mg/mL)	NaCl (mg/mL)	Active lysozyme (%)
1	0	0	0	101 \pm 7
2	1	0	0	109 \pm 3
3	0	2	0	93 \pm 6
4	1	2	0	76 \pm 14
5	0	0	0.88	66 \pm 3
6	1	0	0.88	110 \pm 1
7	0	2	0.88	86 \pm 6
8	1	2	0.88	50 \pm 2

was similar to the control cells cultured in a standard well (data not shown).

4. Discussion

Tissue engineering is a rapidly expanding field that has gained momentum with the therapeutic possibilities offered by stem cells. Indeed, tissue engineering strategies such as 3D-biomimetic surfaces and the prolonged delivery of growth factors may overcome the major limitations in the use of stem cells which are their low survival rate and differentiation capacity after transplantation. A therapeutic tool (PAMs) combining these two strategies has been developed in our laboratory and has shown its potential to enhance the survival, differentiation and regenerative capacities of stem cells and to repair lesioned tissues (Delcroix et al., 2011; Tatard et al., 2004, 2005a,b, 2007a,b). In order to underline these strategies, it is essential to improve protein release from copolymer microspheres, which remains a technological challenge due to the special conformation of proteins. Our study thus focused on the impact of polymer composition on protein release to comprehend the chronology of protein destabilisation during the release step.

PLGA is known for its biodegradability and biocompatibility (Fournier et al., 2003) whereas non-toxic hydrophilic PEG is known for its stabilising effect on proteins (Kissel et al., 1996; Singh et al., 2008; Wade and Weller, 1994). PLGA–PEG–PLGA copolymers were thus chosen to fit these two features. These copolymers were shown to increase drug release by increasing the degradation rate (Li et al., 2000; Youxin and Kissel, 1993) due to their rapid swelling properties (Zange et al., 1997). However, the impact of the molecular weight of PLGA and PEG segments was not studied in the literature. To investigate the effect of ABA composition on the protein release rate, nine polymers were prepared with combinations from 20, 30, and 40 kDa for PLGA segments and 4, 12, 20 kDa for PEG segments.

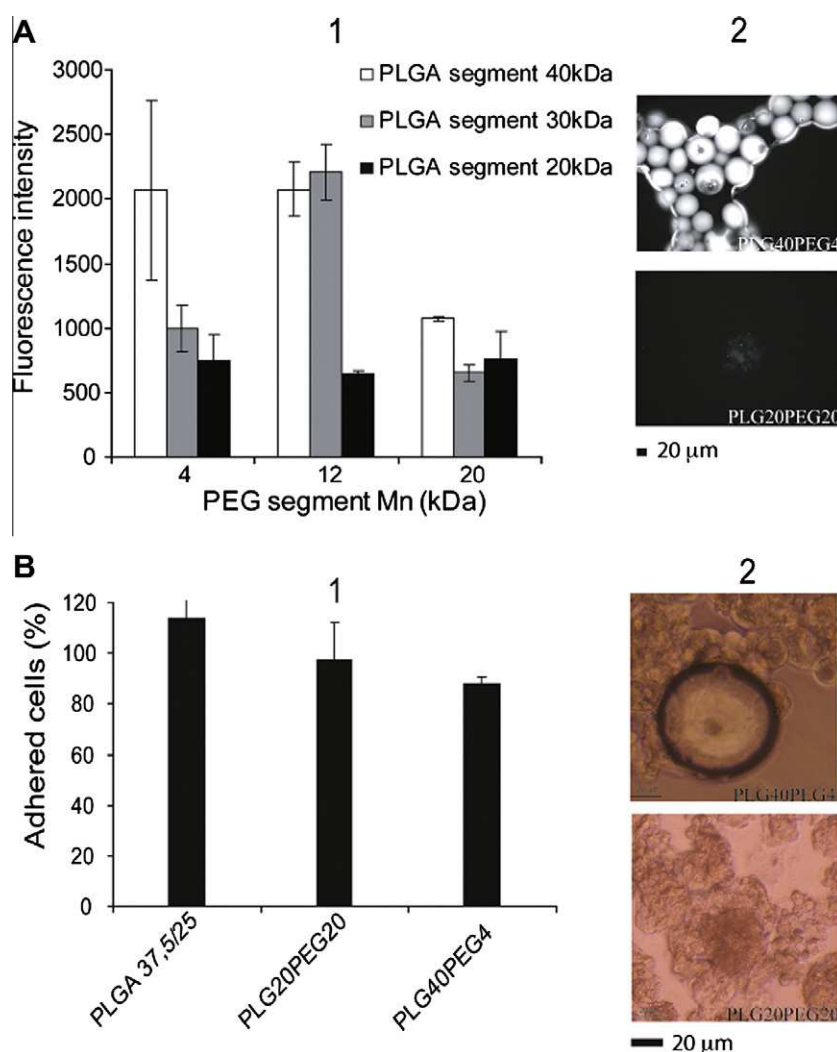


Fig. 7. (A1) fluorescence intensity of fibronectin-coated polymer films incubated with streptavidin–rhodamine and (A2) fluorescence microscopy of fibronectin-coated PLG40PEG4 and PLG20PEG20 microspheres incubated with streptavidin–rhodamine. The bright zone represents the fluorescence of rhodamine. (B1) Illustration of cells adhered to the coated PLG20PEG20, PLG40PEG4 and PLGA 37.5/25 microspheres after 4 h incubation with MSC cells and (B2) their optical microscopy observation (the bar scale represents 20 μm).

Microspheres were prepared with similar protein loadings and were sieved to minimise the number of parameters that can affect drug release (Liggins and Burt, 2004; Tran et al., 2011). It has been stated that the vacuum drying of ABA microspheres during lyophilisation step can induce a porous surface due to the rapid evaporation of water (Morlock et al., 1998). We found that micro-pores could be formed before vacuum drying by microphase separation. During the solvent extraction, the hydrophobic PLGA segments shrank whereas the hydrophilic PEG segments had more affinity with the outer-water phase and moved out to the surface, leading to the formation of porous-structured microspheres. The PEG segments dominated the porosity of these microspheres.

The molecular weights of PEG and PLGA segments presented an important effect on protein encapsulation efficiency. For the 20 kDa PEG segments, the encapsulation yield was dramatically low. The ABA copolymers with the longest segment of PLGA and the shortest PEG segment (PLG40PEG4 and PLG40PEG12) had the highest encapsulation efficiency. The water uptake level into the nascent microspheres increased with PEG segment size leading to protein loss.

The ABA composition also affects the initial stage of *in vitro* release (48 h). Even if no significant differences were detected between the T_g of the raw polymer and the microspheres, the T_g

level was below the *in vitro* study temperature (37 °C), therefore the diffusion of the protein was facilitated out of the rubbery microspheres. Moreover, by increasing the length of hydrophilic segments water uptake by the polymer matrix is promoted, thus favouring the diffusion of the protein towards the outer phase during the hardening process and *in vitro* release.

After 48 h, lysozyme release profiles were continuous up to Day 8 (PLG40PEG20) or Day 36 (PLG40PEG4) before reaching a plateau. We focused on PLG40PEG4 microspheres to elucidate the mechanism of lysozyme instability because it reached a satisfactory hydrophilic-hydrophobic balance. During the first week, although PEG segments gradually leaked out from the ABA copolymers, the microenvironment was still neutral. However, at the same time, the poloxamer gradually ran out, thereby limiting its anti-adsorption protective effect (Paillard-Giteau et al., 2010), and permitting lysozyme to partially adsorb onto the PLGA segment surfaces. It is known that, when a protein is adsorbed on a hydrophobic surface, its conformation slowly changes to maximise the number of exposed hydrophobic regions in contact with the hydrophobic surface (Andrade and Hlady, 1987). During this process, a fraction of lysozyme with exposed hydrophobic regions could make contact with each other and form dimeric aggregations. By Day 8, dimeric aggregations were dissociated by

β -mercaptoethanol implying that disulphide bonds were involved in the lysozyme polymerisation. By Day 22, dimeric aggregations were no longer dissociated by β -mercaptoethanol, so another type of covalent aggregation must be involved in the protein aggregation mechanism at the latter period. From this point of view, the presence of poloxamer in the microsphere is necessary to protect the protein from adsorption and aggregation. However, the monoband of lysozyme was always observed, so lysozyme aggregation could not completely explain the incomplete release of protein.

At a later period, the plateau of protein release correlated in time with the drop of pH in the release medium: from pH 7 to pH < 5. This phenomenon suggests that the incomplete release was partially due to the instability of lysozyme after a long incubation time at 37 °C in the acidic conditions within the microspheres. Although Tris buffer could neutralise the acidity of the release buffer, the pH of the release buffer showed a lag in time with the microenvironment in the microspheres, which began to be acidic from Day 8 onwards. At this time, poloxamer was no longer present, BSA and NaCl diffused into the microspheres from the release buffer creating BSA micro-zones in the microsphere altering the protein microenvironment. These microenvironments correspond the experiment five and six in the Table 2. At pH 2, the study of lysozyme stability in solution versus medium composition showed that without poloxamer lysozyme keeps its biological activity in the BSA micro-zones associated to NaCl, and lost 34% of its biological activity in the BSA-free micro-zones. These results underline the role of the microenvironment on the protein stability particularly at low pH.

To our knowledge, this is the first time that the cause of incomplete protein release from ABA copolymers has been clarified. Various reasons for protein instability accumulated within ABA microspheres can be thus listed as a function of time: protein adsorption, protein aggregation and protein denaturation under acidic conditions. The comprehension of the underlying mechanisms leading to incomplete release will now allow us to overcome these hurdles and create, invent, strategies to attain a complete and sustained release of proteins. One possibility is to optimise the protein microenvironment by retaining poloxamer into the matrix during pH drop.

Further bioconductive characteristics of the microspheres, the 3D biomimetic surface, was also investigated. A first screening of the fibronectin surface of the different microspheres showed that PEG segments present at the surface of the microspheres limited fibronectin adsorption. PEG is well known to minimise the adsorption of plasmatic protein by steric repulsion and there is a direct correlation between the PEG chain-length with the minimisation of protein adsorption (Vonarbourg et al., 2006). In this regard, with the shortest PEG segments, fibronectin was mainly adsorbed to the hydrophobic PLGA segments. For the PLG30PEG12, we suppose that there is no dominant effect from either the PLGA or PEG segments on fibronectin adsorption due to chain length. Therefore, in this case, the rough surface of this polymer probably favoured the entrapment of fibronectin on its surface.

The majority of the cells adhered onto the fibronectin-coated microspheres independent of the amount of fibronectin adsorbed on their surface. The hydrophilic surface facilitates cell adhesion, and thus more than 90% of MSCs adhered to the PLG20PEG20 with a slight fibronectin-coating. Furthermore, it is known that porous microspheres are suitable for the adherence of cells (Senuma et al., 1999). PLG20PEG20, with its high hydrophilic surface and pore morphology, hence has a good level of MSC cell adsorption. Nevertheless, several studies with neural stem cells have demonstrated the interest of fibronectin on cell differentiation (Tatard et al., 2005b; Wijelath et al., 2004). PLG40PEG4 microspheres presenting a biomimetic fibronectin surface thus appear to be more interesting than PLG20PEG20 microspheres for tissue-engineering

approaches. Finally, in the present study, human MSC adhered onto microspheres were still observed after one week of incubation, confirming that all the ABA polymers used in this study showed a good degree of compatibility with cells, as already reported for a mouse cell line (43, 44).

5. Conclusion

A series of biodegradable ABA triblock copolymers were synthesized by varying simultaneously the Mn of PEG and PLGA segments and they were used to prepare PAMs. The introduction of hydrophilic polyoxyethylene B block domains in PLGA chains induced a rapid rate of water uptake and a continuous-release profile. However, higher PEG block copolymer showed shorter time release and was not suitable for the adsorption of the biomimetic surface. The PLG40PEG4 copolymer appeared to be the best candidate polymer and showed the best hydrophilic-lipophilic balance leading to the best encapsulation efficiency (77%), low initial release, and continuous release up to 36 days. Furthermore, the PLG40PEG4 polymer presented a homogeneous fibronectin biomimetic surface and good MSC biocompatibility. Incomplete release due to the time-limited protection of poloxamer, and denaturation of proteins under acidic conditions, was clarified. This study highlights the impact of PEG and PLGA size segments on the acidification rate both in the polymer matrix and in the release medium concurrently to the protein degradation profile. This systematic study has never been reported.

More importantly, this present study emphasises the necessity to both adjust the polymer hydrophilic-hydrophobic balance to the intended protein release profile and to maintain the stabilizer agent within the polymer matrix close to the protein as long as possible. Optimisation of the type (PPO/PEO ratio) and proportion of poloxamer co-precipitated with the protein is now in progress.

This work sets the ground for other studies with therapeutic proteins loaded in PLG40PEG4 microspheres for tissue engineering applications.

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Conclusion

This study allowed us to explore the impact of PLGA-PEG-PLGA copolymer composition and molecular weight on protein release and protein stability during the release step. In this study microspheres formulated using PLGA-PEG-PLGA copolymers were shown to increase active protein release when compared to PLGA microspheres. Protein encapsulation efficiency was dependent of molecular weights of PEG and PLGA segments as the ABA with the longest PLGA segment and the shortest PEG have the highest encapsulation efficiency. Hydrophilicity was increased with PEG segment-size leading to water uptake by the polymer matrix and diffusion of the protein toward the outer phase. These microspheres thus exhibited the more complete release profile. However, a plateau of protein release was observed after 8 days (PLG40PEG20) or 36 days (PLG40PEG4) and was correlated to protein aggregation and denaturation as well as with a drop of pH within the microspheres.

As defined by Tatard et al. (Tatard VM et al. 2005) PAMs have a surrounding biomimetic coating of ECM. It is thus very important to study the protein adsorption at the surface of these hydrophilic polymers. Indeed a compromise needs to be found between adequate protein coating at the surface of the PAMs and hydrophilicity of the polymer to protect the protein limiting the interaction and adsorption to the polymer. PEG is known to minimize protein adsorption by steric repulsion and a direct correlation between the PEG chain length and protein adsorption has been described. Surprisingly, for one of the PEG copolymers no dominant effects on protein adsorption were observed, on the contrary, surface topography and roughness seems to favor FN retention at the surface.

The ABA PAMs allow good hMSCs attachment and are biocompatible as attested by hMSCs viability when adhered for 1 week in vitro culture conditions, suggesting their possible use as drug releasing systems conveying cells releasing GFs. However, although the approach increased protein delivery, more hydrophilic polymers continuously releasing the protein for a longer period with no acidification of the protein microenvironment would be more suitable and need to be investigated.

General Discussion

Current treatments for cardiovascular repair present certain limitations thus, in recent years cell-based therapies have attracted considerable interest as an alternative way of achieving cardiac repair. To successfully lead this therapeutic strategy toward the clinic, research studies need to focalize in resolving several issues, like the choice of the type of cells, the number of injected cells and the time of injection. Moreover, the route of administration and the optimization of their survival, differentiation and retention in the cardiac tissue also need to be addressed. Combining the transplanted cells with a 3D injectable structure providing the biological cues required for optimal graft engraftment was the strategy adopted in this study. We think it is important to provide in situ an appropriate microenvironment to guide and maintain a differentiated phenotype and increase survival of the transplanted cells. This work having for objective at the long run to implement a strategy of cardiac tissue engineering, we made an effort to adopt approaches being able to allow a possible transposition towards the clinic.

Concerning the type of cell, we chose ADSCs, a cell population easy to obtain (simple liposuction), easily expandable, allowing autologous grafting and being able to be directly transferable towards the clinic without ethical concerns. Moreover, some studies have mentioned their mobilization into the infarcted area during myocardial infarction suggesting their role in cardiac repair and confirming the interest raised by these cells (Bayes-Genis, Soler-Botija et al. 2010). Although the literature relates their role on improvement of infarct size and enhancement of the cardiac function, these effects are particularly attributed to paracrine effects rather than cardiomyocyte differentiation. However, some studies have shown integration and differentiation into cardiomyocytes of a few grafted ADSCs in vivo (Cai, Johnstone et al. 2009; Leobon, Roncalli et al. 2009; Bai, Yan et al. 2010; Bayes-Genis, Soler-Botija et al. 2010; Choi, Matsuda et al. 2010; Hamdi, Planat-Benard et al. 2011). Nevertheless, cell-fusion with endogenous cardiomyocytes has sometimes been proposed (Metzele, Alt et al. 2011). In any case it is important to study their cardiac differentiation potential and in this way create a safe biological environment for cardiac transplantation studies.

The cardiac differentiation potential of ADSCs has been evaluated in vitro, and a few studies have even reported a spontaneous differentiation into beating cardiomyocytes. This phenomenon can be partially explained by the demethylated state of some promoters of genes

coding for early transcription factors involved in the commitment into cardiomyocytic lineage, such as GATA4, Nkx2.5 and MEF2C (Daniel Durocher 1997; Black and Olson 1998; Naito, Tominaga et al. 2003; Karamboulas, Dakubo et al. 2006; Yamada, Sakurada et al. 2007; Armiñán, Gandía et al. 2009; Holtzinger, Rosenfeld et al. 2010). These first two factors are involved in the recruitment of MEF2C (Morin S 2000; Vincentz, Barnes et al. 2008). The latter acting as a cofactor of GATA-4 and modulating promoter activity via the GATA binding site (Morin S., Charron F. et al. 2000). Although this demethylated conformation suggests that ADSCs are opened to entry into the differentiation pathway, another promoter having its gene playing a crucial role in the recruitment of these transcription factors and on cardiac development may block the transcription of Nkx2.5, MEF2C and GATA4. We can thus assume that ADSCs progression into beating cardiomyocytes depends largely on the medium composition that moreover differ between all the studies. Many studies use high percentage of FBS, others, media enriched with cytokines such IL3, IL6, BMP4, etc. (Planat-Benard, Menard et al. 2004; Jumabay, Zhang et al. 2009), but one constant in these media is the presence of biological cues. Other studies have employed a demethylating agent but this differentiation is not specific and is due to the open-state of all the DNA promoters (Choi, Disting et al. 2010). Unfortunately, this approach is not clinically transferable and a strategy using physiological growth factors seems to be more reliable.

In this study we have focused on a cocktail of several cytokines adapted from the literature to study the differentiation of ADSCs toward the cardiomyogenic lineage combined or not to another important biological instructive signal, ECM molecules. The set of growth factors/cytokines was composed of TGF β 1, IGF-1, IL-6, FGF-4, BMP-2, VEGF, LIF, α -thrombin and retinoic acid. These factors represent a cocktail of secreted proteins that recapitulate the components of the endodermal secretome critical for cardiogenic induction of the embryonic mesoderm (Behfar and Terzic 2006). Thus, we followed a strategy that mimics the natural embryonic program for uniform recruitment of cardiogenic progenitors from adult stem cells. TGF β -1 has been reported to play important roles in induction of cardiomyocytes differentiation of ADSCs but also MSCs and embryonic stem cells (Gwak, Bhang et al. 2009; Kawaguchi 2011; Yook JY 2011). BMP-2, a member of the same family, and FGF-4 are implied in the induction of cardiac transcription factors in developing embryos and in the induction of the cardiomyogenic differentiation from non cardiac mesodermal cells (Yoon, Min et al. 2005; Lincoln, Alfieri et al. 2006). LIF has been reported to enhance survival of

cardiomyocytes following myocardial infarction (Zou, Takano et al. 2003) and to enhance differentiation of embryonic stem cells. Retinoic acid and IL-6 are required for cardiomyocyte differentiation (Gassanov, Er et al. 2008; Banerjee, Fuseler et al. 2009; Behfar, Yamada et al. 2010; Zhang, Jiang et al. 2010) as well as VEGF (Song, Gehmert et al. 2007). The IGF-1 was shown to enhance survival and proliferation of cardiac stem cells (Opgaard and Wang 2005; Urbanek 2005; Padin-Iruegas, Misao et al. 2009; Penn and Agarwal 2010) but also to enhance cellular engraftment and a-thrombin was shown to induce differentiation of MSCs into myocardial lineage in vitro. We have found that this GF cocktail induced the expression of the earlier transcription factors GATA4 and MEF2C, but the cells remained clearly very immature. This poor differentiation state may be explained in two ways. The first being that the GFs alone was not sufficient to induce a more mature differentiation and the second being that the GFs were applied at the wrong interval of time. In fact, as suggested by literature, these molecules have differentiating effects on several cell types, so we can hypothesize that the ADSCs were not in the proper condition of “gene expression regulation” in which the response to those GFs would be completely effective. In other studies the GFs were applied to cells at defined time-periods when they presented a phenotype ensuring the adequate response to the GF (Behfar and Terzic 2006; Behfar, Yamada et al. 2010).

As the first signals that the cells sense come directly from the surrounding extracellular matrix, we hypothesized that the ECM sensed by the cells could further guide/induce them into the desired pathway as described by the literature (Ross and Borg 2001; Kuppuswamy 2002; Ross 2002; Stupack 2002; Hehlhans, Haase et al. 2007; Streuli 2008; Martino, Mochizuki et al. 2009). We then tested the combination of GF with ECM molecules, as they constitute the natural compound of the heart and instructive molecules to further induce this commitment. FN and LM were studied due to their deregulation during myocardial infarction (Villarreal and Dillmann 1992; Ulrich, Janssen et al. 1997; Pankov 2002; Dijk, Niessen et al. 2008; Malan, Reppel et al. 2009). Finally LM appeared to be the most appropriate ECM molecule for ADSC differentiation in combination with the GFs, as it induces expression of the transcription factors NKX2.5, GATA4 and MEF2C but also the expression of the cardiac contractile sarcomeric protein cTnI. Interestingly, we finally have a more mature differentiating effect with LM after a time period of 2 weeks that is acceptable compared to the literature where contractile proteins such as cTnT were found after 3 weeks (Planat-Benard, Menard et al. 2004; Dijk, Niessen et al. 2008). In this regard, some studies have described some effects on myogenesis. Foster et al. (Foster, Thompson et al. 1987) have

found that a laminin substrate promotes myogenesis in rat skeletal muscle cultures and Hilenski et al. (Hilenski, Ma et al. 1992) have reported that LM influences cytoskeletal and myofibrillar organization in vitro in neonatal rat cardiac myocytes. Further, mutations in the genes coding for the alpha-2 chain of two laminin isoforms have been shown to cause a severe form of congenital muscular dystrophy in humans and mice (Kuang, Xu et al. 1998). The interaction between integrins and laminin can lead to many developmentally relevant processes, such as cell differentiation (Thorsteinsdóttir, Roelen et al. 1999) via their ability to associate with and activate signal transduction pathways (Kuang, Xu et al. 1998; Belkin and Stepp 2000). The role of LM and the mechanistic and signaling pathway employed in cardiac cell differentiation need to be further investigated.

The first objective of cell therapy is to repopulate the fibrotic area with viable cells and to ensure their interaction with the host tissue. When cells are injected alone, the major part is expelled through the blood circulation and approximately 90% of the remaining cells die within the first 24h following transplantation. The lack of anchorage to the ECM, the nutritional status and the acidic microenvironment matrix are some causes of this massive cell death. The loss of contact suppresses also the functional interactions between cells and matrix and blunts the surviving, proliferating and specification signals. For these reasons the PAMs developed in the Angers INSERM Unit were evaluated for cardiac tissue engineering in this study. These PAMs are a biodegradable and biocompatible small-size vector, being able to be injected through a catheter, offering an instructive ECM coating and a 3D support. The results obtained in vitro have shown a 3D effect of the PAMs on ADSC differentiation into cardiomyocytes. Indeed a mature differentiated phenotype with the expression of the cardiac contractile protein cTnI was observed after a short time of one week, with the PAMs providing a LM 3D support in combination with the cocktail of GF, when compared to these biological cues provided in a 2D cell culture condition. Moreover, the cells expressed CX43 suggesting that they could communicate between them to synchronize their development. The effects of the substrate on cell differentiation, but especially the effects of the 3D, are widely reported in the literature (Dutta and Dutta 2009; Kenar, Kose et al. 2011; Karam JP. 2012). The 3D support offered by the scaffold allows cell retention within the infarct area and, particularly, cell survival but also cell proliferation and differentiation. As a matter of fact, we have already shown that PAMs with a biomimetic surface of FN or LM allow cell survival and/or differentiation of the grafted cells within an ischemic tissue and in a neurodegenerative setting (Delcroix, Garbayo et al. 2011; Garbayo, Raval et al. 2011).

Although the small size of PAMs allow catheter injection constituting a real advantage compared to other more invasive procedures, like decellularized matrix, the benefits of this strategy have already been demonstrated and are encouraging. Indeed, these approaches favor the cohesion and the connection of cells in a native extracellular matrix. They overcome the challenge of the “appropriate substrate and mechanosensitive properties” necessary to commit the cells into the desired phenotype and may disturb less the beating heart as described by the literature (Syngelyn JM and Christman KL, 2010). Interestingly, with PAMs as well as with this approach the cells can modulate themselves, the scaffold and secrete their own extracellular matrix. Due to their PLGA composition, PAMs are not the most appropriate in terms of viscoelastic properties to induce myogenic and cardiomyogenic commitment (Engler, Sen et al. 2006; Tse JR 2011; Young and Engler 2011), although improvement in terms of survival was observed. They may be more appropriate for endothelial vascularization, this kind of polymer having been used in this type of approach (Formiga, Pelacho et al. 2010; des Rieux, Ucakar et al. 2011). However, they offer the possibility to continuously release for a prolonged period a GF that can directly act on the grafted cells and modulate at the same time their microenvironment. One possibility for the future could be to combine the PAMs with these decellularized matrices to provide all the instructive cues for the transplanted cells.

We have chosen 3 GFs involved in cardiac regeneration and widely studied in myocardial infarction models, IGF-1, HGF and VEGF. IGF-1 is involved in cell survival and proliferation and some studies have related its role on endogenous CPC proliferation (Urbanek 2005; Davis 2006; Padin-Iruegas, Misao et al. 2009). Moreover, it is one of the components of the GF cocktail that induced ADSC differentiation toward the cardiomyogenic lineage (Behfar, Yamada et al. 2010). HGF is involved in the recruitment of CPCs but also on MSC and ADSC differentiation into cardiomyocytes (Kitta 2002; Forte, Minieri et al. 2006; Roggia, Ukena et al. 2007; Deuse, Peter et al. 2009; Zhu, Zhang et al. 2009). It is also a pro-angiogenic and anti-fibrotic factor. VEGF is known for its pro-angiogenic properties as well as its proliferative and survival potential (Yockman, Choi et al. 2008; Madonna and De Caterina 2009; Zisa, Shabbir et al. 2009; des Rieux, Ucakar et al. 2011). We have thus thought that injection of LM-PAMs releasing IGF-1 and HGF in combination with ADSCs would improve myocardial repair (Fig). The released IGF-1 and HGF may further improve the differentiation of ADSCs toward the cardiomyogenic lineage, while in the same time the

released HGF may recruit the endogenous CPCs and promote neovascularization. Finally, the released IGF-1 may enhance ADSC survival as well as survival and proliferation of the recruited CPCs. The first step of this study implied the development of LM-PAMs releasing the bioactive proteins.

It has been previously shown that protein administration with implantable PLGA microspheres is effective and secure. Moreover, the instability, lack of tissue selectivity, toxicity and carcinogenic risk of GFs administered at high doses have favored the development of systems permitting a localized administration and the prolonged release of physiological doses of proteins. However, their development has been limited because of the destabilizing conditions applied to proteins that occur during the preparation of the microspheres and polymer degradation allowing their release (Giteau, Venier-Julienne et al. 2008). To protect the proteins, we used the s/o/w technique to formulate the PAMs, based on the suspension of a protein nanoprecipitate in an organic solvent. In the absence of an aqueous internal phase, the protein is less sensitive to denaturation by adsorption to the organic phase. Moreover, the structure of the protein in this organic phase is thermodynamically solidified and the changes of conformation cannot occur.

The precipitation procedure has been already developed in the INSERM U1066 and was characterized by A. Giteau (Giteau, Venierjulienne et al. 2008). In this study, we adapted the nanoprecipitation procedure to our needs and further characterized this process. Although it was previously shown that temperature has limited effect on protein precipitation, we decided to work at a fixed temperature of 4°C. Furthermore, an increase in protein concentration is known to favor its precipitation. We tried to define the optimal protein concentration allowing precipitation. In this study we have found a minimal protein concentration that guarantees the protein precipitation at a fixed temperature of 4°C now helping the screening of precipitation conditions for other proteins. It is also known that pH, as well as ionic strength, acts on protein precipitation. Two proteins with very different isoelectric points will behave differently at the same pH, so we adapted the pH to the isoelectric point. Indeed, at a pH equal to the isoelectric point, the protein remains in a more stable conformation because the interaction with the aqueous medium is limited.

Salt-induced precipitation is an extensively used method in biotechnology for separating target proteins from multi-component protein solutions as the first purification step. Sodium

chloride, which decreases protein solubility with minimal denaturant effects, was selected in the previous studies due to its possible use in parenteral pharmaceutical formulations (Giteau, Venierjulienne et al. 2008; Paillard-Giteau, Tran et al. 2010; Tran, Karam et al. 2012). The precipitation method developed by A. Giteau et al (Giteau, Venierjulienne et al. 2008) further employs a non-toxic organic solvent, glycofurol, to induce the formation of fine nanosized proteins (Giteau, Venierjulienne et al. 2008). We played on the increase of ionic-strength required to induce salting-out. We further hypothesized that the protein being more stable and interacting less with the aqueous phase at pH=isoelectric point there would be less competition for water molecules with the salt and would undergo more easily the salting-out. Additionally, the protein was co-precipitated with a stabilizer agent, poloxamer 188, which protects the protein and also increases the release profile. Poloxamer prevents self-association of the protein particles (even at low concentrations) (Paillard-Giteau, Tran et al. 2010) and allows a more homogeneous protein distribution within the microspheres. On the other hand, by hydrophilization of the polymer surface, poloxamer contributes to a reduction in non-specific adsorption of the protein on the PLGA surface (Giteau, Venier-Julienne et al. 2008; Tran, Karam et al. 2012) during microsphere hydration. Finally, the gelling properties of poloxamer may also play a key role during the release period increasing the wettability of the microspheres by creating pores within the polymer, favoring the release of the protein. The ratio protein/poloxamer/polymer was here investigated to find a compromise between the burst release and the encapsulation efficiency. Finally a ratio w/w of protein poloxamer of 1/20 was found to be the more appropriate for a better release profile. Adapting this method to every protein, at a temperature of 4°C with a defined concentration (superior to 5.5 mg/ml) and a pH closes to the isoelectric point, we could precipitate a novel therapeutic protein by varying only the ionic strength and defining the appropriate “precipitation buffer”. The limit of this method resides on the fact that we have only found a minimal protein concentration to precipitate the protein. The maximum threshold required for precipitation needs to be explored as too elevated protein concentrations could favor irreversible aggregation rather than precipitation.

In this study we obtained an encapsulation efficiency of approximately 80% and a continuous release for up to 3 weeks of VEGF, IGF-1 and HGF. Moreover, the released factors were bioactive and induced HUVEC and NIH3T3 proliferation. Using these PAMs with our INRC collaborators in cardiac tissue engineering approaches, several effects have been observed. FN-PAMs releasing VEGF were first used in vitro in combination with eEPCs to study their

effect on survival and differentiation of EPCs.. Our results indicate that eEPCs were able to adhere to PAMs and VEGF releasing FN-PAMs favor this adhesion. Moreover, endothelial-like phenotype and cell survival were improved with eEPCs adhered to FN-PAM-VEGF (Musilli, Karam et al. 2012). Finally, these PAMs induced the proliferation and migration of a more mature endothelial phenotype. In a future implementation for heart repair, this approach could then induce transplanted eEPC survival and differentiation, while stimulating endogenous cell survival, proliferation, migration and vascularization of the ischemic area. The second approach using FN-PAM-VEGF combined them with MSCs in an in vitro model of post-ischemic heart reperfusion. This strategy had for future objective the improvement of cell survival within the ischemic microenvironment. The results showed that PAMs releasing VEGF mainly hampered post-MSC death induced by hypoxia/reperfusion through the activation of anti-apoptotic proteins (Perrelli M-G, Penna et al. submitted,). PAMs releasing VEGF could be proposed as a novel approach for enhancing MSC survival and regeneration in a hostile environment of post-ischemic tissue. Finally proposed VEGF-PAM as a tool to protect the grafted cells against apoptosis and induce their proliferation. In vivo studies have to been done to confirm the results obtained with these two distinct in vitro approaches.

Using LM-PAMs releasing HGF and IGF-1 combined to ADSCs we here showed that the cells were induced to a cardiomyogenic lineage allowing us to obtain cells expressing cardiac contractile proteins in a similar manner as with the ADSC/LM-PAM complexes combined to the GF cocktail. Moreover, CX43 was highly expressed in these conditions and the cells expressing this gap-junction protein were found homogeneously distributed within and all around the complexes. In parallel, together with our INRC collaborators, we investigated the effects of the implantation of ADSCs adhered onto PAMs releasing HGF and IGF-1 on cardiac repair after MI. Indeed, interesting effects have been described in the literature when IGF-1 and HGF have been injected as a solution in the infarcted heart, such as the improvement of cardiac function and recruitment of CPCs in the ischemic area (Urbanek 2005). Transplantation of ADSCs with PAM-HGF and PAM-IGF-1 in the ischemic rat heart, allowed the improvement of ventricular ejection fraction and decrease in infarct size. Additionally, ADSCs injected with PAMs survived and homed into the host myocardium. Interestingly new vessels were observed around the ADSCs-PAMs complexes confirming the HGF effects on angiogenesis. Surprisingly, PAMs releasing HGF and IGF-1 increased the arrhythmia in the infarcted heart. Some studies have shown that ADSCs alone do not induce

arrhythmia (Fotuhi, Song et al. 2007; Bai and Alt 2010) as confirmed by our studies. Moreover, the released GF are not described in the literature as inducers of ADSCs differentiation into undesirable cells such as adipocyte that could induce arrhythmia. We can then suppose that the presence of PAMs could disturb the electric propagation, because they are not good conductors.

To overcome this problem, we have combined the PAMs with a thermosensitive HG, Pluronic F 127 or poloxamer 407. HG have been extensively studied for the heart and offer the advantage to have enough flexibility to respond synchronically with the myocardium contraction and effectively transfer mechanical stimuli from the myocardial microenvironment to the transported cells, providing appropriate cues to develop a contractile phenotype and communicate with other cells. It appears that cells sense the substrate strength and convert it into signals guiding survival, proliferation, migration and differentiation (Assoian and Klein 2008; Jacot, McCulloch et al. 2008; Peyton, Kim et al. 2008; Tse JR 2011). Many studies have shown the effect of the elastic properties of HG support on cell differentiation (Jacot, McCulloch et al. 2008; Tse JR 2011). Moreover, the HG could favor PAM's retention within the heart following injection but also enhance ADSCs survival as observed in our study. In vitro results have demonstrated that PAMs integrated with this thermosensitive HG can have a Young modulus matching the properties described by Engler et al. (Rehfeldt, Engler et al. 2007; Engler, Humbert et al. 2009; Reilly and Engler 2010; Tse JR 2011; Young and Engler 2011) to induce myogenic differentiation. Combining the P407 HG with LM-PAMs releasing IGF-1 and HGF having ADSCs adhered onto them, an increase in mRNA levels of cardiac proteins was observed. An in vivo study needs to be performed to investigate the effects of P407 HG/PAMs on arrhythmia as well as to confirm the better integration and differentiation of ADSCs within the infarcted area.

Unfortunately, we observed a slower release profile of the protein encapsulated into P407 HG/PAMs compared to the standard release medium, probably due to a less hydrophilic environment and less water molecules available. To improve this release profile, PAMs with a faster degradation could be developed. In fact, the hydrophobic nature of the PLGA limits the escape of its degradation products contributing to the incomplete release of the protein. The PLGA can be modified in order to obtain a more hydrophilic copolymer (Tran, Karam et al. 2012). The direct introduction of a hydroxyl group on the glycolic acid has demonstrated an increase in lysozyme release. The PLGA-PEG-PLGA (ABA), a more hydrophilic copolymer than PLGA, was used to increase protein delivery. Moreover, the PEG itself stabilizes

proteins. Additionally, PEG is a more elastic and soft polymer and has already been used in scaffold tailoring for the heart (Gupta MK, Walthall JM et al. 2011, Kadner K, Dobner S. et al. 2012). So, we can hypothesize that a copolymer of ABA embedded or not within an HG would be more adapted to develop more flexible PAMs and better respond to heart constraints, while improving the protein release profile. In our study, we have investigated the impact of the molecular weight of the segments that compose polymer on lysozyme release. We have found that the augmentation of the molecular weight of the PEG and diminution of the molecular weight of the PLGA, both increase the water uptake and the protein release. Furthermore, the encapsulation efficiency and the surrounding biomimetic coating also depend on the variation of the PEG and PLGA segments. Interestingly these biodegradable triblocks copolymers are also biocompatible when cultured with hMSC and allow cell adhesion, rendering feasible the use of such polymer in the heart. However, an incomplete protein release persists. Currently, biocompatible copolymers triblocks of PLGA-P188-PLGA are under investigation in the lab. Interestingly, the P188 is used to protect the cells against cell membrane electroporation (Collins JM 2007). Moreover, it offers the advantage to limit the protein adsorption but also to stabilize the pH inside the microsphere thus limiting protein unfolding and denaturation.

In conclusion, we have developed PAMs releasing HGF, VEGF and IGF-1 combined to ADSCs for cardiac repair. However, their pertinence in a strategy trying to further induce ADSC engagement could be enhanced by PAMs releasing TGF β -1. Indeed, TGF β -1 has been proposed as a potent inductor of cardiac differentiation and our results confirm this hypothesis.. Actually, the more appropriate approach could be a PAM releasing several GFs in order to better control cell behavior. However, due to the importance of the “therapeutic window of application” of GFs to efficiently direct cell differentiation, the pertinence of a PAM releasing sequentially several GFs for a prolonged period could be investigated (Fig 4). The possibility to use cells that are more capable to give rise to functional cardiomyocytes is another alternative that could be investigated. Two cell populations could be proposed. The first are, CPCs, being the only cells capable to give fully differentiated and functional cardiomyocytes. Interestingly, laminin has been described as the ECM molecule for cardiomyocyte development and activation compared to fibronectin and RGD-peptide (LaNasa SM, Bryant SJ. 2009) while cardiosphere secreting laminin have shown better engraftment and are more potent to enhance LEVF and more resistant to death and oxidative

stress than monolayer-cultured cells. LM-PAMs releasing IGF-1 could be used to improve cardiac function, promoting CPCs survival and proliferation. In this regard, a study has described the effect of IGF-1 self-assembling peptide nanofibers on recovery of cardiac function enhancing engraftment of exogenous CPCs and activation of endogenous CPCs (Padin-Iruegas, Misao et al. 2009).

A last strategy could be to prepare the ischemic environment prior to PAM transplantations. Recently, an enhanced-permeability and retention-like (EPR) behavior within the ischemic area has been described. Some groups have started to investigate this EPR-like state for imaging using nano-compounds (Lukyanov, Hartner et al. 2004; Sun, Lin et al. 2012). It would be interesting to assess and confirm the retention of some nanoparticles developed in the lab within the infarcted heart. If it worked, we can imagine that following myocardial infarction, an injection of nanoparticles containing an agent limiting scar formation and/or modulating the inflammation response would prepare the cell-PAM-GFs complexes and allow better engraftment and response.

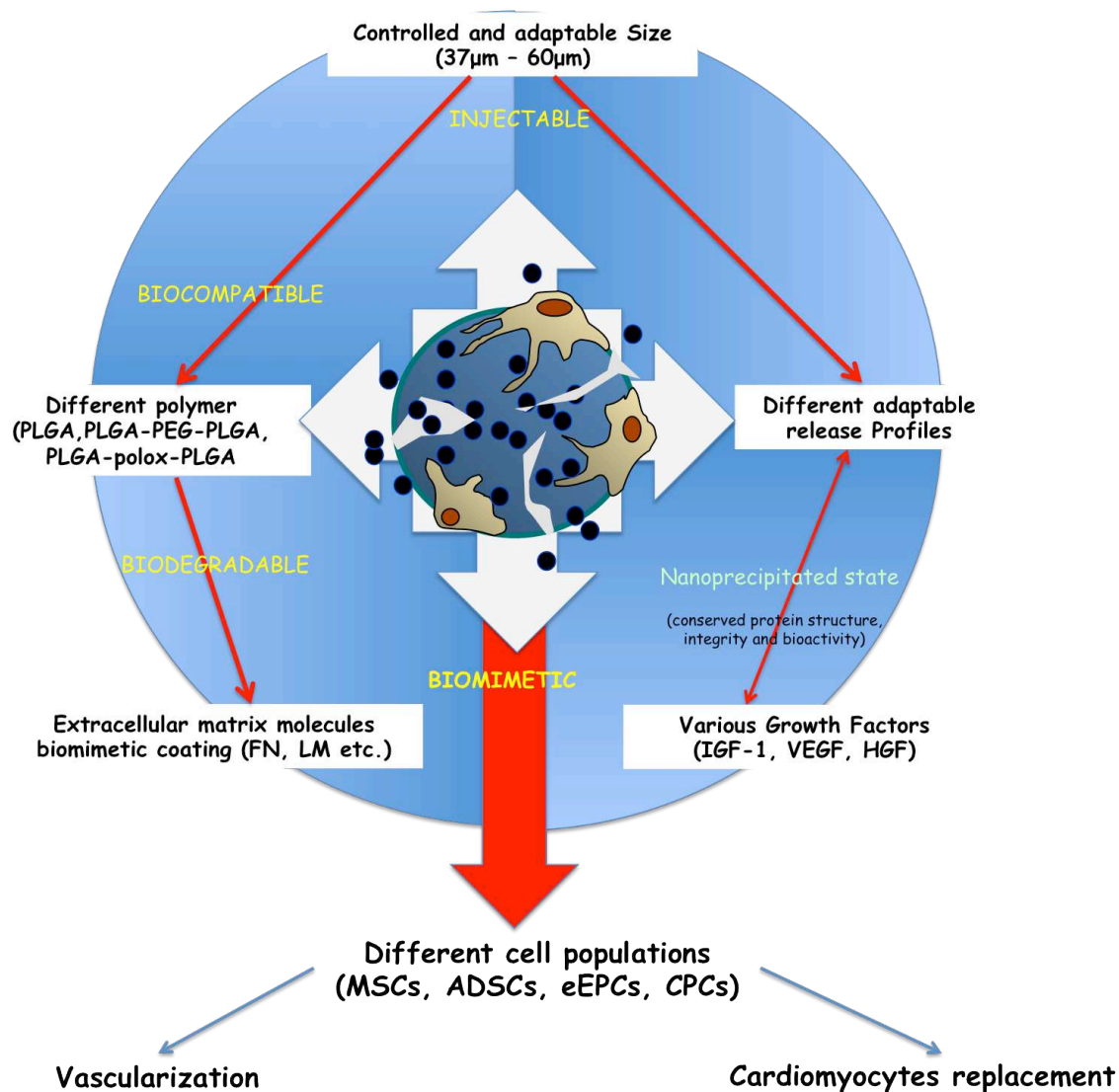


Fig 6: Finally, we can described the PAMs as a potential as modulable with the appropriate ECM biomimetic surface for cell survival and/or differentiation and also as an adaptable system that can release more or less protein depending on the cell type.

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Annexes

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Pharmacologically Active Microcarriers for Endothelial Progenitor Cell Support and Survival

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PHARMACOLOGICALLY ACTIVE MICROCARRIERS FOR ENDOTHELIAL PROGENITOR CELL SUPPORT AND SURVIVAL

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The aim of the present study was to investigate whether human early endothelial progenitor cells (eEPCs) could be efficiently cultured in pharmacologically active microcarriers (PAMs) coated with fibronectin (FN-PAMs), with or without controlled delivery of VEGF-A. Our data indicate that eEPCs were able to adhere to empty FN-PAMs within a few hours. FN- PAMs realising VEGF-A increased the ability of eEPCs to adhere to them and strongly supported endothelial-like phenotype and cell survival. Moreover, the release of VEGF-A by FN-PAMs stimulated *in vitro* HUVEC migration and proliferation.

These in vitro data strongly sustain the use of FN-PAMs for supporting eEPCs growth and their combined effect on progenitor and mature endothelial cells seems to be of potential interest for in vivo applications.

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Abstract

The regenerative potential of endothelial progenitor cell (EPC)–based therapies is limited due to poor cell viability and minimal retention following application. Neovascularisation can be improved by means of scaffolds supporting EPCs. The aim of the present study was to investigate whether human early EPCs (eEPCs) could be efficiently cultured on pharmacologically active microcarriers (PAMs), made with poly(D,L-lactic-coglycolic acid) and coated with adhesion/extracellular matrix molecules. They may serve as a support for stem cells and may be used as cell carriers providing a controlled delivery of active protein such as the angiogenic factor, vascular endothelial growth factor-A (VEGF-A). eEPC adhesion to fibronectin-coated PAMs (FN-PAMs) was assessed by means of microscopic evaluation and by means of Alamar blue assay. Phospho ERK_{1/2} and PARP-1 expression was measured by means of Western blot to assess the survival effects of FN-PAMs releasing VEGF-A (FN-VEGF-PAMs). The Alamar blue assay or a modified Boyden chamber assay were employed to assess proliferative or migratory capacity, respectively. Our data indicate that eEPCs were able to adhere to empty FN-PAMs within a few hours. FN-VEGF-PAMs increased the ability of eEPCs to adhere to them and strongly supported endothelial-like phenotype and cell survival. Moreover, the release of VEGF-A by FN-PAMs stimulated *in vitro* HUVEC migration and proliferation.

These data strongly support the use of PAMs for supporting eEPC growth and survival and for stimulating resident mature human endothelial cells.

Key words pharmacologically active microcarriers; early endothelial progenitor cells; cell survival; cell adhesion; endothelial cell proliferation and migration; VEGF-A.

Abbreviations:

ECM: extracellular matrix

EPCs: endothelial progenitor cells

FN: fibronectin

FN-PAMs: fibronectin-coated pharmacologically active microcarriers

FN-VEGF-PAMs: fibronectin-coated pharmacologically active microcarriers releasing VEGF-A

HUVECs: human umbilical vein endothelial cells

PAMs: pharmacologically active microcarriers

PARP-1: poly (ADP-ribose) polymerase 1

PLGA: poly(D,L-lactic-coglycolic acid)

VEGF-A: vascular endothelial growth factor-A

1. Introduction

Ischaemic tissue disease remains one of the primary causes of morbidity and mortality. Conventional therapies are not yet sufficient to promote adequate recovery of the blood flow in ischaemic areas [1]. Initially, growth factor-based approaches without additional cell application were widely used to enhance neovascularisation, but rapid protein degradation *in vivo* hinders sustainable success [2]. More recently, cell-based therapies have attracted great interest, since improved neovascularisation in both experimental hind limb ischaemia models and clinical studies have demonstrated [3,4]. Early endothelial progenitor cells (eEPCs) are able to promote vasculogenesis: they can be isolated from blood, bone marrow, and blood vessels [5,6], expanded *ex vivo*, and then transplanted into damaged tissue. However, this technique has some drawbacks that limit the real efficacy of the treatment, such as the death of a large percentage of transplanted cells within a few hours from their injection and their clearing through both lymphatic and blood vessels [7]. Vasculogenic progenitor cell therapy for ischaemic diseases still requires further optimisation to justify its clinical application and the outcome of patients treated with cell therapy is still poor. Optimisation protocols for enhanced cell therapy have been proposed [8,9]. The disadvantages associated with cell injection could be overcome by using pharmacologically active scaffolds which allow cell delivery and enhance the survival of stem cells.

Among the various polymers that are commonly used for the production of scaffolds suitable for regenerative medicine, biodegradable and biocompatible microparticles made of poly(D,L-lactic-coglycolic acid) (PLGA) [10] commercially available product with current human application, have been used [11,12]. These carriers with a biomimetic surface may be loaded with growth factors stimulating either transported stem cells or resident mature cells [10]. These molecules may improve survival and differentiation of the cells, and may also affect the immediate environment, thus allowing better graft integration. Cell carriers or microspheres delivering growth factors have been used in animal models of neurodegenerative diseases [13-16]. Pharmacologically active microcarriers (PAMs) transporting stem or progenitor cells with a biomimetic surface and

delivering growth factors have also been effectively used for tissue repair in Parkinson's disease [17,18] and to promote cartilage formation [19]. However, no information is available on the use of PAMs for the support eEPCs.

The aim of the present study was to investigate whether eEPCs could be cultured onto PAMs with a fibronectin biomimetic surface (FN-PAMs) through evaluation of their adhesion, survival, and differentiation. We also compared eEPC adhesion and phenotype when cultured with FN-PAMs loaded with VEGF-A (FN-VEGF-PAMs). The effect of FN-PAMs delivering VEGF-A on human mature endothelial cell activation was also investigated.

Our data demonstrate that FN-PAMs were a good support for eEPCs. Moreover, the release of VEGF-A by FN-PAMs supported the eEPC phenotype, increased their survival and stimulated mature endothelial cell migration and proliferation.

2. Materials and Methods

2.1. Preparation of polymeric biodegradable microspheres of PLGA

Poly(lactic-co-glycolic acid) (PLGA)-microspheres of an average diameter ranging from around 30 -60 μm were prepared using a previously described emulsion solvent extraction-evaporation process [18,20]. The total protein loading was 0.6% w/w of the amount of polymer, *i.e.* 0.1% VEGF₁₆₅ (VEGF-A) (Peprotech, France) and 0.5% HSA. First, NaCl and glycofurol, a water-miscible protein non-solvent, were used to precipitate the proteins separately, as previously described [18]. For VEGF-A an NaCl solution at 0.01M at 4°C, containing a protein-poloxamer 188 excipient in an additive to protein ratio of 20:1 was added and mixed to glycofurol to form a 1 ml suspension. The same procedure was used for HSA with an NaCl solution at 0.3M. The nanoprecipitates were then carefully dispersed in the organic solution, water was added and the resulting o/w emulsion formed the imprint of the microparticles which were filtered after organic solvent extraction, as previously described [18]. Unloaded microparticles were prepared in the same manner without adding the protein.

To obtain PAMs, PLGA microspheres were coated with fibronectin-like sequence (Sigma, Saint-Quentin Fallavier, France). A combination of FN with the highly charged poly-D-lysine molecule (Sigma, Saint-Quentin Fallavier, France) was used to favour cell attachment to the PAM surface. Briefly, microspheres were suspended in Dulbecco's phosphate buffered saline (DPBS) and sonicated until full dispersion of the microspheres. They were then incubated with a mixture of both molecules ($9 \mu\text{g.mL}^{-1}$ FN and $6 \mu\text{g.mL}^{-1}$ PDL for a final concentration of the coating molecules of $15 \mu\text{g/mL}$) by continuous stirring at 15 rpm for 90 min as previously described [18]. PAMs were then freeze-dried and stored at 4°C for the experiments or immediately used for release kinetics studies. These fibronectin-coated microspheres were named FN-PAMs.

2.2. Protein encapsulation efficiency

Protein encapsulation yield was determined considering both the VEGF-A biologically active entrapped protein and the total protein. Protein PLGA microspheres (5 mg) were dissolved in 1 mL acetone in silanised glass tube for 1h, the entrapped protein was separated from the dissolved polymer by centrifugation (15-min, 14,000 rpm) and the acetone was removed. To ensure that the entire polymer was dissolved, the step was repeated and the pellet resuspended in PBS. The encapsulation efficiency was measured using a protein dosage kit, NanoOrange test (Invitrogen, France) and VEGF-A ELISA kits (Peprotech, France).

2.3. Release kinetics, *in vitro* study

The *in vitro* release profile of protein from PLGA microspheres was determined by adding 250 μL of PBS buffer, pH 7.4, containing 1% w/v BSA to 1.5 mg of microspheres to eppendorf tubes. The tubes were closed and incubated in a shaking water bath (37°C , 125 rpm). The tubes were centrifuged for 5-min at 3,000 rpm and 250 μL of the supernatant were collected for analysis and replaced by fresh buffer. The percentage of released VEGF-A was measured by ELISA (Peprotech).

2.4. Early endothelial progenitor cell isolation and characterisation

Early endothelial progenitor cells (eEPCs) were obtained as previously described [21]. Mononuclear cells were isolated using Ficoll density-gradient centrifugation (Ficoll-Paque®) from healthy human peripheral blood and then plated on fibronectin-coated tissue culture dishes in endothelial basal medium (EGM-2 Bullet-Kit Cambrex) containing VEGF, fibroblast growth factor-2, insulin-like growth factor-1, epidermal growth factor, and 10% foetal bovine serum (complete medium). After 7 days of culture, non adherent cells were discarded, while the adherent ones were characterised by direct fluorescent staining to detect dual binding of FITC-UEA-1 (VECTOR Laboratories, Peterborough, England) and DiI-AcLDL (Molecular Probes, Leiden, The Netherlands), to confirm the EPC phenotype and by the expression of endothelial markers such as VEGF-A-R2, Ve-cadherin, E-selectin (Figure 1).

2.5. Cell seeding on PAM

Cells, ranging from 5×10^4 to 18×10^5 , were seeded in ultralow attachment 24-well in 1 ml of EBM-2 supplemented with 10% FCS and growth factors (complete medium) in the presence of 0.5 mg empty FN-PAM and were left in the incubator for 2 days without any change of the medium. After 6-15, 24 and 48h the adhesion of EPCs to PAM was evaluated under the inverted microscope. To assess EPC adhesion to VEGF-A-loaded PAM (FN-VEGF-PAMs), cells were seeded in 1ml of EBM-2 basal medium in the presence of 10% FCS but without any growth factor supplemented.

2.6. Analysis of adherent viable cells

Cell distribution and adherent viable cells on PAMs were evaluated by means of Alamar blue assay. Following 24 or 48h of incubation with microparticles, eEPCs attached on PAMs were recovered and divided from un-attached ones by means of a rapid and slow centrifugation (1000rpm, 30 sec). Then, pellets were placed in Phosphate buffer saline (PBS) and plated on a 96-well microplate in

the presence of 10 μ L of Alamar blue reagent (Invitrogen), kept at 37°C for 2 hours and then the fluorescence was measured at 540nm using a Wallac Victor 2 (Perkin Elmer).

2.7. Immunocytochemical analysis

After indicated time points eEPCs cultured on PAMs were fixed in 4% paraformaldehyde for 10 min, washed with PBS and quenched for 20 min with 4% BSA. The cells were labelled overnight at 4°C at manufacturer-recommended concentrations with the fluorescent antibodies: anti-VE-cadherin-FITC (Chemicon; Temecula, CA) and anti-KDR-PE (R&D Systems, Minneapolis, MN). For direct fluorescent staining to detect dual binding of FITC-UEA-1 and DiI-AcLDL, cells were first incubated with DiI-acLDL (Molecular Probes, Leiden, The Netherlands) at 37°C and later fixed with 4% paraformaldehyde for 10 min. Then cells were reacted with FITC-UEA-1 (VECTOR Laboratories, Peterborough, England) for 1h. Samples were viewed with a fluorescent microscope.

2.8. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated with collagenase perfusion of term umbilical cord vein [22]. HUVECs were grown in M199 medium supplemented with 20% FCS, antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin), 2 mM glutamine on 0.1% gelatin-coated plates, kept in a humidified incubator at 37°C in 5% CO₂ and split twice a week 1:2 using trypsin-EDTA solution. Cells between passages 2 and 5 were used. The cell type was characterised by “cobblestone” cell morphology under phase contrast microscopy and by immunohistochemical assay with a monoclonal anti-human factor VIII-related antigen antibody (DAKO). More than 95% of the cells were positive for von Willebrand factor.

2.9. Migration assay

A modified Boyden chamber was used to evaluate HUVEC migration (48-multiwell plates; Neuroprobe) [23]. Briefly, polyvinyl-pyrrolidone-free polycarbonate filters, 8 μ m pore size, were

coated with 100 $\mu\text{g.mL}^{-1}$ collagen type I and 10 $\mu\text{g/ml}$ fibronectin. Cells were suspended (50 μL , 1.2×10^4 cells) and added to the upper wells. FN-VEGF-PAMs (0.5 mg) or recombinant VEGF-A were added to the lower wells in 30 μL , then migration was evaluated by incubating the chamber at 37°C for 4 h. Migrated cells were methanol-fixed, stained with Diff-Quik and counted using a microscope (400x magnification) in 10 random fields per well. Each experimental point was measured in triplicate.

2.10. Proliferation assay

The biological activity of the released VEGF-A was confirmed using a HUVEC proliferation assay; HUVECs were cultured and passaged in standard endothelial cell medium conditions containing the growth supplement. The HUVECs were then plated (5×10^3 cells) onto 24-well plates, with filtered supplement-free medium conditioned with recombinant VEGF-A (rVEGF-A) at different concentrations and the effect on HUVEC proliferation was investigated at several times: 24h, 48h, 5 days and 1 week by the Alamar Blue assay (Invitrogen). The best effect was observed with 4 ng.mL^{-1} of rVEGF for 5 days. The effect of bioactive VEGF-A released from the microspheres on HUVEC proliferation was performed with the different aliquots collected throughout time and diluted to 4 ng.mL^{-1} according to the ELISA results and compared to the supplement-free medium alone or supplement-free medium containing 4 ng.mL^{-1} VEGF-A.

2.11. Western blot analysis

Adherent eEPCs to PAMs were lysed in buffer followed by centrifugation at 14,000 x g for 10 min at 4°C as previously reported [24]. Proteins were run on 10% SDS-PAGE gels, then transferred to a polyvinylidene difluoride (PVDF, Millipore) membrane and treated with anti-p-ERK $_{1/2}$ [Thr²⁰²/Tyr²⁰⁴] (mouse monoclonal IgG1, 1:2000, Cell Signaling Technology), poly (ADP-ribose) polymerase anti-PARP-1 (rabbit polyclonal 1:1000, Cell Signaling Technology) and anti- β -tubulin (mouse monoclonal clone tub 2.1, SIGMA, 1:1000) antibodies, followed by secondary

antibodies (anti-rabbit IgG peroxidase-linked antibody from donkey 1:10000 or anti-mouse IgG1 peroxidase-linked antibody from goat, 1:10000). Immunoreactive proteins were detected by enhanced chemoluminescence (ECL).

2.12. Statistical analysis

Data are reported as means \pm SEM. Statistical analysis was performed using Student's *t*-test for unpaired data. $P < 0.05$ was considered significant.

3. Results

3.1. eEPC adhesion to empty PAMs

Early EPCs at increasing concentrations, ranging from 5×10^4 to 18×10^5 , were seeded in the presence of 0.5 mg empty FN-PAMs, in complete medium. The attached cells were observed under the microscope after 6, 12, 24, 48h and 5 days. Results showed that the optimal EPC concentration (1.25×10^5 /0.5 mg PAM) attached to FN-PAMs within a few hours (Figure 2A) and increased in a time-dependent manner, with maximal effect after 48h (Figure 2E). It is possible, indeed, to observe many cells attached to the microspheres that formed connections and bridges with other adherent and non-adherent cells. Moreover, the adhesion was improved when FN-PAMs were further coated with full length fibronectin (10 μ g/ml), as it is shown in the Figure 2D and 2F (+ 29% over FN-PAMs).

3.2. VEGF-A release profile and effect of the released protein on HUVEC proliferation

Microspheres ranging around 40-60 μ m were obtained. The encapsulation yield of FN-VEGF-PAMs was $97.4 \pm 18.7\%$. The 40 μ m FN-VEGF-PAMs exhibited a burst release of VEGF-A within the first 24h and then a slow and continuous release for at least 3 weeks consisting in a cumulative release of $444,57 \pm 85,44$ ng/mL for 2,5 mg (Figure 3A). The release kinetics for the amount of FN-VEGF-PAMs used in this study (0.5 mg), corresponded to approximately 90 ng/mL of VEGF

released during 3 weeks. The bioassay performed with the HUVEC cells confirmed that the VEGF released from 40-60 μ m FN-VEGF-PAMs was released under a bioactive conformation during the entire period. Briefly, the VEGF collected from each sample of the kinetics release assay (diluted to 4 ng/mL) was able to stimulate HUVEC proliferation for a period of 5 days in a similar manner as the recombinant VEGF at 4 ng/mL (Figure 3B).

3.3. eEPC adhesion to VEGF-A-releasing PAM (FN-VEGF-PAMs)

A time-course of eEPC adhesion to VEGF-A-loaded microspheres (FN-VEGF-PAMs) was assessed by seeding 125,000 cells with 0.5 mg FN-PAMs in medium supplemented with 10% FCS but without any growth factor inside. Early EPCs were able to adhere to FN-VEGF-PAMs earlier than to empty FN-PAMs (Figure 4). The prompt adhesion was evident within 6 h (Figure 4B vs A) and this difference was more apparent after 12h incubation (Figure 4D vs C). After 24h incubation (Figure 4 E,F) it was possible to observe many cells adherent to VEGF-A-releasing FN-PAMs, which formed bridges with other cells and microspheres (Figure 4F).

Following 48 h incubation, the quantification of adhesion to empty and FN-VEGF-PAMs was assessed by means of alamar blue assay. As shown in Figure 5, eEPCs significantly increased their adhesion to FN-VEGF-PAMs when compared to empty FN-PAMs. The number of attached cells to FN-VEGF-PAMs was almost two-fold increased compared to FN-PAMs ($P < 0.05$, Figure 5). Based on these results, the possible pro-survival or anti-apoptotic effects of FN-VEGF-PAMs was investigated by means of the detection of phospho-ERK_{1/2} and cleaved PARP-1. eEPCs grown for 2 days on FN-VEGF-PAMs displayed a smaller cleaved PARP-1 expression (Figure 6A) and increased phosphorylation of ERK_{1/2} (Figure 6B) compared to eEPCs attached to empty FN-PAMs, suggesting a protective role of VEGF-A released by microspheres on endothelial progenitor cell apoptosis.

3.4. Phenotype of adherent eEPC to empty and FN-VEGF-PAMs.

Based on the results reported above, the eEPC phenotype was assessed following their adhesion to microspheres. Cells were culture with loaded and empty FN-PAMs in 10% FCS medium without any supplemented growth factor. Following 48h incubation, adherent cells were positive for either Ve-cadherin (Figure 7A) or KDR (Figure 7B), although the number of double-positive cells was higher for FN-VEGF-PAMs than for empty ones (Figure 7). Only the cells attached to VEGF-A-releasing FN-PAMs were strongly positive for endothelial markers for up to 5 days of culture. Furthermore, loaded-PAMs were completely covered and enclosed by eEPCs, which formed connections with other cells and microspheres. Conversely, in the absence of a slow release of the growth factor from the microspheres (empty FN-PAM), few cells maintained an endothelial phenotype (Ve-cadherin⁺ KDR⁺) after 5 days of culture (Figure 7).

3.5. Effect of FN-VEGF-PAMs on eEPC and mature endothelial cell migration

The ability of FN-VEGF-PAMs to stimulate mature endothelial cell and eEPC migration through their release of VEGF-A was tested. The VEGF-A released from FN-PAMs was able to significantly induce mature endothelial cell migration and this effect was comparable to that obtained with an effective concentration of human recombinant VEGF-A (Figure 8A). Early EPCs did not respond to either 10 ng/mL human recombinant VEGF-A or FN-VEGF-PAMs (Figure 8B). As is well known, eEPCs migrate in response to a VEGF-A concentration as high as 50 ng/mL [24].

4. DISCUSSION

The formation of a functional vessel network via angiogenesis represents a crucial and critical step in the wound healing process and in the repair of ischaemic tissue. Effective angiogenesis occurs as a result of the proliferation of pre-existing mature endothelial cells in response to stimuli released in the wound site, but may also involve the direct or indirect action of EPCs. Direct incorporation of EPCs into neo-vessels has been demonstrated together with the production of paracrine signals

locally released [5,25]. To that end, great efforts have made to improve EPC recruitment and incorporation into sites where angiogenesis is needed. Since the discovery of EPCs [26], a variety of delivery techniques has been used to enhance angiogenesis, including endogenous upregulation [27], systemic delivery [28] and local injection [29]. Although the functional benefit of EPC therapy in the setting of myocardial and hind limb ischaemia has been shown, few have been able to demonstrate a long-term effect, due to high percentage of EPC death and systemic dispersion. Short of sustained survival of transplanted EPCs, neovasculature fails to recruit the perivascular cells and does not resemble mature microvessels. Despite great progress, the field of angiogenesis is viewed as a top priority in tissue regeneration and tissue engineering, and also the area of least progress in the past decade [30].

The use of biomaterials as a scaffold for EPC delivery has been proposed and diverse biomaterials have been used to stimulate angiogenesis necessary for wound healing [31], skin regeneration [32], hind limb ischaemia [33] and myocardial infarction [34,35]. Pharmacologically active scaffolds have also been proposed for enhancing angiogenesis through the stimulation of delivered EPCs and cells in the microenvironment [33,36]. Recently in a murine model of hind limb ischemia it has been demonstrated that a sustained release of VEGF-A from PLGA-based formulations stimulated the angiogenic process [37]. However, it has not been yet demonstrated that pharmacologically active microcarriers can support human EPC adhesion and survival.

In our study, fibronectin-coated microcarriers made with poly(D,L-lactic-co-glycolic acid) (PLGA), either empty or loaded with VEGF-A, have been examined as an optimized culturing scaffold for early EPCs (eEPCs). We demonstrated that FN-PAMs are a good support for eEPC culture, since cells attach to them within a few hours and maintain their phenotype for up to 5 days of culture. Despite some limitations, “early” endothelial progenitor cells (eEPCs), can easily be obtained in few days by *ex-vivo* differentiation of peripheral blood mononuclear cells (PBMCs) and have been demonstrated to stimulate angiogenesis in *in vivo* animal models and in humans [38,39]. It is well

known that despite their poor integration into neovessels, they are able to release many growth factors which strongly stimulate resident cells which lead to the angiogenic process [40,25].

Our data also demonstrate that the slow release of VEGF-A from FN-PAMs, strongly sustained eEPC phenotype and survival, in *in vitro* culture, without any addition of other growth factors. eEPCs, indeed, were Ve-cadherin⁺ and KDR⁺ up to 5 days in culture, while eEPCs cultured with empty FN-PAMs without any growth factor supplemented, were less able to attach to them and to maintain an endothelial phenotype. Adhesion experiments with labelled cells (i.e. Alamar blue assay) also demonstrated an increased eEPC adhesion to VEGF-A loaded FN-PAMs (FN-VEGF-PAMs), compared to empty ones. Since it is well known that eEPCs do not proliferate [41,5], the reason for this increased cell number was investigated. We found that cleaved poly (ADP-ribose) polymerase (PARP-1) was reduced in lysates of eEPCs attached to VEGF-A-loaded microspheres. It is well known that during apoptosis, PARP-1 is specifically cleaved from its enzymatically active form into two fragments of 24 kDa and 89 kDa by execution caspases 3 and 7, and the cleavage of PARP-1 has been shown to be a reliable marker for apoptosis in a wide variety of cell types [42]. It is conceivable that the VEGF-A released from microcarriers has anti-apoptotic/pro-survival effects on EPCs. The increase in phospho-ERK_{1/2} in eEPCs cultured with FN-VEGF-PAMs may confirm this hypothesis.

Tissue engineering, which combines cells with a supportive scaffold providing a 3D structure, may help to improve cell engraftment after transplantation. Scaffolds providing a biomimetic surface of different extracellular matrix (ECM) molecules or their derived peptides, that stimulate cell survival and differentiation, may further enhance cell engraftment [43]. In this regard, fibronectin has been demonstrated to be the best ECM surface for culturing eEPCs [26,41], since it facilitates stem precursor attachment to culture dishes and promotes stem cell differentiation toward an endothelial phenotype [44].

PLGA microspheres have previously been used for many applications, including bone repair [45] and for neurodegenerative disorders, by using them as a pharmacological tool [46] and/or

pharmacological scaffold for cell therapy [18;19,18]. The effect of microsphere delivered Nerve Growth Factor (NGF) has also been tested for sustaining peripheral nerve regeneration in a rat model [47]. PAMs are biodegradable and non-cytotoxic poly(lactic-co-glycolic acid) microspheres, coated by a biomimetic surface and releasing a therapeutic protein, which acts on the cells conveyed on their surface and on their microenvironment. The ability to release angiogenic factors might be a good strategy for stimulating either delivered EPCs or resident cells involved in the angiogenic process. Our data demonstrate that VEGF-A released by PAMs is able to sustain eEPC phenotype and survival, but not their migration; the amount of the growth factor released within few hours is not enough for stimulating their migration, which is known to be induced for VEGF-A concentration as high as 50 ng/ml [21]. FN-VEGF-PAMs were also able to stimulate mature endothelial cell growth and migration. The proliferative effects on HUVECs were still evident up to 21 day, thus demonstrating that VEGF-A released from the microparticles was under a bioactive conformation during the entire period.

In conclusion, this combined effect of pharmacologically active microcarriers on progenitor and mature endothelial cells seems to be of potential interest for in vivo applications.

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ACCEPTED MANUSCRIPT

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Figure legends

Figure 1: Phenotype of early EPC. A-D) Representative histograms of flow cytometry analysis of endothelial markers in eEPCs. E-F) EPC characterisation by immunofluorescence: endocytosis of acLDL (E) and binding to UEA-1 (F). Magnification $\times 400$.

Figure 2: eEPC adhesion to empty FN-PAMs following 6 h (A), 12 h (B), 24h (C) and 48h (E) to FN-PAM and to FN-PAM further coated with full length fibronectin after 24h incubation (D and F). Magnification $\times 200$.

Figure 3: Quantitative and functional characterization of FN-VEGF-PAMs. A) Representative *in vitro* release of VEGF-A from 1.5mg PAMs during 21 days. The release kinetic was evaluated by quantifying the total VEGF-A released at each time-point by ELISA (mean \pm SD of 2 experiments). B) Representative bioactivity of released VEGF-A with a HUVEC cell culture assay. Proliferation rate of HUVECs evaluated by Alamar blue after 5 days in: i) supplement-free medium alone (day 0), ii) supplement-free medium containing 4 ng.mL⁻¹ standard VEGF-A (100% of HUVEC proliferation), iii) supplement-free medium conditioned with VEGF-A released from FN-VEGF-PAMs at several time-points (d=days) diluted at 4 ng/mL (based on the ELISA results). Mean= average proliferation rate at all the time-points (mean \pm SD, n= 2).

Figure 4. Time course of eEPC adhesion to FN-PAMs (A, C, E) and FN-VEGF-PAMs (B, D, F). Magnification $\times 200$.

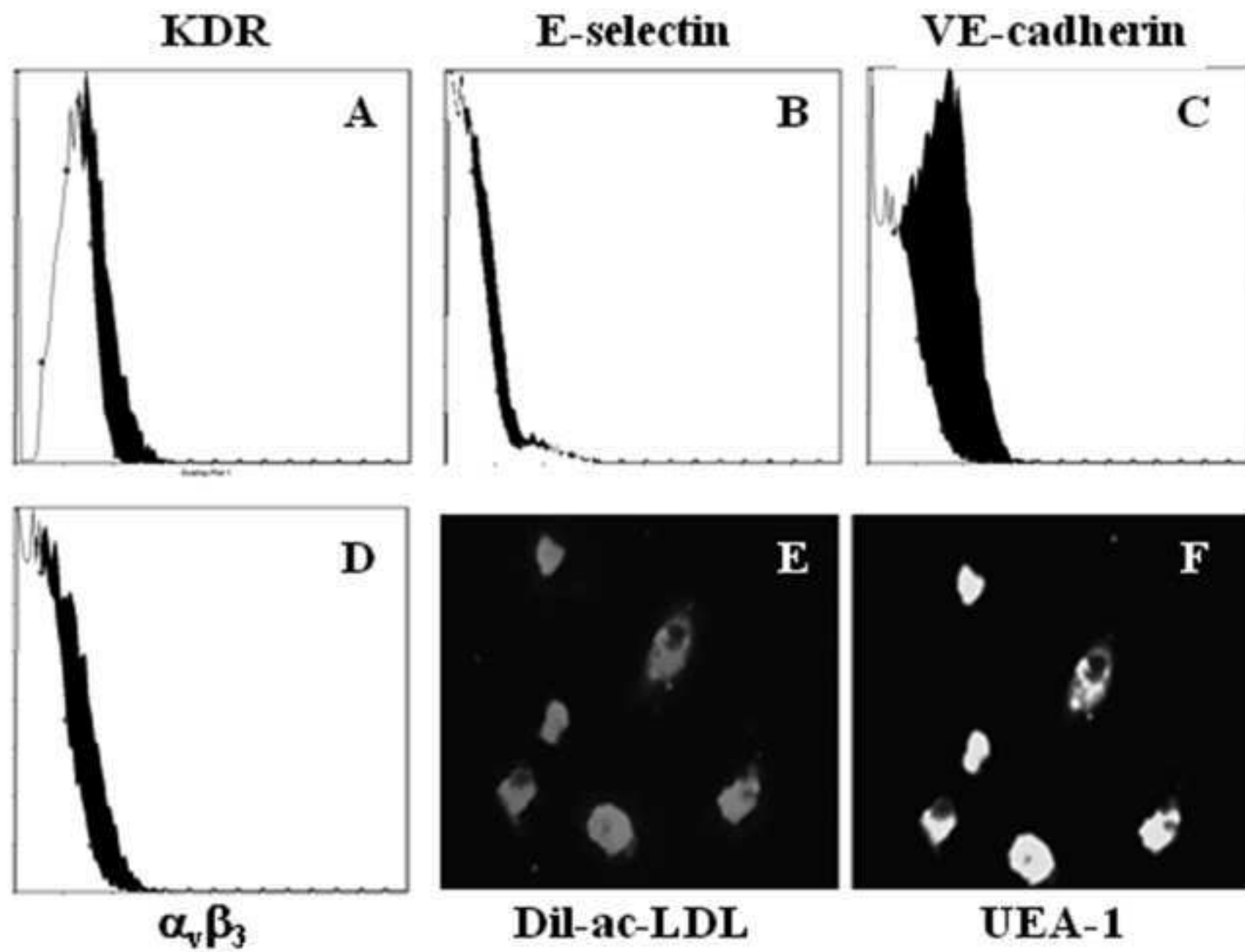
Figure 5. Quantification of adherent EPCs. Fluorescent EPCs adherent to empty (FN-PAMs) and VEGF-A-loaded PAMs (FN-VEGF-PAMs) were measured. Mean \pm SE of 3 experiments. $P < 0.05$ vs FN-PAMs.

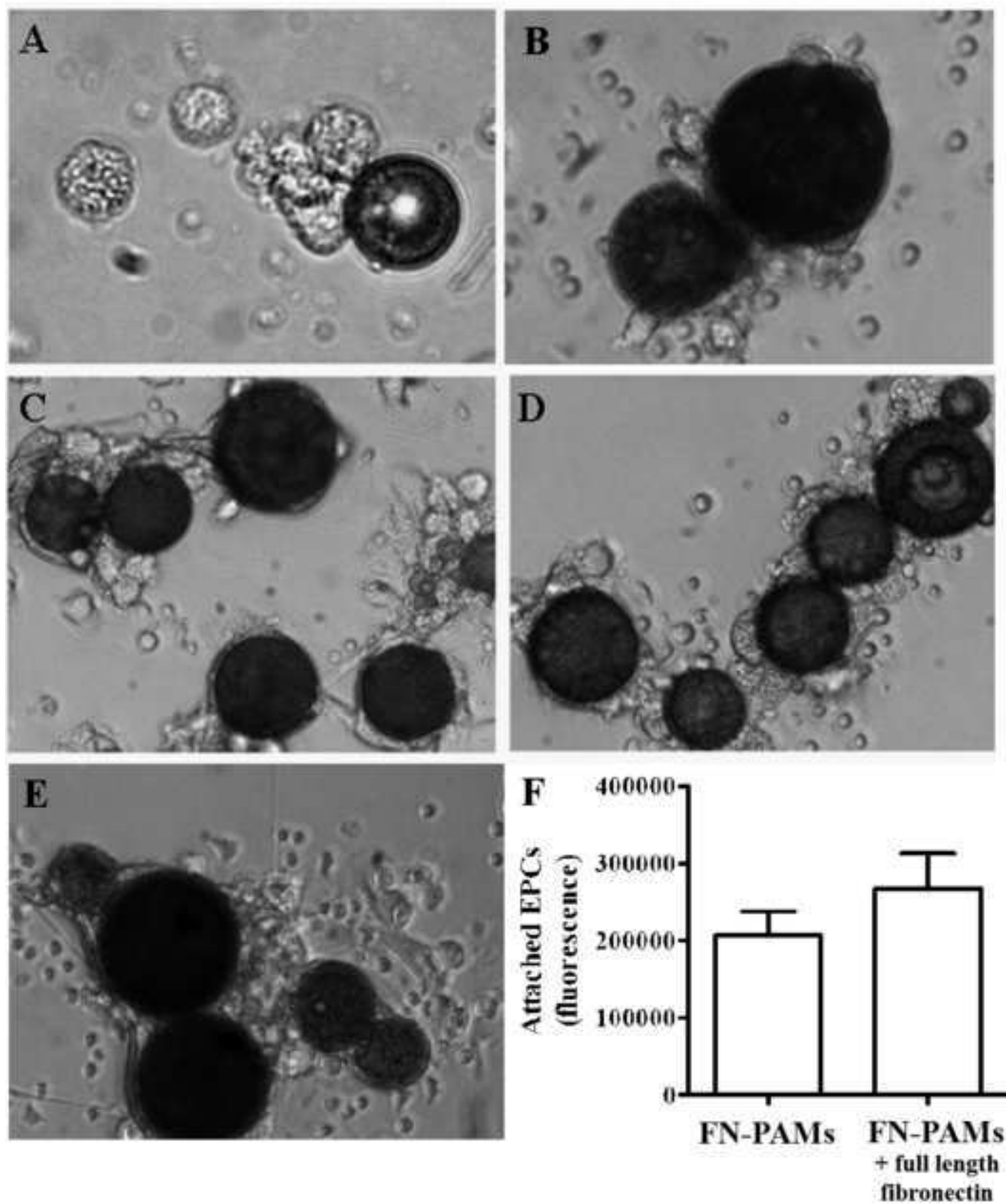
Figure 6. Effect of FN-VEGF-PAMs on cell apoptosis. eEPCs attached to PAMs were lysed and cell lysates were run on 10% polyacrylamide gel electrophoresis. A) cleaved-PARP1 (clv PARP-1) expression on eEPCs attached to empty (FN-PAMs) or VEGF-loaded PAMs (FN-VEGF-PAMs). B) ERK_{1/2} phosphorylation on EPCs attached to empty or FN-VEGF-PAMs. Data were normalised with β -tubulin and are expressed as an immunoreactive band densitometry (OD). * $P < 0.05$ vs empty PAMs (FN-PAMs), mean \pm SE, n=3.

Figure 7. Endothelial phenotype of adherent eEPCs following 48h and 5 days of culture with empty and VEGF-loaded PAMs. Ve-cadherin⁺ eEPCs (A) and KDR⁺ eEPCs (B) detection. Magnification x400.

Figure 8. Effect of FN-VEGF-PAMs on HUVEC (A) and eEPC (B) migration. HUVECs were stimulated with 0.5mg VEGF-loaded PAMs for 5 h. VEGF-A (10 ng/mL) was added as positive control. eEPCs were allowed to migrate for 18h in response to either 0.5 mg FN-VEGF-PAMs or 10 ng/mL VEGF-A. Mean \pm SE, n=3 in triplicate- * $P < 0.05$ vs unstimulated cells (control, ctr).

Figure 1





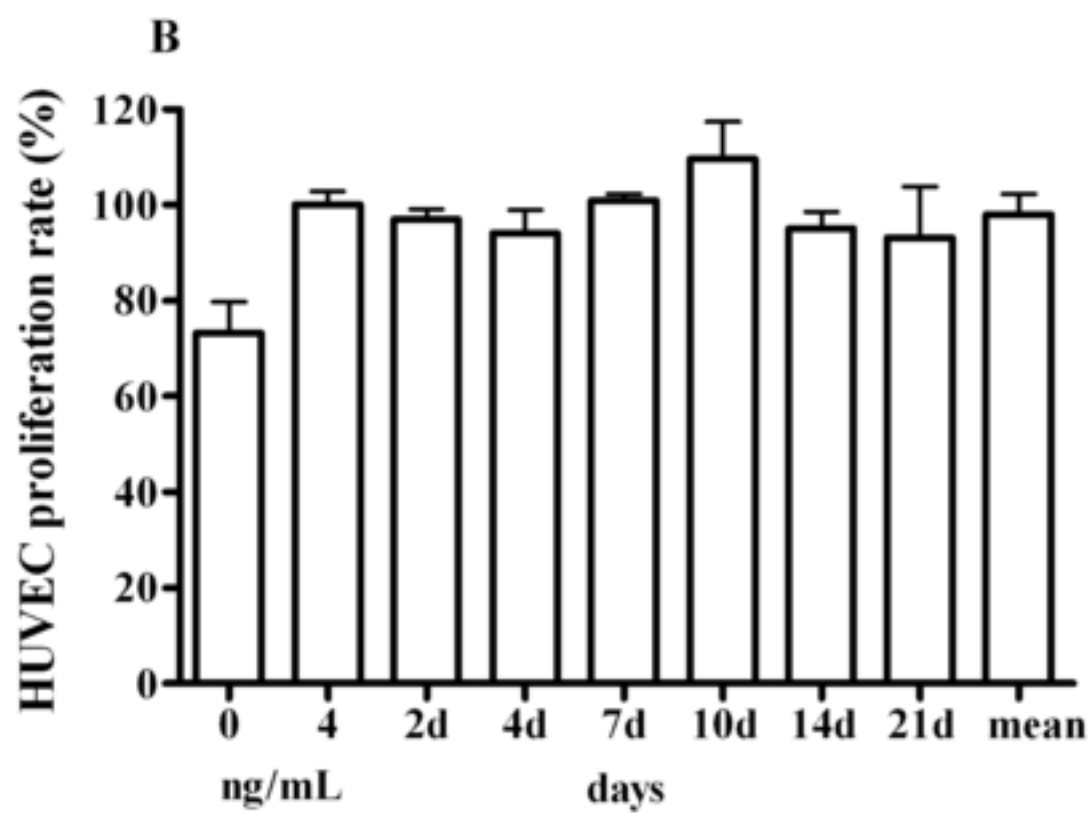
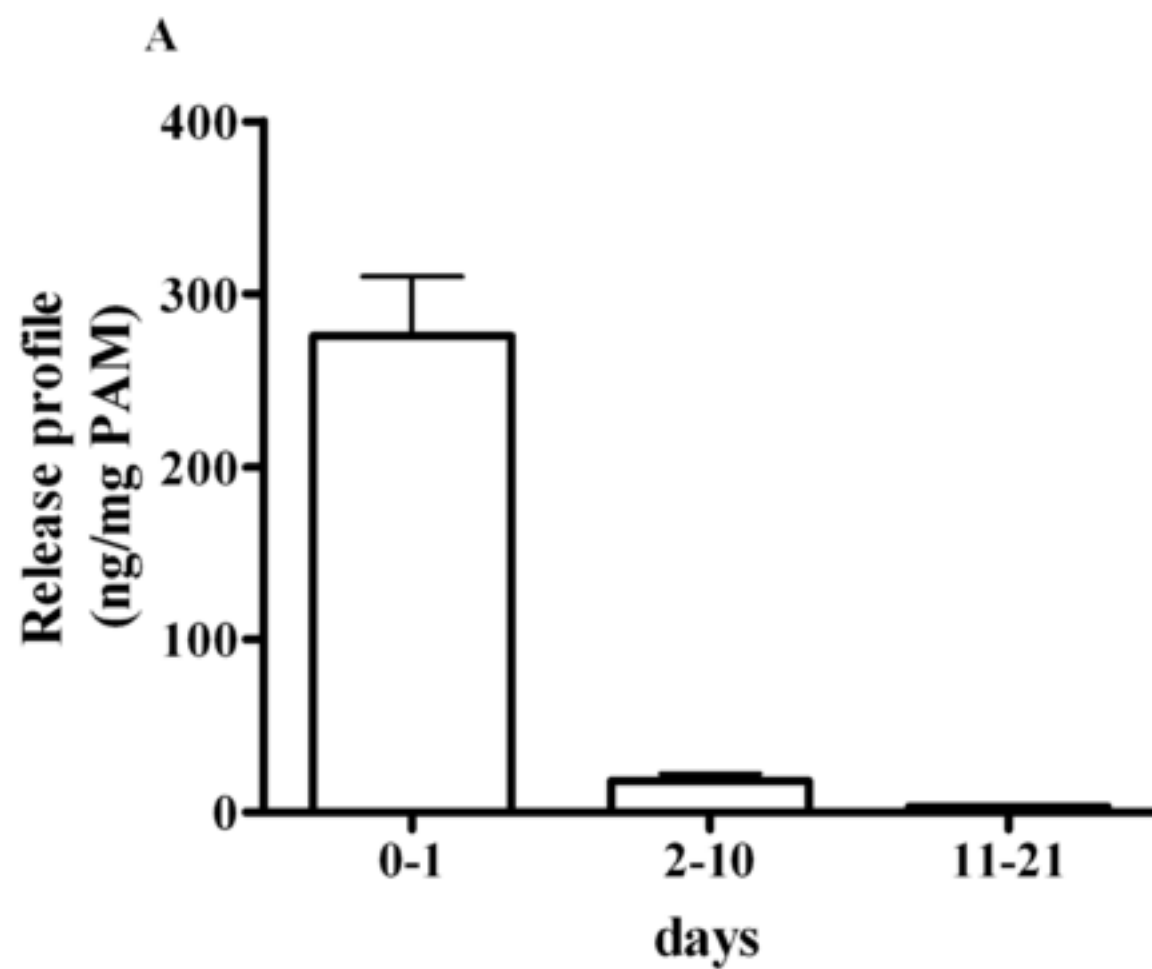


Figure 4

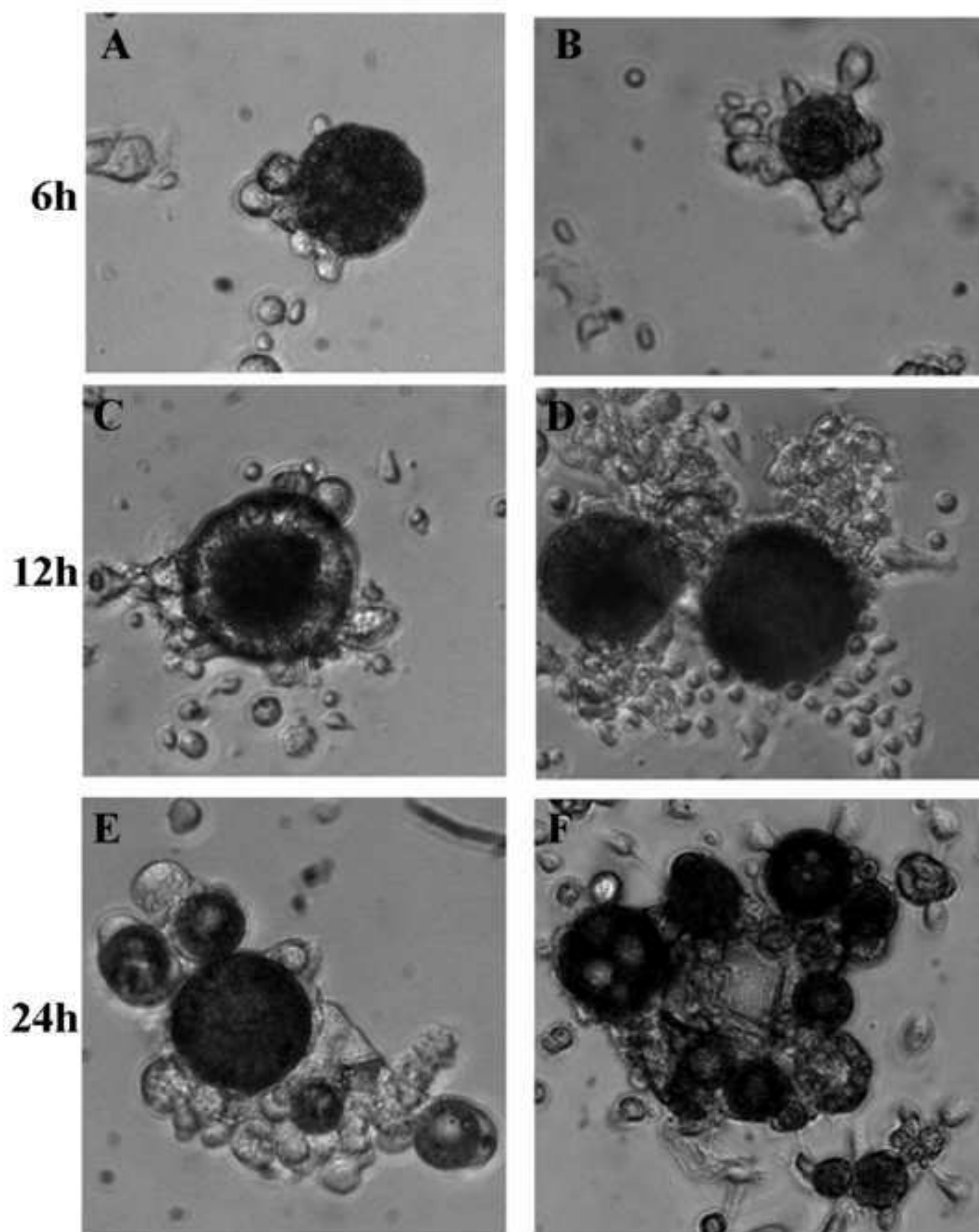


Figure 5

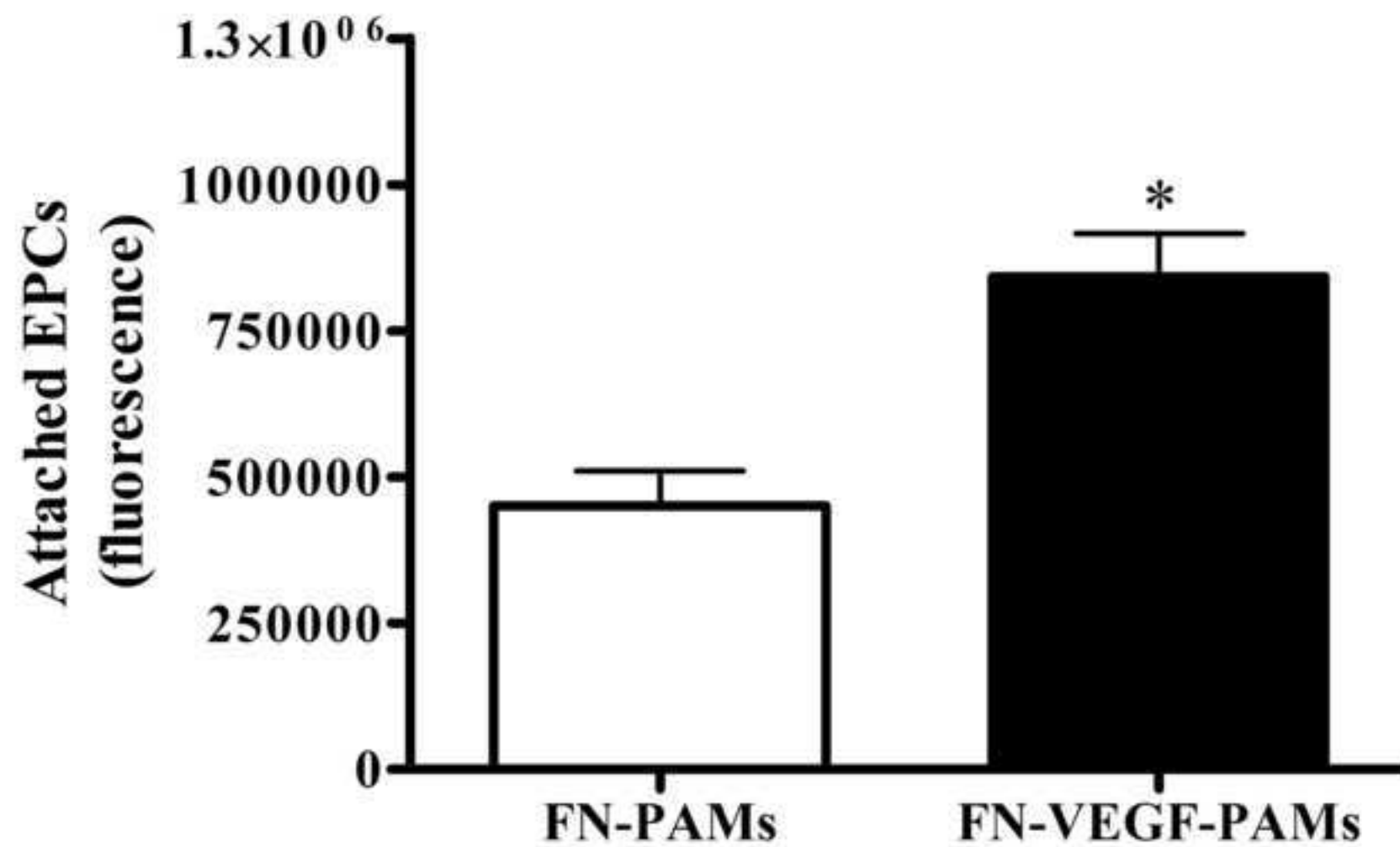
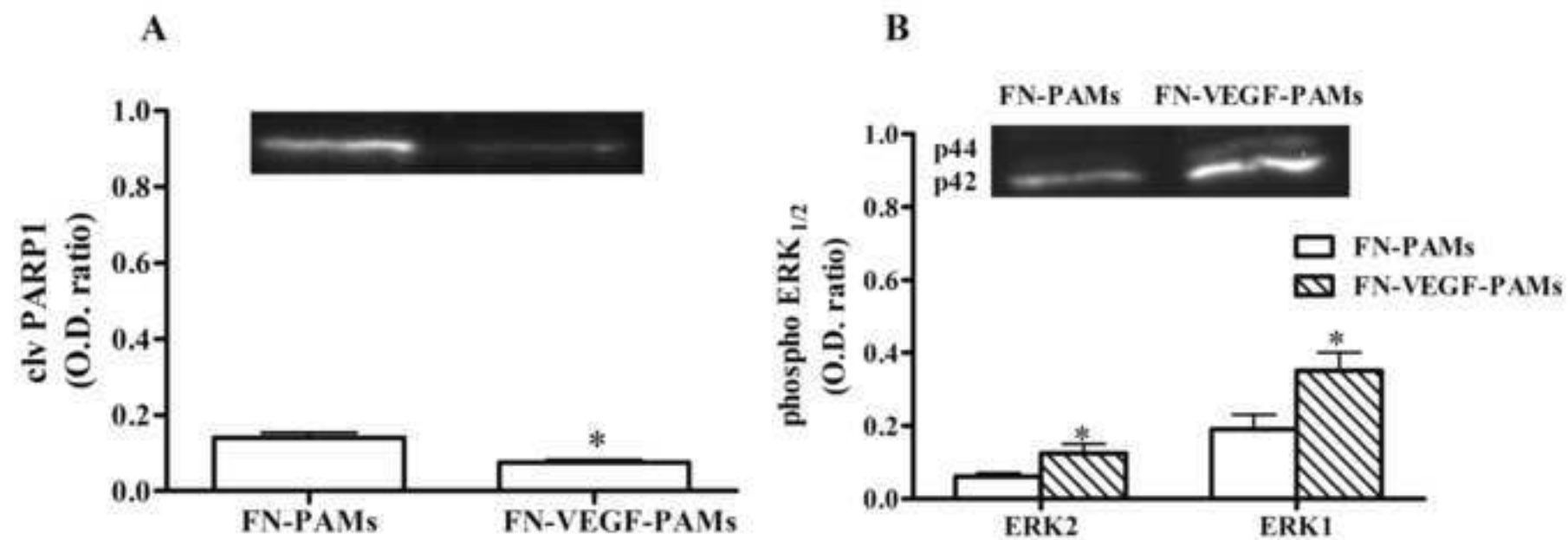
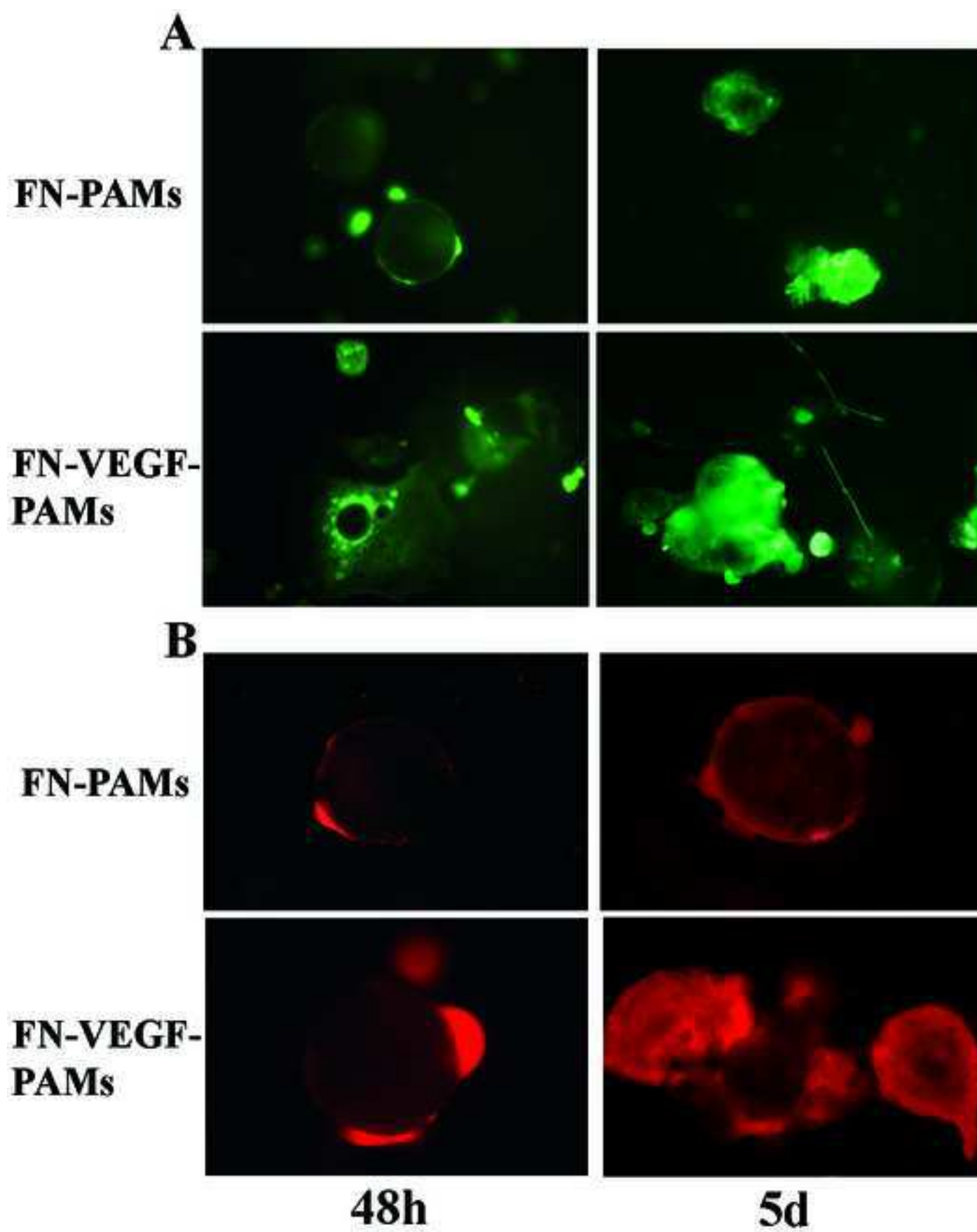
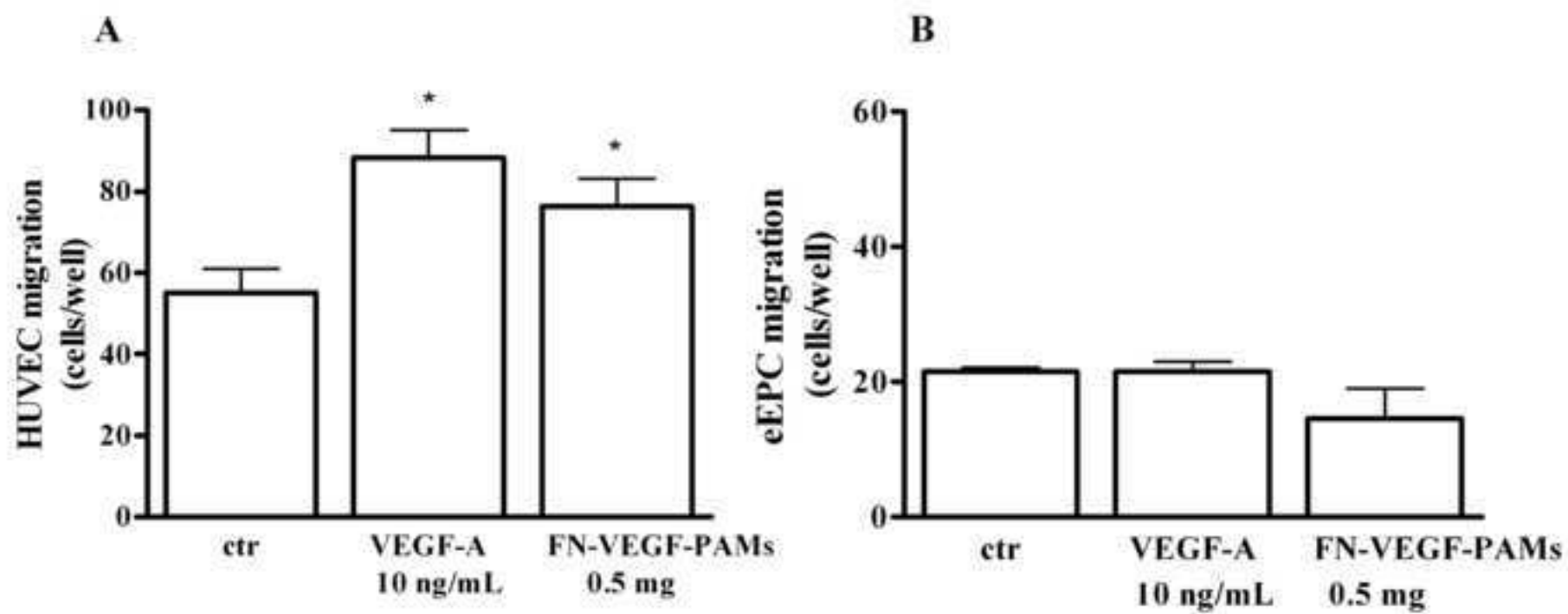
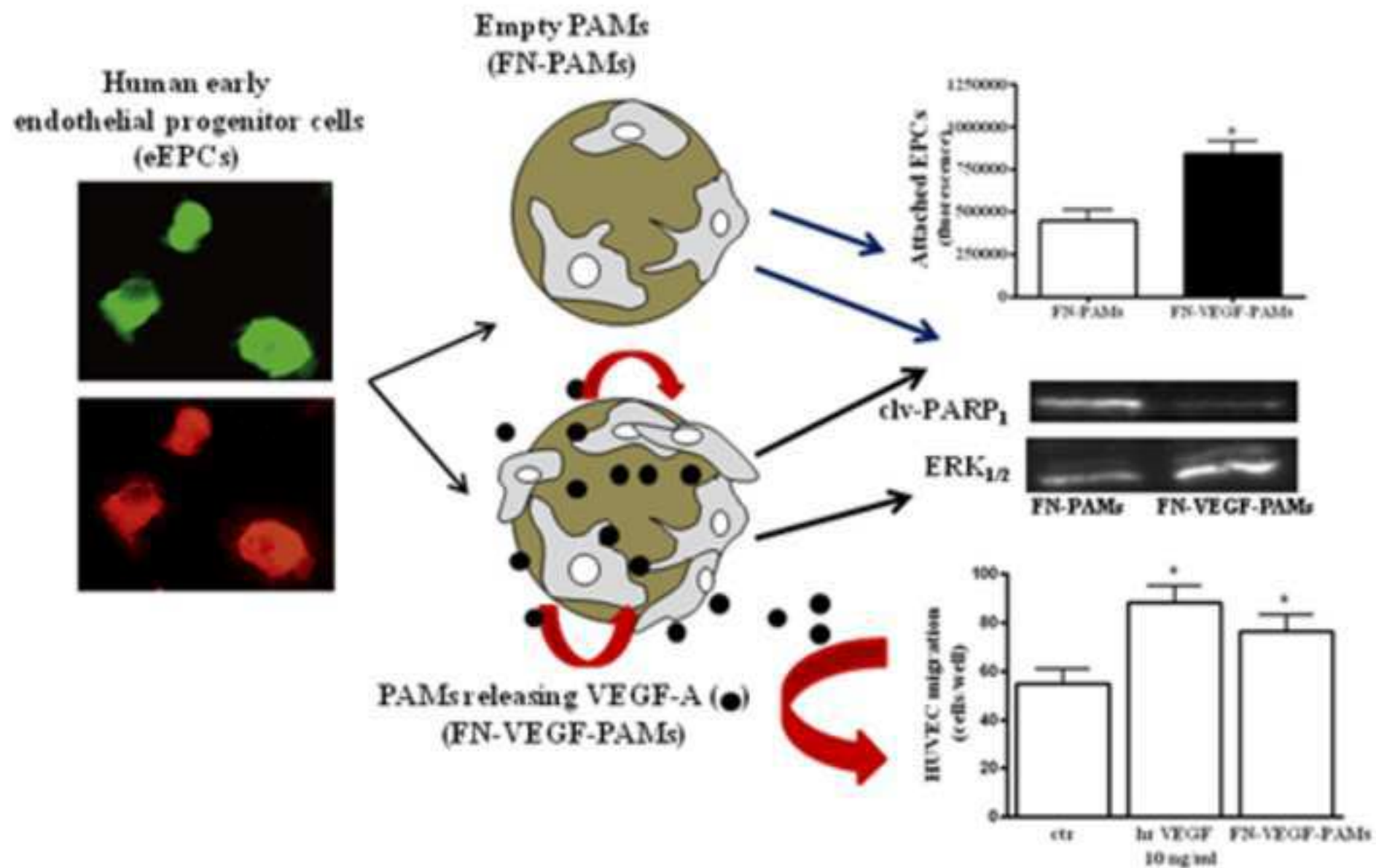


Figure 6











Pharmacologically active microcarriers influence VEGF effects on mesenchymal stem cells survival

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**Pharmacologically active microcarriers influence
VEGF effects on mesenchymal stem cells survival**

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Running head: VEGF, microcarriers and MSC survival

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Abstract

Vascular-endothelial-growth-factor (VEGF) as such or slowly released by fibronectin-coated pharmacologically-active-microcarriers (fPAM-VEGF) could differently affect survival kinases and anti-apoptotic mediator (*e.g.* Bcl-2). Therefore VEGF or fPAM-VEGF could differently enhance cell proliferation, and/or resistance to hypoxia/reoxygenation (H/R) of mesenchymal-stem-cells (MSCs). To test these hypotheses MSCs were incubated for 6-days without VEGF or with VEGF alone or with fPAM-VEGF. In addition, MSCs pre-treated for 24-h with VEGF, fPAM-VEGF or empty PAMs (fPAMs) were subsequently exposed to H/R (72-h 3% O₂ and 3-h of reoxygenation). The cell-proliferation and post-hypoxic vitality were determined. Cell proliferation increased about two-fold ($p<0.01$) 6-days after VEGF treatment, but by a lesser extent (a 55% increase) with fPAM-VEGF ($p<0.05$). While pre-treatment of MSCs with VEGF confirmed a stimulation of cell-proliferation ($p<0.01$), pre-treatment with fPAM-VEGF protected MSCs against H/R. Accordingly, VEGF increased the amount of phospho-ERK-1/2, phospho-Akt and phospho-PKC ϵ compared to the untreated cells or fPAM-VEGF. Only fPAM-VEGF significantly increased the amount of Bcl-2. While VEGF enhances MSC proliferation in normoxia, fPAM-VEGF mainly hampers post-hypoxic MSC death. These different effects underscore the necessity of approaches suited to the various conditions. The use of fPAM-VEGF can be considered as a novel approach for enhancing stem cell survival and regeneration in hostile environment of post-ischemic tissue.

Key words: Microspheres; Drug release; Growth factor; Hypoxia; Transplantation; Stem cells.

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1. Introduction

Loss of cells in the absence of appropriate tissue repair characterizes many organ diseases. The high mortality rate of cells during diseases, including ischemia, is not counteracted by the limited intrinsic regeneration capacity of many adult organs. Many investigators attempted to modify the ratio “organ tissue damage/repair” by means of stem cell based regenerative therapies during the last decade [1,2]. Results from recent clinical studies with infarcted patients showed improved cardiac function following injection of adult stem cells from different sources including bone marrow and peripheral blood [2-4].

Mesenchymal stem cell (MSCs) transplantation is a promising strategy. However, cell replacement therapy is limited by the poor vitality of transplanted cells, especially in hypoxic environment. It has been reported that the capacity of transplanted cells to survive and subsequently integrate into host tissue may be so poor that about 99% of transplanted MSCs are lost during the first 24-h after transplantation [5]. Nevertheless, after infusion or injection into an ischemic tissue, MSCs face a hostile, inflammatory environment that may strongly limit their function and survival. Another important problem during the implantation of stem cell in the injured organ is the formation of new vasculature. In fact, it is important that during the repair of the ischemic tissue an appropriate angiogenesis occurs [6].

Experimental observations have shown the plasticity of MSCs indicating that in appropriate conditions these cells can repair a damaged tissue [2,7]. The influence of endogenous factors such as cytokines, growth factors, and the local cellular milieu upon stem cells remains poorly understood [8-16]. Besides being a promoter of angiogenesis, the vascular-endothelial-growth-factor (VEGF), is considered a multifunctional growth factor; in

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3 particular, it has been shown to promote myocardial protection in the short term by
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5 decreasing cardiomyocyte apoptotic signaling and in the long term by increasing
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7 neovascularization and tissue perfusion [17,18]. MSC themselves are capable of producing
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9 VEGF both in normo- and in hypoxic conditions [19]. However, evidently, this production
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11 is not enough to promote their protection when transplanted [5]. Exogenous VEGF can
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13 directly augment myocardial function during acute ischemia/reperfusion [20]. Moreover,
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15 VEGF can induce the activation of survival protein kinases including Akt pathway, as well
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17 as the activation of antiapoptotic and growth/hypertrophy signaling pathways [21,22].
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19 Accordingly, one would expect that VEGF may increase the progeny formation of MSCs.
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21 However, the size of the progenitor's cell compartment is governed by the balance between
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23 the cell gain (self renewal) and the cell loss (apoptosis).
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29 Therefore, an imbalance in renewal and apoptosis will result in an elevation or a fall in the
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31 progenitor cell mass [23]. It can be expected that the limitation of cell death in a hostile
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33 environment may require a prolonged activity of the protective agent. Since VEGF may
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35 affect both self renewal and apoptosis, and since pharmacologically-active-microcarriers
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37 (fPAMs) have been developed, which allow *in situ* prolonged/controlled VEGF delivery and
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39 cell adhesion by their fibronectin biomimetic surface, we hypothesize that there are
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41 substantial differences on whether this growth factor is applied as a free compound or
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43 complexed to fPAMs for a sustained release.
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48 In the present study we compared *in vitro* whether or not fPAMs delivering VEGF (fPAM-
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50 VEGF) induces a different protective/proliferative effects compared to VEGF alone. In
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52 particular, we tested whether MSC survival in hypoxic conditions is differently influenced
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54 by fPAM-VEGF and VEGF pre-treatment.
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2. Materials and Methods

2.1 Formulation of PAMs Releasing VEGF

Poly(lactic-co-glycolic acid) (PLGA)-microspheres of an average diameter of 60 µm were prepared using an emulsion solvent extraction-evaporation process previously described [24]. The PLGA-copolymer is with a lactic:glycolic ratio of 37.5:25 (MW: 25,000 Da) (Phusis, Saint Ismier, France). The total protein loading was 0.6% w/w with respect to the amount of polymer, *i.e.* 0.1% VEGF₁₆₅ (Peprotech, France) and 0.5% HSA. First, NaCl and glycofurol, a water-miscible non-solvent of proteins, were used to precipitate the proteins separately, as previously described [25] and adapted to each protein. For VEGF a NaCl solution at 0,01M at 4°C, containing a protein-poloxamer 188 excipient in an additive to protein ratio of 20:1 was added and mixed to glycofurol to form a 1 ml suspension. The same procedure was used for HSA with a NaCl solution at 0,3M. Thirty min later, the protein nanoparticles were recovered by centrifugation (10,000 g, 30-min). They were then carefully dispersed in the organic solution (667 µl; 3:1 methylene chloride:acetone) containing 50 mg polymer and emulsified in a poly (vinyl alcohol) aqueous solution (90 ml, 4% w/v) maintained at 1°C and mechanically stirred for 1-min (Heidolph RZR2041, Merck Eurolab, Paris, France). After addition of 33 ml of deionized water and stirring for 10-min, the resulting o/w emulsion was added to deionized water (167 ml) and stirred at 550 rpm further for 20-min to extract the organic solvent. Finally, the formed microparticles were filtered on a 5 µm filter (HVLP type, Millipore SA, Guyancourt, France), washed with 500 ml of deionized water and freeze-dried. Unloaded microparticles were prepared in the same manner without adding the protein.

The microspheres were coated with 12 $\mu\text{g/mL}$ of poly-D-lysine and 18 $\mu\text{g/mL}$ of fibronectin (Sigma, Saint-Quentin Fallavier, France) as previously describes [26]. Briefly, microspheres were placed under rotation with the fibronectin-poly-D-lysine mixture for 90-min at 15 rpm in an incubator and were then freeze-dried and stored at 4 C for the experiments or immediately used for release kinetics studies. These fibronectin-coated microspheres are named fPAMs.

2.2 Protein encapsulation efficiency

Protein encapsulation yield was determined considering both the VEGF biologically active entrapped protein and the total protein, Protein PLGA microspheres (5 mg) were dissolved in 1 ml acetone in silanized glass tube for 1h, the entrapped protein was separated from the dissolved polymer by centrifugation (15-min, 14,000 rpm) and the acetone was removed. To ensure that the entire polymer was dissolved, 1ml of acetone was added and the solution was left to stand for one more hour, and then totally removed after centrifugation (15-min, 14,000 rpm). The pellet was resuspended in PBS. The encapsulation efficiency was measured using a protein dosage kit, NanoOrange test (Invitrogen, France) and VEGF ELISA kits (Peprotech, France).

2.3 Release kinetics, in vitro study

The *in vitro* release profile of protein from PLGA microspheres was determined by adding 250 μL of PBS buffer, pH 7.4, containing 1% w/v BSA to 2.5 mg of microspheres into eppendorf tubes. The tubes were closed and incubated in a shaking water bath (37°C, 125 rpm). The tubes were centrifuged for 5-min at 3,000 rpm and 250 μL of the supernatant were collected for analysis and replaced by fresh buffer. The percentage of released VEGF was measured by ELISA (Peprotech).

To confirm the biological activity of the released VEGF a bioassay was performed using a human umbilical vein endothelial cells (HUVECs) proliferation assay; HUVECs were cultured and passaged in standard endothelial cell medium conditions containing the growth supplement. The HUVECs cells were then plated (5×10^3 cells) onto 24 well plates, with filtered supplement-free medium conditioned with recombinant VEGF (rVEGF) at different concentrations and the effect on HUVEC proliferation was investigated at several time-points 24h, 48h, 5 days and 1 week by the Alamar Blue assay (Invitrogen). The best effect was observed with 4ng/mL of rVEGF for five days. The effect of bioactive VEGF released from the microspheres on HUVEC proliferation was performed with the different aliquots collected throughout time and diluted to 4 ng/mL according to the ELISA results and compared to the supplement-free medium alone or supplement-free medium containing 4 ng/ml VEGF.

2.4 MSC isolation and cell culture

Stem cells were extracted from bone marrow of femurs of Wistar rats 6-12 months of age (weight 450-550 g) (Janvier, France). MSCs were extracted by inserting a 21-gauge needle into the shaft of the bone and flushing with a solution of minimum essential medium eagle α (α -MEM) and 20% fetal bovine serum (FBS) (Sigma-Aldrich) implemented with 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Lonza); the cell suspension was filtered and cultured at 37°C. After 24-h the medium was replaced with α -MEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. We allowed MSCs to grow up to passage 3 (P3), replacing the medium every 2-3 days as reported in the literature [27-30].

To verify that the cell population we used for cultures was composed of MSCs, we showed that the cells were CD90 positive and CD34/CD45 negative [27-29]. Moreover, previous differentiation experiments performed in our laboratories showed the MSC potential to differentiate into adipocytes [29], osteoblasts and muscle cells [27].

The MSCs were then included in the study and used accordingly to the protocols described below.

Rats were used in accordance with the Italian law (DL-116, Jan. 27, 1992) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The project was approved by the Italian Ministry of Health, Rome, and by the ethics review board of the University of Turin. Rats were anesthetized by i.p. injection of urethane (1 g/kg) and killed by decapitation [30].

2.5 MSC adhesion to microspheres

fPAMs and fPAM-VEGF were used according to the following protocol: 0.5 ml of α -MEM with 10% FBS was added into the eppendorf containing the microspheres (0.5 mg) and incubate for 15-min in order to resuspend them. Just before cell attachment, the solution containing microspheres was vortexed, put in an ultrasound bath for 30-sec and vortexed again. Then the microsphere suspension was put into ultra low attachment cluster plates (Corning) and 9.0×10^4 MSCs in 0.5 ml of culture medium were added.

2.6 Normoxic and hypoxic experimental conditions

1. Normoxia: i.e. standard conditions (normoxic: 21% O₂ and 5% CO₂) to study cell survival; these gas concentrations are identical to those used during MSC culture from isolation to P3.

2. Hypoxia/reoxygenation (H/R): *i.e.* hypoxic mixture (3% O₂ and 5% CO₂) for 72-h and subsequent reoxygenation (21% O₂ and 5% CO₂) for 3-h to study cell survival.

2.6 Experimental Groups:

2.6.1 Study of viability in standard-normoxia with and without treatment with VEGF, fPAMs or fPAM-VEGF

We carried out experiments with MSCs exposed to agents for 3 or 6 days. Therefore the groups considered in this study were the following:

- a) Control: MSCs were kept under standard conditions for 3-days (N-MSC-3) or 6 days (N-MSC-6);
- b) fPAMs: 0.5 mg fPAMs were added to culture medium together with 9.0×10^4 MSCs and kept under standard conditions for 3-days (N-fPAM-3) or 6 days (N-fPAM-6);
- c) fPAM-VEGF: 0.5 mg VEGF₁₆₅/fPAM were added to culture medium together with 9.0×10^4 MSCs and kept under standard conditions for 3-days (N-fPAM-VEGF-3) or 6 days (N-fPAM-VEGF-6);
- d) VEGF: 9.0×10^4 MSCs were cultured and kept under standard conditions, and then 9 ng/day VEGF₁₆₅ were added to the cell culture for 3-days (N-VEGF-3) or 6 days (N-VEGF-6).

2.6.2 Study of survival after H/R with and without pre-treatment with VEGF, fPAMs or fPAM-VEGF

In this protocol, experiments were carried out with MSCs alone or MSC pretreated with the studied factors (fPAMs, fPAM-VEGF or VEGF₁₆₅). In particular, the MSCs were pre-treated with VEGF₁₆₅ (9 ng), fPAMs (0.5 mg) or fPAM-VEGF (0.5 mg) for 24-h. Before

exposure to hypoxic condition, MSCs were incubated with trypsin-EDTA 0.25% solution and subjected to mild centrifugations (900-1000 rpm for 5-min) in order to separate cells from fPAMs or fPAM-VEGF. Then the medium was changed and cells were cultured in 2% FBS without treatment factors. Thereafter, subgroups of cells were subjected to a protocol of hypoxia/reoxygenation (72/3 hours) in a hypoxic chamber (INVIVO₂ 200, Belsar). Therefore the groups considered in the study of cell survival were the following:

- a) Untreated MSCs kept under standard conditions for 3-days (MSC-3-N), and untreated MSCs exposed to H/R (MSC-3-H);
- b) MSCs pre-treated with fPAM and kept under standard conditions (fPAM-3-N), and MSCs pre-treated with fPAMs and exposed to H/R (fPAM-3-H);
- c) MSCs pre-treated with fPAM-VEGF and kept under standard conditions (fPAM-VEGF-3-N), and MSCs pre-treated with fPAM-VEGF and exposed to H/R (fPAM-VEGF-3-H);
- d) MSCs pre-treated with VEGF and kept under standard conditions (VEGF-3-N), and MSCs pre-treated with VEGF and exposed to H/R (VEGF-3-H).

In brief, in each of the above 4 groups, for comparative purpose we considered normoxic (-N) and hypoxic (-H) protocols.

2.7 Cell survival

At the end of experiments cell survival was assessed using the cell viability test 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit [31].

2.8 Western blotting

In order to compare the effects of fPAM-VEGF and VEGF on critical proteins for cell survival and apoptosis, the analysis of western blotting was performed on MSCs exposed to normoxic conditions after 3-days of treatment.

About 50 µg of protein extracts were separated by SDS-10% PAGE and transferred to PVDF membranes (GE Healthcare). The membranes were incubated overnight with the following antibodies: anti-Akt (Cell Signaling), anti-PKCε (Cell Signaling), anti-ERK1/2 (Cell Signaling), and phosphorylated forms anti-phospho-Akt (Ser 473 Cell Signaling), anti-phospho-PKCε (Ser 729 Upstate), anti-phospho-ERK1/2 (Thr 402-Tyr 204 Cell Signaling) and Bcl-2 (Cell Signaling); all antibodies were diluted according to manufacturer's instructions. Western blotting analysis was displayed by the Immuno-Star HRP substrate (BioRad) and quantified by Kodak Image Station 440CF. The quantification of protein used was performed according to Bradford method [32].

To confirm equal amounts of protein loaded membranes were incubated with anti-β-actin (Sigma). The image analysis was performed using the Kodak 1D 3.5 software [33]. We normalized the expression of kinases for each condition to its matched loading control β-actin and then where normalized with respect to the mean values of N-MSC-3.

2.8 Statistical analysis

Data were expressed as mean ± SEM and reported as percentage control. The values were analyzed using ANOVA and Newman-Keuls Multiple Comparison Test as post-ANOVA test, and were considered significant for p<0.05.

3. Results

3.1 VEGF encapsulation and release kinetics from fPAM-VEGF

The encapsulation yield of total protein into fPAMs was 67.8% and biologically active VEGF into fPAM-VEGF was 59.4%. A nice continuous release of VEGF from fPAMs was observed for at least 3 weeks consisting in a cumulative release of 300ng/mL and representing 21% of the entrapped protein (Fig. 1). The VEGF collected from each sample of the kinetics release assay (diluted to 4ng/mL) was able to stimulate HUVEC proliferation for a period of 5 days in a similar manner as the recombinant VEGF at 4ng/mL (data not shown). The bioassay performed with the HUVEC cells confirmed that the VEGF was released under a bioactive conformation during the entire period. One mg fPAM was loaded with 1.0 μ g VEGF₁₆₅. Based on the release kinetics study of VEGF₁₆₅, fPAM-VEGF released 17.8 ng VEGF₁₆₅/mg fPAM/day during the first week. Since we used 0.5 mg of fPAM, the dose of VEGF was about 9 ng/day. Therefore, the doses of VEGF₁₆₅ employed in the two conditions with the cells (fPAM-VEGF or VEGF only) are identical. This concentration of VEGF₁₆₅ is similar to that used in other cellular models [34].

3.2 Cell proliferation analysis in normoxic conditions

In Fig. 2, data are presented as percent variation with respect to mean value of cell count in control (N-MSC-3). We found that there were no significant differences in cell viability between N-MSC and N-fPAM groups both after 3 and 6 days.

The treatment for 3-days with fPAM-VEGF and VEGF (N-fPAM-VEGF-3 and N-VEGF-3) did not induce significant variations in cell viability with respect to N-MSC-3. However after 6 days, cell numbers were significantly increased by N-fPAM-VEGF-6 ($155\pm6\%$) and even more by N-VEGF-6 ($216\pm4\%$). As can be seen in Fig. 2, p is <0.001 between N-VEGF-6 and all other conditions, and p is <0.05 between N-fPAM-VEGF-6 and other

conditions. No cell death was observed throughout the experiments, so that the differences in cell number are likely due to different cell proliferation.

These results suggest that *VEGF has a more potent proliferative effect than fPAM-VEGF after 6 days treatment in normoxia.*

3.3 Survival analysis after H/R in pretreated cells

In Fig. 3, data are presented as percent variation with respect to mean value of cell count in control conditions (MSC-3-N). The analysis of viability in normoxia showed that 24-h pre-treatment with either fPAMs or fPAM-VEGF did not influence cell numbers; however, VEGF-pretreatment (VEGF-3-N) increased cell number. These data support the idea that *free-VEGF has a major pro-proliferative effect.*

In the absence of pre-treatment, the H/R protocol induced a 34% reduction in MSC number ($p<0.01$, MSC-3-N vs MSC-3-H). In MSCs pretreated with fPAMs a similar percent reduction (-27%) of cells was observed ($p<0.05$, fPAM-3-N vs fPAM-3-H). While the pre-treatment with fPAM-VEGF was able to counteract hypoxia effects ($P=NS$ between fPAM-VEGF-3-N and fPAM-VEGF-3-H), VEGF pretreatment did not; in fact a 38% reduction in MSC number was observed in VEGF-3-H ($p<0.01$ vs VEGF-3-N), whereas pretreatment with fPAM-VEGF preserved cell survival in post-hypoxia.

These results suggest that *pre-treatment with fPAM-VEGF limits cell mortality after H/R.*

3.4 Western Blot analysis

Starting from different levels of total kinases, 3-days after treatment the phospho-ERK 1/2 levels were greatly and similarly increased in both VEGF and fPAM-VEGF groups, whereas the phospho-Akt and phospho-PKC ϵ abundances were higher after treatment with VEGF than with fPAM-VEGF.

Fig. 4 shows the total and phospho-kinase bands (central panels) with relative normalized ratio (left panels) and mean values of normalized phospho-enzyme levels (right panels).

The analysis for Akt (Fig. 4, Top Panels) evidenced a reduced phospho/total ratio of Akt in fPAM-VEGF and a preserved ratio in VEGF only. The level of phospho-Akt was lower in treated groups (both VEGF and fPAM-VEGF). Importantly, phospho-Akt level was higher in VEGF than in fPAM-VEGF group.

The analysis of ERK1/2 (Fig. 4, Middle Panels) showed an increase for phospho/total ratio in VEGF group. However, the phospho-ERK1/2 levels were similarly increased in both fPAM-VEGF and VEGF.

The analysis for PKC ϵ (Fig. 4, Bottom Panels) evidenced an increased phospho/total ratio in both fPAM-VEGF and VEGF; however phospho-PKC ϵ level resulted lower than the MSC group, in both fPAM-VEGF and VEGF. Yet the phospho-PKC ϵ level was higher in VEGF than fPAM-VEGF group.

After 3-day treatment the studied marker of anti-apoptotic activity, involved in the survival mechanisms (Bcl-2, Fig. 5), was preserved by fPAM-VEGF and decreased by free VEGF.

4. Discussion

The major new finding in this study is that sustained VEGF release by fPAM-VEGF induces a greater resistance of MSCs to H/R, whereas free VEGF induces a greater increase in MSC proliferation in normoxia.

Accordingly Akt and PKC ϵ are more phosphorylated/activated by free VEGF than by fPAM-VEGF, whereas MSCs treated with fPAM-VEGF show a higher level of the anti-apoptotic factor Bcl-2 with respect to VEGF only. These results on proliferative mediators

and anti-apoptotic agents corroborate the different effects of the two modalities of VEGF application (free VEGF *vs* fPAM-VEGF) on cell proliferation in normoxia and cell survival in hypoxia. In fact, while both treatment and pre-treatment with free VEGF increase cell proliferation more than fPAM-VEGF in normoxia, only pre-treatment with fPAM-VEGF limits cell mortality after H/R. Since pro-proliferative kinases were more activated by free VEGF than fPAM-VEGF, we observe an enhanced proliferation with free VEGF. Moreover, the levels of phospho-ERK 1/2 - involved in the survival mechanisms - are similarly increased by both treatments (free VEGF and fPAM-VEGF), and the anti-apoptotic activity (Bcl-2) is better preserved by fPAM-VEGF than free VEGF; thus, we suggest that these factors may be responsible of the greater resistance to hypoxia challenging by MSCs pre-treated with fPAM-VEGF.

Although some of the studied factors may exert both pro-proliferative and anti-apoptotic effects (*e.g.* phospho-Akt), we can observe that specific anti-apoptotic agent, namely Bcl-2, is increased by fPAM-VEGF, whereas agents with proliferative properties, such as phospho-Akt and phospho-PKC ϵ , are higher when VEGF is given alone. The phospho-ERK 1/2 is similarly increased by both treatments; whereas ERK 1/2 shows an increase in phospho/total ratio in VEGF group only.

These results suggest a stronger anti-apoptotic activity by fPAM-VEGF with respect to VEGF alone, whereas VEGF alone stimulates mainly proliferative agents. Therefore in normoxia the cell number is increased greatly by VEGF; whereas in H/R cell death is mainly limited by fPAM-VEGF. Moreover, as said, we should consider that although Akt is both a proliferative and an anti-apoptotic agent, Bcl-2 is an anti-apoptotic factor specifically activated against H/R [35]. Therefore, it is likely that VEGF alone promotes proliferation in

normoxia (*via* Akt phosphorylation) thus increasing the number of viable cells. Yet fPAM-VEGF increases cell survival by reducing apoptosis (*via* Bcl-2 preservation).

The efficiency of the transplanted stem cells is limited by several factors, including a hostile environment such as that occurring in inflammation, reoxygenated or aging tissue and the lack of growth factors. The development of biopolymer-based growth factor delivery for tissue repair may overcome these hurdles and be beneficial even in the case of reduced availability of stem cell to implant.

Several kinds of scaffolds composed of synthetic or natural polymers have been tested with different experimental models in preclinical and clinical studies. Pharmacologically-active-microcarriers (PAMs) have been developed which combine, in an adaptable and simple device, *in situ* controlled drug delivery and implantation of cell adhered onto biomaterials-based scaffolds. The PAMs used in the present study are biocompatible and biodegradable microspheres made of poly (D,L-lactide-co-glycolide acid (PLGA)) with a biomimetic surface of ECM molecules supplying a three-dimensional structure for the cell both *in vitro* and *in vivo* after transplantation. They may also be engineered to release a therapeutic factor in prorogated manner [36]. Therefore these combined parameters may promote or maintain cell survival, differentiation and integration in the host tissue after complete degradation of the carrier. In particular, adhesion to structural glycoproteins of the ECM seems to be necessary for cell survival and fibronectin is considered as a protective factor for many adherent cells [37]. Moreover, it has been recently suggested a novel pro-survival pathway involving integrin receptors and proteins of Bcl-2 family which can be stimulated by fibronectin [38]. Thus, we cannot rule out that the differences observed between VEGF and

fPAM-VEGF groups inherent their effects on MSC survival can be due, at least in part, to the interaction of VEGF with fibronectin.

VEGF production is a crucial component of stem cell-mediated cardioprotection as evidenced by a reduction in post-ischemic myocardial functional recovery following intracoronary infusion of MSCs with targeted VEGF suppression using siRNA [39]. Here using either VEGF alone or in combination with fPAMs (fPAM-VEGF) we observed an enhanced proliferation of MSCs in normoxia. However, VEGF is more effective than fPAM-VEGF in inducing proliferation. On the other hand, in hypoxic conditions fPAM-VEGF was more protective than VEGF against H/R challenging. Differences between free VEGF and fPAM-VEGF may be due also to the VEGF kinetics release elicited by the fPAM (see Fig. 1). Since it has been underlined that reduced proliferation might favor stem cell differentiation, whereas highly proliferating stem cells hardly differentiate [40,41], we suggest that functionalized fPAM inducing a limited proliferation and an increased resistance to hypoxia/reoxygenation may be a novel approach for enhancing stem cell survival and differentiation and, thus, regeneration of damaged tissue. For instance, fPAM-VEGF can be used when the cell loss affects the heart, to limit heart failure [42]. In fact MSCs can differentiate into vascular endothelial cells and cardiomyocytes and can improve heart function even via vascular paracrine signaling effects [3,4,43,44].

5. Conclusions

Our data suggest that *in vitro* treatment with free VEGF enhances MSC proliferation. In this response ERK 1/2 pathway may play a pivotal role. Yet, controlled release of VEGF from fPAM-VEGF limits mainly MSC death *via* both ERK 1/2 and Bcl-2 mechanisms, thus enhancing MSC survival in post-hypoxic environment.

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3 It is now clear that in cell transplantation therapies different approaches are required
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5 depending on the different conditions. Our data suggest that the use of fPAM complexed
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7 with growth factors can be considered a new approach to improve stem cells survival and
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9 regeneration in hostile environments such as inflammatory and post-ischemic tissue.
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15 16 **Acknowledgments**

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30 *The authors confirm that there are no conflicts of interest.*
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Figure Legends

Figure 1: Illustrative Kinetics release of VEGF from fPAM and bioassay. The cumulative release of VEGF from fPAM up to 4 weeks was 21% that represent about 300 ng/ml of the entrapped protein. Each point represents the mean of triplicate experiments. VEGF collected from each sample of the kinetics release assay was able to stimulate HUVEC proliferation as an equal amount of native VEGF for a period of 7 days (data not shown).

Figure 2: Cell growth in normoxia with and without factors (free-VEGF, fPAMs or fPAM-VEGF) after 3 and 6 days. Data are % variation with respect to mean value of N-MSC-3.
* $p < 0.05$ vs other groups; ** $p < 0.001$ vs all other groups. $n = 5$ for each condition.

Figure 3: Cell growth in normoxia for 3-days (first four bars), and cell survival after 72-h hypoxia and 3-h reoxygenation, of MSC pre-treated or not with factors (free-VEGF, fPAMs or fPAM-VEGF) for 24-h. Data are % variation with respect to mean value of MSC-3-N.
* $p < 0.01$ vs MSC-3-N. $n = 5$ for each condition.

Figure 4: Western blot analysis of Akt (top panels), ERK1/2 (middle panels), and PKC ϵ (bottom panels) after 3-days treatment with active factors (free-VEGF and fPAM-VEGF). The panels on the left are normalized phospho/total kinase ratios. The central panels are representative bands of total and phospho-kinases (some of the presented bands were not juxtaposed in the original film). The panels on the right show the normalized mean values of phospho-enzyme only. We normalized the expression of total kinases and phospho-kinases for each condition to its matched loading control β -actin and then where normalized with respect to the mean values of N-MSC-3.
* $p < 0.05$ respect to N-MSC-3; ** $p < 0.001$ respect to N-MSC-3. $n = 4$ for each condition.

Figure 5: Representative blots of and mean levels of the anti-apoptotic factor, Bcl-2 after 3-days treatment with active factors (free-VEGF and fPAM-VEGF). We normalized the expression of Bcl-2 for each condition to its matched loading control β -actin and then where normalized with respect to the mean values of N-MSC-3.

* $p < 0.05$ respect to N-MSC-3; ** $p < 0.001$ respect to N-MSC-3. $n = 4$ for each condition.

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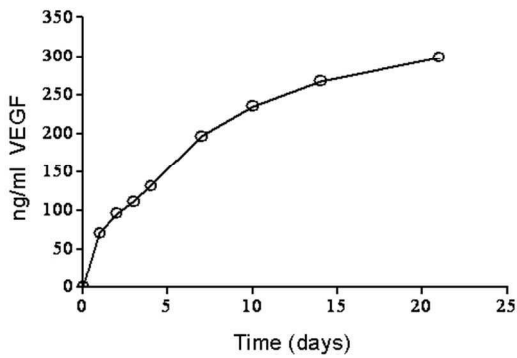


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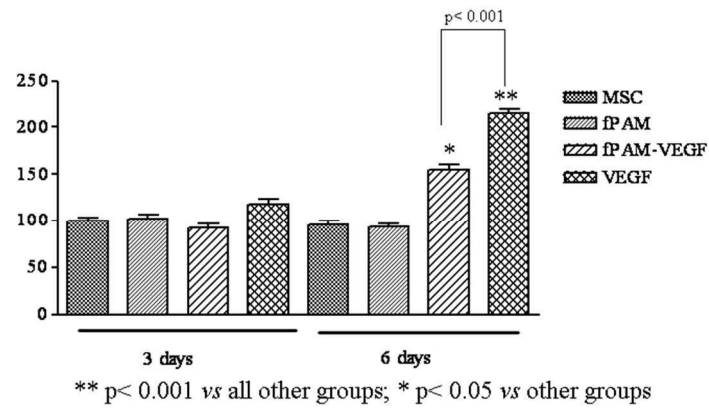


Figure 2: Cell growth in normoxia with and without factors (free-VEGF, fPAMs or fPAM-VEGF) after 3 and 6 days. Data are % variation with respect to mean value of N-MS-C-3.

* p < 0.05 vs other groups; ** p < 0.001 vs all other groups. n = 5 for each condition.
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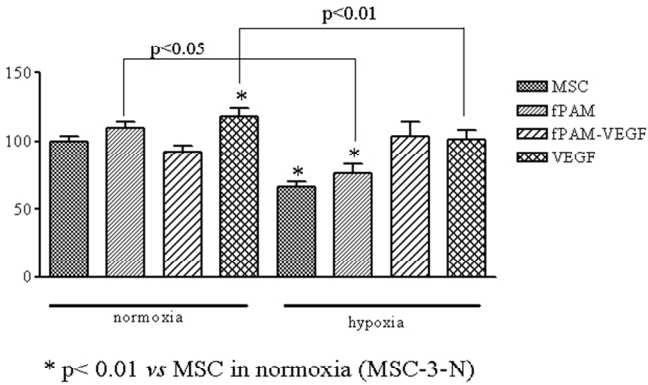


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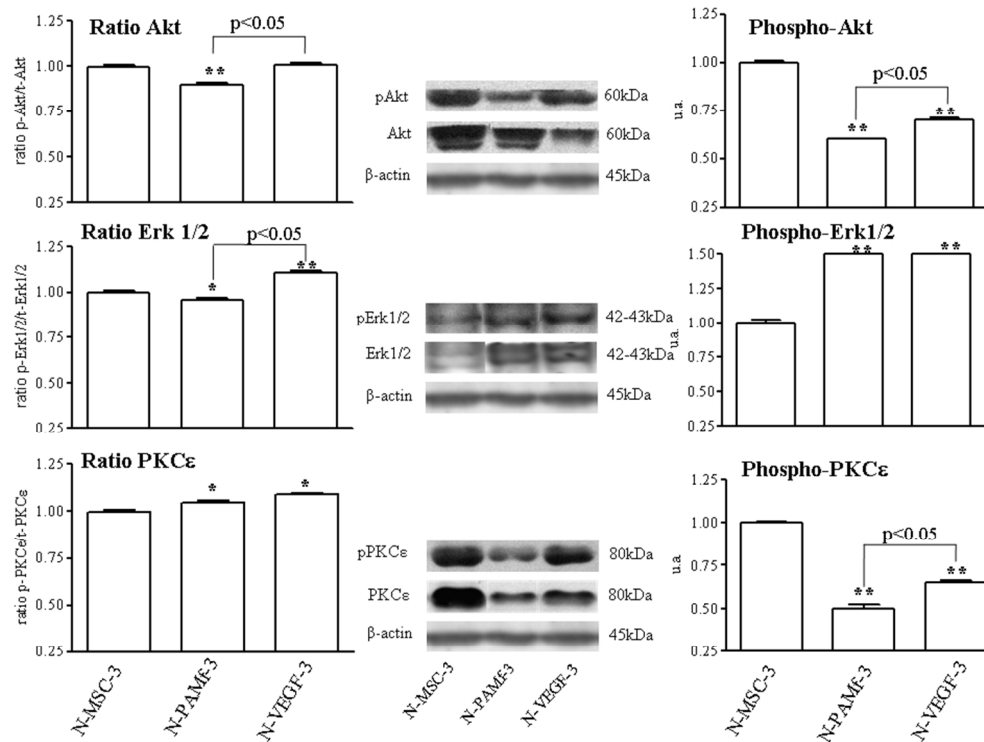


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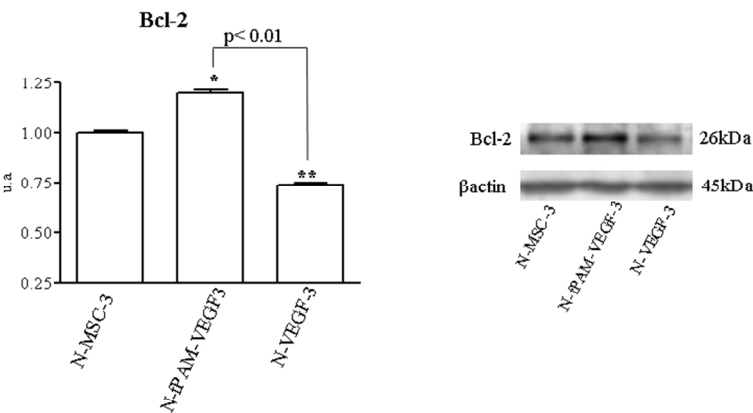


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Résumé

La thérapie cellulaire constitue une stratégie prometteuse dans le traitement de l'infarctus du myocarde. Afin de mieux contrôler la survie, la différenciation et l'intégration des cellules greffées, nous avons tenté une approche d'ingénierie tissulaire en associant les cellules à un microvecteur comportant une surface biomimétique et pouvant libérer un facteur de croissance (FC), les microcarriers pharmacologiquement actifs (MPA). Parmi les cellules utilisées dans une telle approche, les cellules souches adultes dérivées du tissu adipeux (ADSC) ne soulèvent pas de problèmes d'ordre éthique et permettent de réaliser des greffes autologues. Ces cellules sont largement étudiées pour la régénération de nombreux tissus en vertu de leurs propriétés immuno-modulatrices, de leur capacité à sécréter des FC et chimokines, mais également de leur large potentiel de différenciation.

Dans une première étape, nous avons étudié l'effet des molécules de la matrice extracellulaire et des MPA sur la différenciation en cardiomyocytes des ADSC en présence d'un cocktail de FC. Nous avons ainsi pu observer que l'apport du cocktail de FC permettait aux cellules de s'engager dans la voie de différenciation cardiaque après 2 semaines. En comparant l'effet de la laminine (LM) et de la fibronectine sur cette différenciation, nous avons pu observer que la LM permettait d'induire une différenciation dans une cellule plus mature et que ceci était potentialisée par un enrichissement du milieu en TGF β 1. Finalement, l'apport de MPA recouverts de LM favorisait une différenciation plus rapide des cellules en présence du cocktail.

Les MPA peuvent également libérer un facteur de croissance de manière prolongée au cours du temps. Par conséquent, nous avons encapsulé 3 protéines, le VEGF, le HGF et l'IGF-1, et d'étudier leur effet sur les comportements des ADSC. Nous avons ainsi pu observer que des MPA libérant du HGF et de l'IGF-1 induisaient une différenciation de ADSC dans la voie cardiaque et que cette différenciation était également observée lorsque les complexes ADSC-MPA étaient intégrés dans un hydrogel thermosensible. Cependant, nous avons aussi observé que la quantité de protéines libérées à partir des MPA était plus faible dans le gel.

Nous avons donc cherché dans une dernière partie à améliorer le profil de libération des protéines à partir des MPA en changeant la composition du polymère. Nous avons ainsi utilisé différents copolymères triblock PLGA-PEG-PLGA pour formuler des microsphères et évaluer leur rôle sur la libération de la protéine mais aussi sur la stabilité de celle-ci durant la dégradation du polymère.

Mots clés : thérapie cellulaire, ingénierie tissulaire, ADSC, infarctus du myocarde, microcarriers pharmacologiquement actifs, laminine, fibronectine, VEGF, HGF, IGF-1,

Summary

Cell therapy constitutes a promising strategy for the treatment of myocardial infarction. To better control the survival, the differentiation and the integration of the grafted cells, we have used a tissue-engineering approach associating the cells with a microvector comprising a biomimetic surface and able to release a growth factor (GF), the pharmacologically active microcarriers (PAM). Among the cell used for such approach, adipose-derived stem cells (ADSC) do not raise ethical concerns/issues and allow autologous transplantation. These cells are widely studied for the regeneration of various tissues due to their immunoregulatory properties, their potential to secrete GFs and chemokines but also their large potential of differentiation.

In a first step, we have investigated the effect of extracellular matrix molecules and PAMs on ADSCs differentiation into cardiomyocytes in combination with a GF cocktail. We have thus observed that the GFs cocktail allowed cell commitment into the cardiac lineage after 2 weeks. Comparing the effects of the laminin (LM) and fibronectin (FN) on this differentiation, we found that LM allowed ADSCs differentiation into a more mature phenotype. Moreover, the enrichment of the GF cocktail with TGF β 1 potentiated the LM effect. Finally, providing PAM covered with LM allowed an earlier differentiation into cardiac lineage in combination with the cocktail.

PAMs can release a GF in a prolonged manner. Consequently, we encapsulated 3 proteins, the VEGF, the HGF and the IGF-1, and assessed their effect on ADSCs behavior. We observed that PAMs releasing HGF and IGF-1 induced ADSCs cardiomyogenic differentiation. Moreover, we also observed ADSCs cardiac differentiation markers expression, when ADSC-PAMs complexes are integrated within a thermosensitive hydrogel. However, we also found that the amount of protein released from PAMs decreased within the gel.

In a last part we have sought to improve protein release profile from PAMs changing polymer composition. So, we used various triblock copolymers of PLGA-PEG-PLGA to formulate microspheres and then evaluated their role on protein release but also on protein stability during polymer degradation.

Keywords: Cell therapy, tissue engineering, ADSCs, myocardial infarction, pharmacologically active microcarriers, laminin, fibronectin, VEGF, HGF, IGF-1.