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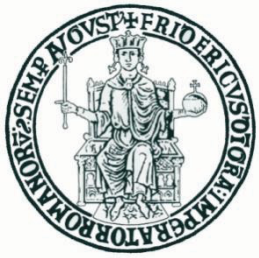

School of Engineering

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Ph.D in Engineering of Materials and Structures

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ENGINEERING CARDIAC MICROTISSUE *IN VITRO*: EFFECT OF THE SCAFFOLD AND CULTURE CONDITIONS ON THE FINAL PROPERTIES OF CARDIAC MICRO-MUSCLES

Ph.D Thesis

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Chapter 3

A bottom-up approach to cardiac tissue engineering: how to build-up a tissue equivalent in vitro

Abbreviations

MI: Myocardial Infarction
CM: Cardiomyocyte
CF: Cardiac Fibroblast
EC: Endothelial Cell
ECM: Extracellular Matrix
EE: Endocardial Endothelium
CTE: Cardiac Tissue Engineering
SMC: Smooth Muscle Cells
Cx43: Connexin 43
BrDu: 5-Bromo-2'-deoxyuridine
μ-TPs: Microtissue Precursors
HD-μTPs: Human Dermal Micro-tissue Precursors
HDF: Human Dermal Fibroblasts
GPMs : Porous Gelatin Micro-beads
GAL: Glyceraldehyde
PSR: Picro Sirius Red
SR: Sarcoplasmic Reticulum
NMC: Non-myocardyal cells

Chapter 1

RESEARCH INTRODUCTION, HYPOTHESES AND OBJECTIVES

1.1 The Need for Engineered Cardiac Tissue

Heart disease remains the leading cause of morbidity and mortality in developed countries [1]. Thus, there is an urgent demand for more efficacious pharmaceuticals to treat heart disease and new methods to repair damaged cardiac tissue. The limited ability of cardiac tissue to regenerate [2] and the scarcity of organs for transplantation [3] contribute to the need for a larger pool of transplantable cardiac tissue. Tissue engineering offers the possibility of creating functional tissue equivalents for scientific studies and tissue repair [4]. Representative examples include in vitro cultured blood vessels [5], liver [6], kidney [7], muscle [8], bone [9], neuronal tissue [10], and cartilage [11].

1.2 Research Overview and Hypothesis

Most living tissues are composed of repeating units on the scale of hundreds of microns, which are different cell types with well defined three-dimensional (3-D) micro-architectures and tissue-specific, functional properties. To generate thick and functional engineered tissues, the recreation of these structural features is of great importance in enabling the resulting function [12]. Recent efforts [13,14] have been concentrated on bottom-up approaches aimed to generate a larger tissue construct by the assembly of smaller building blocks, which mimics the *in vivo* tissue structure of repeating functional units. In this PhD thesis a novel bottom-up approach has been applied to produce functional cardiac tissue, starting from the outcomes of recently published works [14,15,16].

The overarching goal of this work was to create *in vitro* functional cardiac µ-tissue by coupling engineered porous µ-scaffold with neonatal rat cells. We hypothesized that such cardiac µ-tissue construct could be used as a functional building unit to obtain a 3D cardiac tissue *in vitro.* The µ-scaffolds, consisting of gelatine porous micro-beads with a diameters distribution of 75-150 µm, were colonized by cardiac cell population in dynamic cell seeding condition by means of spinner flask bioreactor. To optimize the micro-tissue functions we varied several culture parameters: spinner culture conditions (duration and type of flow regime) as well as the initial composition of cardiac cell population. We have successfully established that µ-scaffold construct embedded with a specific cardiac cell composition exhibited the important properties of native cardiac tissue, including the assembly of differentiated cardiac cell populations into a 3D syncytium, as well as electrophysiological functionality and responsiveness to external electrical stimulation. Furthermore, it has been tested the possibility to produce a 3D cardiac tissue constructs, of defined size and shape, by exploiting the biological sintering capability of the micro-tissues (µTPs). We conjectured that the cardiac tissue engineered construct developed could be used as a biological model for studying cardiac tissue development and/or disease processes in vitro, and eventually as an implant to repair injured myocardium.

1.3 Research Objectives

Overall Objectives

The overall objective of this project was to create 3D cardiac tissue using cardiac µTPs as building blocks. To achieve this we had to 1) devise a method to realize cardiac µTPs, 2) to characterize cardiac µTPs using functional *in vitro* assays, and 3) design a method to achieve a 3D cardiac tissue by assembly the micro-modules that act as building blocks for the fabrication of the corresponding macro-tissue. These three objectives will be covered in the following section.

Specific Objective 1: Fabrication of cardiac μ-TP

Cardiac μTPs constructs were fabricated using an engineered gelatine porous micro-bead. The first step was the optimization of cell seeding parameters. In the standard conditions [14], HD-μTPs cultivation was initiated by inoculating HDF a density of 10 cells bead^{-1}, the culture suspension in the spinner flask was stirred intermittently at 30 rpm (5 min stirring and 30 min static incubation) for the first 6h post-inoculation for cell adhesion, and then continuously agitated at 30 rpm. In the case of cardiac cell, we started the process by inoculating in the spinner flask cardiac cells and gelatin porous micro-beads at the ratio of 1000 cells for bead. Compared with previously described spinner culture conditions used to generate dermal-like tissue, in the case of cardiac cell culture, the ratio between cells and beads must be higher due to lower cardiomyocytes proliferation. Moreover, the seeding parameters were slightly different because of the difference in the cell adhesion time between dermal fibroblasts and cardiomyocytes. Indeed, dermal fibroblasts adhere within 4 hours, cardiomyocytes took at least 3 days to adhere and to spread on the substrate. For this reason, the spinner culture was carried by using a cycle of 10 minutes at rest and 30 minutes at 30 rpm for the first 3 days of culture. After this time, the dynamic culture switched to continuous stirring at the velocity of 30 rpm up to 9 days of culture. Furthermore, we assessed three different cell composition harvested from neonatal rat heart with different culture medium in order to promote the realization of a μTP having properties close to native cardiac tissue. Three different experiments were performed for the seeding phase; isolated cells were pre-plated onto tissue culture polystyrene to reduce the initial non-myocardial cells (NMC) contamination by exploiting the differential attachment time between myocardial and non-myocardial cells. By pre-plating the whole cell population for one hour it was obtained a cell population named cardiomyocite-rich (CM-R). By pre-plating for three step of one hour it was obtained a cell population named cardiomyocytes-extra rich (CM-ER). The population CM-R will be named along the paper CM-R(+) if used in combination with 5-bromo-2deoxyuridine (BrDu) or CM-R(-) it used in absence of BrDu[17]. As consequence, different features characterized each formulation of μTP. The choice of the first cell population, allowed obtaining a μ-scaffold well colonized by cardiac cells and the functional cardiac μTPs. We hypothesized that the presence of BrdU in the

culture medium was crucial to maintain a balance between the cardiac cells proliferation and the μ-tissue viability.

Specific Objective 2: Characterization of cardiac μ-TP

The functionalities and morphology of cardiac μ-TP have been characterized during culture in order to assess its time evolution. Electrical responsiveness and contractility were measured as primary indicators of micro-tissue functionality. We investigated the μTP self-beating capability along the culture time and verified the electrical coupling and electrical synchronization between μ-tissues by means of voltage sensitive probes. Furthermore, immunohistological analysis showed that connexin43, a gap junction (GJ)-related protein, was detected between μTPs during the synchronization and it was assessed a correlation between the connexin 43 amount in the ECM and μTP electrical activity. In particular higher was the beating frequency, higher was the cx43 amount. In addition, it was showed that the μTPs were responsive to external electrical stimulation: oscillating electrical field induced a synchronous contraction in the μTP. Finally, we studied the capability self-aggregation of the micro-tissues and the ability of fusion between them in the time.

Specific Objective 3: 3D Cardiac tissue equivalent in vitro

Cardiac cells were dynamically seeded on micro-scaffold for 3 days in order to realize cardiac micro-tissues. The micro-modules realized have been used as building blocks for the fabrication of the corresponding macro-tissue. In fact, after 3 days of seeding, cardiac-μTPs suspension was transferred from the spinner flask to the maturation chamber to allow their molding in disc-shaped construct. After 7 days of culture we obtained the 3-D cardiac tissue, that showed a macroscopic and homogeneously beating distributed throughout on the patch and the histological analysis showed similarities with the native tissue.

1.4 Thesis Organization

The thesis is organized into self contained chapters. The first chapter outlines the aim of the thesis. While, chapter 2 goes on to review and present the current methods and challenges in cardiac tissue engineering as well as relevant background literature. In chapter 3 we discuss the fabrication of μTP and their behaviour in the time. Finally, chapter 4 focuses on the realization of 3D cardiac tissue.

Reference

[1] Hennekens CH. 1998. Increasing burden of cardiovascular disease: Current knowledge and future directions for research on risk factors. Circulation 97:1095–1102.

[2] Canale ED, Campbell GR, Smolich JJ, Campbell JH, Oksche A, Vollrath L. 1986. Handbook of Microscopic Anatomy, Volume II/7: Cardiac Muscle. Berlin: Springer-Verlag.

[3] Stevenson L, Warner S, Steimle A, Fonarow G, Hamilton M, Moriguchi J, Kobashigawa J, Tillisch J, Drinkwater D, Laks H. 1994. The impending crisis awaiting cardiac transplantation. Modeling a solution based on selection. Circulation 89:450–457.

[4] Langer R, Vacanti JP. 1993. Tissue engineering. Science 260:920–926.

[5] Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, Langer R. Functional arteries grown in Vitro. Science 284:489–493.

[6] Bader A, Knop E, Fruhauf N, Crome O, Boker K, Christians U, Oldhafer K, Ringe B, Pichlmayr R, Sewing KF. 1995. Reconstruction of liver in vitro: geometry of characteristic flat bed, hollow fiber, and spouted bed bioreactors with reference to the in vivo liver. Artif Organs 19:941–950.

[7] Humes HD. 1996. Tissue engineering of a bioartificial kidney: A universal donor organ. Transplant Proc 28:2032–2035.

[8] Vandenburgh H, Del Tatto M, Shansky J, Lemaire J, Chang A, Payumo F, Lee P, Goodyear A, Raven L. 1996. Tissue-engineered skeletal muscle organoids for reversible gene therapy. Hum Gene Ther 7:2195–2200.

[9] Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. 1997. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. J Biomed Mater Res 36:17–28.

[10] Woerly S, Plant GW, Harvey AR. 1996. Neural tissue engineering: From polymer to biohybrid organs. Biomaterials 17:301–310.

[11] Cao Y, Vacanti JP, Paige KT, Upton J, Vacanti CA. 1997. Transplantation of chondrocytes utilizing a polymer–cell construct to produce tissueengineered cartilage in the shape of a human ear. Plast Reconstr Surg 100:297–302.

[12] Hugo Fernandes,Lorenzo Moroni,Extracellular matrix and tissue engineering applications. Journal of Material Chemistry.

[13] V. Mironov, R. P. Visconti, V. Kasyanov, G. Forgacs, C. J. Drake and R. R. Markwald, Organ printing: tissue spheroids as building blocks, Biomaterials, 2009, 30(12), 2164–2174.

[14] Palmiero C, Imparato G, Urciuolo F, Netti P. Engineered dermal equivalent tissue in vitro by assembly of microtissue precursors. Acta Biomater. 2010 Jul;6(7):2548-53. 20.

[15] Urciuolo F, Imparato G, Palmiero C, Trilli A, Netti PA. Effect of process conditions on the growth of three-dimensional dermalequivalent tissue obtained by microtissue precursor assembly. Tissue Eng Part C Methods. 2011 Feb;17(2):155-64.

[16] Imparato G.,Urciuolo F.,Casale C,Netti PA 2013 The role of microscaffold properties in controlling the collagen assembly 3D dermis equivalent using modular tissue engineering.Biomaterials 34(32):7851-61.

[17] P Simpson and S Savion Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Cross-striations, ultrastructure, and chronotropic response to isoproterenol. *Circ Res.* 1982;50:101-116

Chapter 2

THE HEART, CURRENT METHODS AND CHALLENGES IN CARDIAC TISSUE ENGINEERING

2.1 The Heart

The heart is a muscular organ about the size of a fist, located just behind and slightly left of the breastbone. The vertebrate heart is principally composed of cardiac muscle and connective tissue; it is an involuntary striated muscle tissue specific to the heart and is responsible for the heart's ability to pump blood. The heart pumps blood through the network of arteries and veins called the cardiovascular system. The heart has four chambers (Fig.1):

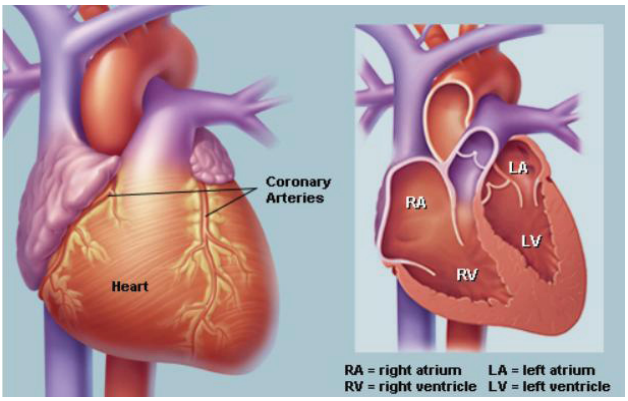

Figure 1: Heart organization

•The right atrium receives blood from the veins and pumps it to the right ventricle.
•The right ventricle receives blood from the right atrium and pumps it to the lungs, where it is loaded with oxygen.

•The left atrium receives oxygenated blood from the lungs and pumps it to the left ventricle.

•The left ventricle (the strongest chamber) pumps oxygen-rich blood to the rest of the body. The left ventricle's vigorous contractions create our blood pressure.

The coronary arteries run along the surface of the heart and provide oxygen-rich blood to the heart muscle. A web of nerve tissue also runs through the heart, conducting the complex signals that govern contraction and relaxation. The heart is organized as a complex arrangement of both cellular and acellular components. The myocytes are arranged in layers as originally described by Streeter [20] and more recently as laminae [21]. These laminae are organized into layers of myocytes two to five cells thick and surrounded by an endomysial collagen network. Fibroblasts form an interconnected network of cells that lies within the endomysial collagen network that surrounds the groups (lamellae) of myocytes [22, 23, 24, 21]. This arrangement of fibroblasts *in vivo*, with interconnected cellular processes forming a network of cells within the collagen network, is analogous to the organization of fibroblasts in a collagen gel. This *in vivo* arrangement can allow the fibroblasts to contract the endomysial collagen, exerting force on the myocytes. Changes in fibroblast contractility have been suggested to impact myocardial relaxation. The distribution of endothelial cells and smooth muscle cells is confined to the vasculature. Importantly, the intercellular connections of fibroblasts appear to be via at least two different types of cell-cell molecules: connexins and cadherins. In mice and rats, the connexins that connect fibroblasts to myocytes are connexin43 (Cx43), while connexion 45(Cx45) connect myocytes, although the distribution of these connexins may vary in different species [23,25].

2.2 Tissue Engineering in vitro

There is an increasing demand in regenerative medicine to repair and restore the function of injured, degenerated, or congenitally defected tissues. In a wide range of pathology, neither native nor purely artificial implantable materials can adequately replace or repair these damaged tissues. Tissue self-repair capability is limited to the case of bone [6] or skin [7,8] where the damage is not invasive such as a small injury or a superficial wound. In many tissues such as myocardium [9] and cartilage [10] or in the case of large bone defect and deep skin wound, the self-repairing capability is lost and surgery becomes necessary. To overcome such limitations, tissue engineering focuses on the *in vitro* fabrication of living and functional tissue that can be implanted in the damaged zone to restore the healthy status. The classical tissue

engineering approach (herein referred to as “top-down”) is based on the concept of seeding cells into preformed, porous, and biodegradable polymeric scaffolds that act as a temporary template for new tissue growth and reorganization (Fig.2).

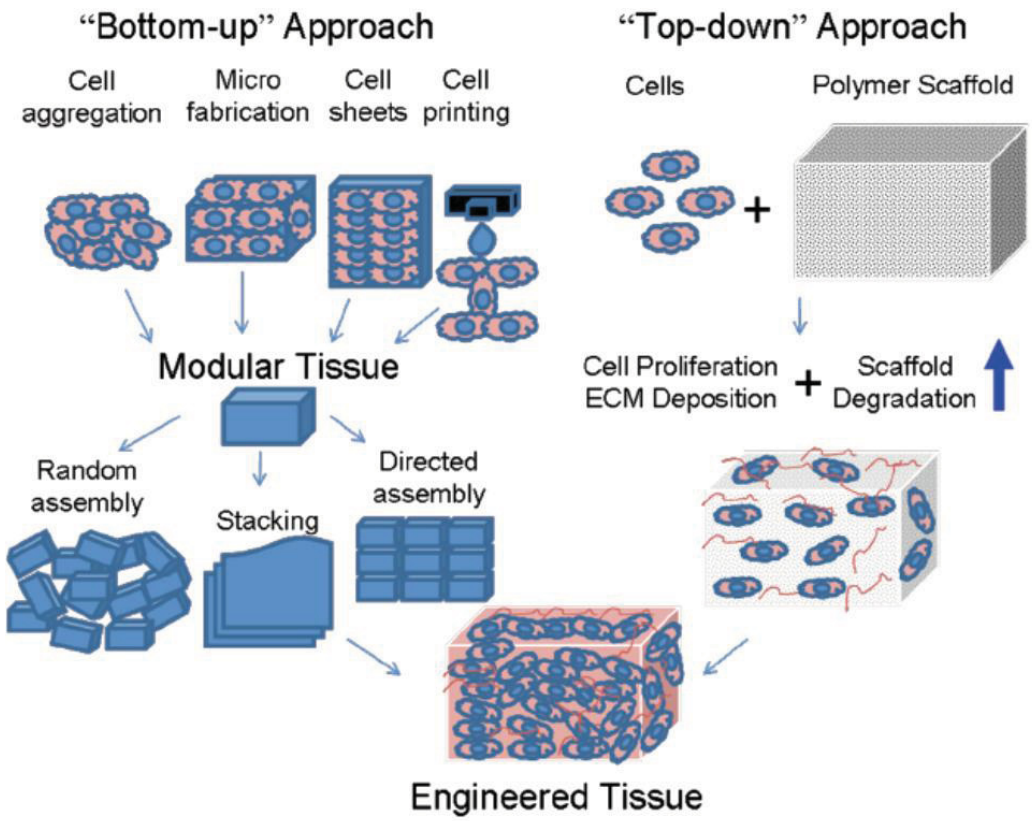

Figure 2: Bottom-up & Top-down approaches to tissue engineering. In the bottom-up approach there are multiple methods for creating modular tissues, which are then assembled into engineered tissues with specific microarchitectural features. In the top-down approach, cells and biomaterial scaffolds are combined and cultured until the cells fill the support structure to create an engineered tissue [4].

Such cellular construct is then processed in bioreactors that provide a viable molecule microenvironment and simulate physiological conditions that furnish suitable stimuli for cell survival, differentiation, and extracellular matrix (ECM) synthesis [11]. The main drawbacks of this approach are related to:

- the difficulty in reproducing adequate microenvironmental conditions in a three-dimensional (3D) thick structure at the pericellular level;
- recreating the architecture of native tissue;
- problems in selecting the ideal biomaterial scaffold for a given cell type;

- time constraints in achieving a high enough cell density and the homogeneous cell distribution necessary to construct a viable tissue.

By studying the nature of living tissues, it is possible to observe that most of them are composed of repeating units on the scale of hundreds of microns, with well-defined 3D micro-architectures and tissue-specific functional properties. The recreation of these structural features is becoming significant in enabling the resulting tissue function in vitro [12]. In light of this observation and to overcome the limitation of top-down tissue engineering, recent efforts have been devoted to bottom-up [13,14] approaches aimed at generating a larger tissue construct by assembling smaller building blocks (Fig.2) that mimic the in vivo tissue structure of repeating functional units. These building blocks can be created in a number of ways, such as self-assembled cell aggregates [15,14], microfabrication of cell-laden microgel [12], creation of cell sheet [17] and microfabrication of cell seeded micro-beads [18, 19]. Once obtained, these building blocks can be assembled in larger tissue through a number of methods including random packing, stacking of layers, or direct assembly [74]. Templates are rather cheap and allow rapid production of big quantities of Microscale Building Blocks of defined shape and size. Their assembly into ordered constructs requires further development (Fig. 3).

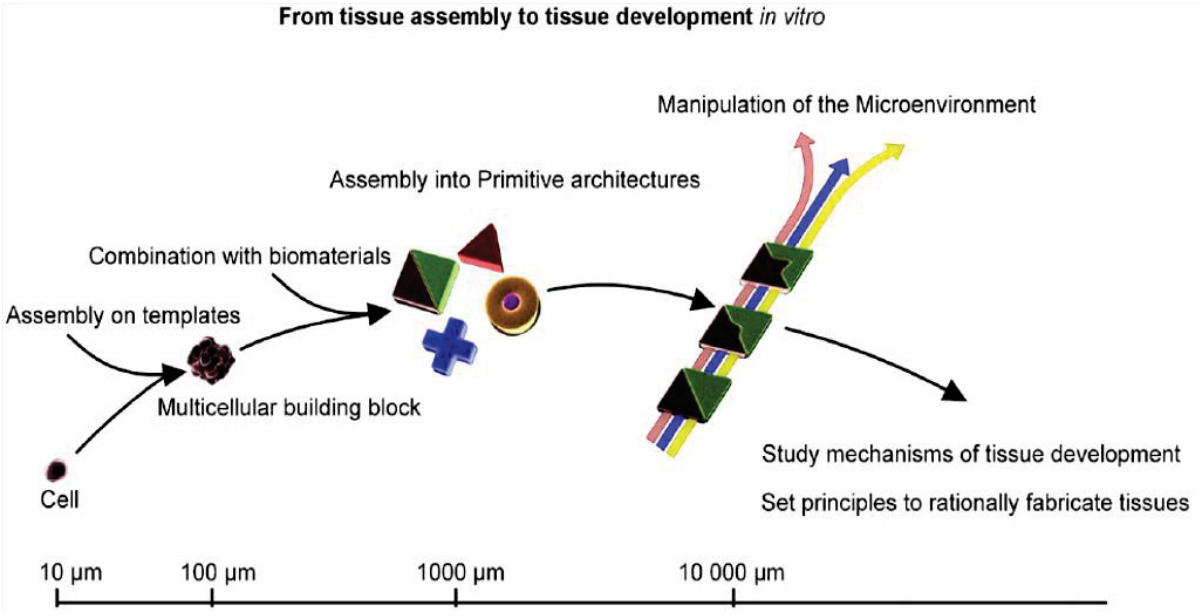

Figure 3: from tissue assembly to tissue development *in vitro*. Tissue fabrication methods allow the assembly of cells into primitive tissue compartments, which are prone to remodeling. The tissue geometry along with the manipulation of the environment at a microscale further promotes the self-organization of cells into more complex tissues *in vitro*. [4].

Examples of constructs resulting from a bottom-up approach include structures from various single cell types and from mixtures of different cell types by pouring different types of cell beads into a single PDMS mold (fig 4A). Although approximately 100000 monodisperse cell beads are required to fabricate millimetrescale 3D tissue. The primary advantages of this method are rapid production of millimetre-thick 3D cell structures, homogeneous cell density, and tissue formation without necrosis in a period of less than a week because of the supply of the cell culture medium through cavities between cell beads. In addition, when the millimetre-thick 3D cell structure was composed of HepG2 cells and NIH 3T3 cells, albumin secretion increased daily from the HepG2 cells in the 3D tissue, but not in the 2D culture system. However, the construction of the vessel network in the 3D cell structures is required to maintain cell viability over a long period. Adaptations of this method will allow the formation of capillary networks in the structure because methods using the mold can integrate cell-laden fibers as capillaries into bead-based cell structures [103]. As another method of a bead-based assembly, the printing of cellular spheroids or cell-laden hydrogel beads can be used to sequentially stack these materials layer-by-layer

[104,105]. Combined with computer-aided printing systems, the printing approach enables the rapid construction of complex 3D structures with different types of cells.

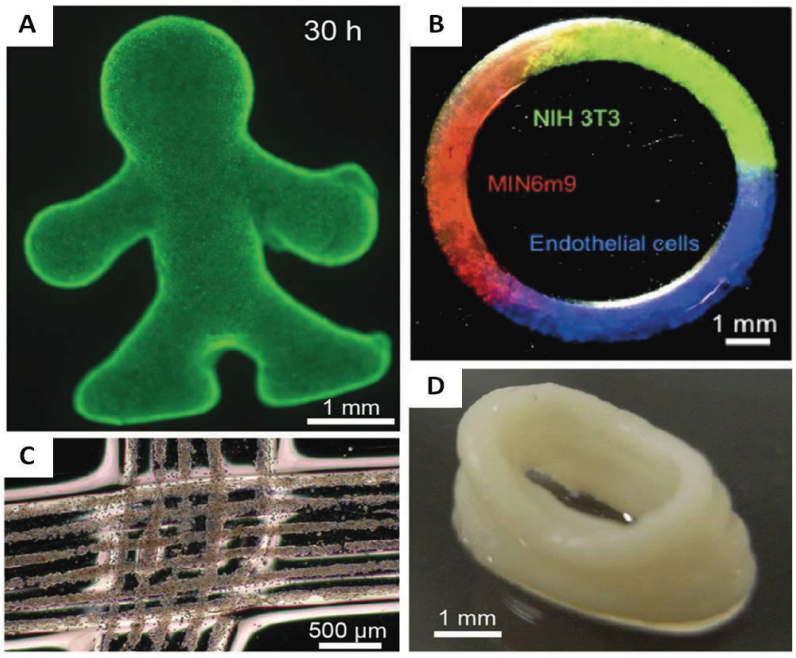

Figure 4: 3D organotypic construct (i) Fluorescent image of a macroscopic structure with a complex shape. Live cell staining indicates that almost all cells within the structure are alive.(j) 3D cell structures formed by knitting cell-laden hydrogel fibers. The fibers are composed of collagen and alginate gel encapsulating HeLa cells.34 (k) Ring-shaped cell structure fabricated by printing cell beads. Various cell types are spatially coded within the structure.30 (l) Tube-shaped structure prepared by printing cell beads.30.[28].

For example, the printing of cellular spheroids or cell-laden collagen beads has been used to reproducibly construct ring-shaped structures containing multiple cell-laden beads in specific designed locations (Fig.4B) and hollow tubes (Fig. 4D) [104,105,106]. A fiber-based assembly method was recently proposed for the construction of 3D cell structures with complex shapes. Assemblies of cell-laden hydrogel fibers can be formed by weaving these fibers together, much as threads are woven to form cloth [107]. These approaches produce a metastable multicellular construct that will remodel over time according to biological and physical principles (i.e. migration of the cells, shrinkage of the hydrogel). Shapes and patterns are not inevitably translated to the final tissue. Designs must thus focus on promoting proper remodeling into the final architecture. Clearly, understanding and promoting tissue self organization would tremendously improve tissue microfabrication. The study of

the emergence of forms and functions *in vitro* tissue models is still in its infancy. It is likely that those strategies of auto assembly can only reach a limited complexity and should be followed by more complex manipulations of the microenvironment. The rapid development of complex microfluidic systems, microbioreactors and detecting tools allows the long term culture of microscale tissues in precisely defined microenvironment. Microbioreactors permit the long term culture in controllable and continuous environment using minute amounts of biological factors [107] and to include parameters like shear stress, interstitial flow [108,109] or gradients of soluble factors [110,111]. They present great possibilities to culture micro-tissues in controlled, heterogeneous environments. Mechanisms underlying tissue organization such as the development, maintenance of tissues architecture and function are highly conserved through organisms and are better understood now than two decades ago [112] it appears clearer that, beyond genetic regulation, the tissue architecture and microenvironment feeds back to promote its development, maintain its integrity and function [113,114]. Thus, to promote *in vitro* tissue developments, of special interest are (i) the creation of multicellular architectures prone to remodeling and (ii) strategies and tools to manipulate the microenvironment and promote *in vitro* organization.

2.3 Engineered Cardiac Muscles for Functional Cardiac Regeneration

The heart is one of the most important organs in the entire human body, owes its specialized functions to the unique complement of cells and their complex arrangements. Its contractile function derives primarily from cardiomyocytes (CMs). The myocardium is composed of tightly packed cardiomyocytes connected via gap junctions and supported by resident fibroblasts and vascular cell populations, which act as supporting cells by maintaining the extracellular matrix (ECM) as well as guiding electrical current propagations throughout the heart. Together these three cell types form a mechanically and electrically coupled unit that generates the force necessary to pump blood throughout the body. Until the recent discovery of resident cardiac progenitor cells [1,2] the myocardium was thought to be composed entirely of terminally differentiated cells. However, in their native state, these progenitor cells do

not contribute sufficiently to the repairing of major cardiac trauma. Regenerative potential of the heart is severely limited, predominantly because of a low proliferative capacity of CMs and propensity to scar formation following injury. A constant supply of oxygen and nutrient is required to sustain the high metabolic demand of CM. For this reason, any disruption of circulation to the heart muscle, such as that seen during a myocardial infarction (MI), will lead to rapid cell death and tissue necrosis. In most cases, the cell debris triggers an inflammatory response that is characterized by the infiltration of monocytes and macrophages. The necrotic tissue is then replaced by granulation tissue consisting mainly of fibroblasts and collagen. Finally, this tissue is remodelled to form a stiff and non-contractile scar tissue. In end-stage heart failure and many congenital cardiac anomalies, the requirement for heart transplantation is inevitable. While various surgical procedures and pharmacological therapies can prolong the life span, neither provides a cure. Heart transplantation results in an effective treatment; however, there is a shortage of donor organs. In addition, organ rejection and/or failure, life-long requirement for immunosuppression therapy and complications of that therapy further threaten the long-term success of heart transplants [3]. An alternative cardiac tissue source is required to address a growing need. This alternative source of cardiac tissue is most likely to arise from the collective efforts of the tissue engineering field, where through a multi-disciplinary approach to *in vivo* and *in vitro* tissue development, new organs may be generated for transplantation and tissues for reconstruction. The National Science Foundation (USA) defined tissue engineering as, ‘an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function [4]. The final product of tissue engineering developed by the scientists and engineers will require to produce implantable cardiac tissue in vitro by combining CM and other supporting cell types in an appropriate scaffold. The resulting engineered cardiac tissue should possess similar mechanical and functional properties as native cardiac tissue. The presence of functional cells should have the capability to secrete ECM and growth factors to encourage beneficial host remodeling, which will significantly contribute to the long term integration of the implant [5].

2.4 Cell Sourcing for Cardiac Tissue Engineering

Cell sourcing is perhaps the most important consideration when designing tissue regeneration strategies. An optimal cell source should be easily obtainable, the choice of cell can directly impact on ECM remodeling, the functioning of the regenerated tissue and ultimately host response. The cells selected should also be matched with suitable scaffolds, culture conditions and delivery methods in order to maximize its therapeutic potential. In cardiac tissue engineering, the cells of interest are primarily cardiomyocytes, cardiac fibroblasts, and endothelial cells. The following sections will outline the features of these cell types and their most common sources.

Cardiomyocytes

The most impressive feature of the heart is its ability to contract continuously and synchronously during a person's lifespan. Heart muscle cells, called cardiomyocytes (CM), are the primary cell type responsible for the generation of contractile force. The developmental origin of CM can be traced back to the mesoderm layer of the gastrulated embryo [20]. The heart itself is formed by two separate waves of myogenesis known as the primary and secondary heart fields. The primary heart field originates from the anterior lateral mesoderm and give rise to the left ventricle of the mature heart, while the secondary heart field develops into the right ventricle inflow and outflow tracks [21]. The secondary heart field also gives rise to some of the non-myocyte component of the heart including cardiac fibroblast (CF), smooth muscle cells (SMC) and endothelial cells (EC).

Cardiomyocytes share many similarities with skeletal muscle cells; both are striated, multinucleated, and excitable cells. In comparison to skeletal muscle cells, CM are more resistant to fatigue, contain more mitochondria, and are more adapted to aerobic respiration. Nevertheless, perhaps the most distinct anatomical feature of CM are the intercalated discs found between adjacent cells. Intercalated discs are areas of cell membrane rich in desmosomes and gap junctions. Desmosomes are cadherin family transmembrane proteins that are associated with intermediate filaments. Through homophilic binding, desmosomes also confer mechanical cell adhesion and integrity between CM. On the other hand, intercellular communications are mediated

by gap junctions. Transmembrane proteins called connexins form hemi-channels on the membranes of adjacent cells. These connexins can align and form a direct channel between the cytoplasm of these adjacent cells, allowing membrane depolarization to propagate from one cell to the next, thus facilitating action potential propagation over the myocardium. The ultimate goal of cell sourcing in tissue engineering is to find a cell type that can differentiate into all the necessary cells found in an organ in order to recapitulate organogenesis during development. Embryonic stem cells (ESC) derived from the inner cell mass of the blastocyst seems to be the best choice for tissue engineering. These cells are pluripotent and have strong proliferative capacity compared to adult stem cells. However, pluripotency is not without its downfalls. Direct injection of undifferentiated ESC can lead to teratoma formation [22]. Therefore, the phenotype of ESC and ESC derived cells must be carefully regulated. Selective differentiation of ESC have been shown to be a promising path towards a steady source of CM [23,24]. Laflamme *et al*. demonstrated that the injection of differentiated human ESC in a rat model maintains cardiac phenotypes and continues to proliferate over 4 weeks [25]. In addition, the same group has demonstrated the utility of human ES derived CM in tissue engineering application by fabricating contractile cardiac constructs [26]. Although ESC derived CM improved cardiac function *in vivo*, their allogeneic nature poses a significant obstacle towards clinical application. The recent discovery of induced pluripotent stem (iPS) cells derived from adult fibroblast may be the answer for an autologous and pluripotent cell source. The Yamanaka group pioneered the technique in restoring pluripotency of somatic cells to the same level as ESC by ectopically expressing Oct3/4, Sox2, Klf4 and c-Myc [27]. It can be assumed that current methods being used to drive ESC toward CM differentiation can similarly be applied to iPS cells. Like other stem cells, the *in vivo* tumorgenicity and immune response toward iPS cells will ultimately determine their clinical usefulness. Further studies are needed to answer these questions. From a clinical therapy perspective, the ideal CM for tissue engineering should retain contractile phenotype indefinitely, cause minimal immune response in the host, and participate in functional remodeling by organizing into contractile bundles [28]. Much of these criteria can be satisfied by using CM differentiated from stem or progenitor cells. Studies in stem cell lineages have identified populations of adult stem cells in tissues, such as adipose [29], skin, dental pulp [30] and amniotic fluid [31,32], some of which are capable of differentiating into

beating CM [33]. However, the most studied population for cardiac regeneration in clinical application to date is the bone marrow derived messenchymal stem cells (MSC). These cells inhabit the stromal space of bone marrow. They can be obtained through direct bone marrow aspiration or from peripheral blood. In both cases, the MSC reside as a subpopulation of cells within the mononuclear cell fraction [34]. Under the right condition, these MSC can be induced to express cardiomyogenic markers [35] and play a cardioprotective role to nearby cardiomyoblasts [36]. In clinical studies, intravenous or intramyocardial injections of MSC in acute MI have been shown to increase ejection fractions and vascularity around the infarct area [37,38]. However, few MSC differentiate into CM lineage and those that are committed have little to do with the functional improvements that are observed [39]. Therefore, it appears that much of the benefits of MSC in acute MI are derived from their cytoprotective and angiogenic roles [40,41]. Although not a viable CM source for therapeutic applications, primary rat neonatal ventricular cardiomyocytes have served as a useful cell model that has greatly contributed to the understandings of CM physiology and development. Structurally, neonatal CM lack a well developed T-tubule system, the deep invagination of the sarcolemma, found in adult CM [43]. This leads to a more heterogeneous cytosolic Ca^{2+} concentration compared to adult myocytes and contributes to its unique excitation-contraction coupling characteristic.

Cardiac Fibroblasts

The cardiac fibroblast (CF) constitutes the bulk of non-myocyte cell population in the heart [44]. They are traditionally defined as cells of mesenchymal origin that produce interstitial collagen [45,46] and maintain ECM components, including collagen, fibronectin, and laminin. Fibroblasts tend to lack a basement membrane and display multiple processes or sheet-like extensions. These cells contain an oval nucleus, extensive rough endoplasmic reticulum, a prominent Golgi apparatus, and abundant cytoplasmic granular material. Unlike cardiomiocytes and endothelial cell, the cardiac fibroblast is found natively in the stromal space and lack basement membranes. This feature gives CF its unique ability to migrate and populate injury sites, such as a myocardial infarct, and quickly restore tissue volume and ECM proteins [47]. In the developing fetal heart, CF contributes to ECM rich structure including valves and

atrial ventricular walls. As the heart matures, a 3D network of collagen and CF, known as the cardiac skeleton, begins to take shape. This network allows CF to exert forces on the myocytes, as well as, to respond to external stimuli through degradation and synthesis of ECM. These processes help maintain the mechanical integrity of the heart through cell-cell and cell-ECM interactions. More importantly, CF forms heterotypic gap junctions with neighboring CM or CF, whereby the conductivity of these junctions can be modulated by the differential expression and coupling of connexins isoforms, including Cx45, Cx43 and Cx40 [48,49]. The cell-cell interaction between CF and CM helps to ensure the long range synchronization of myocardium contraction. Cardiac fibroblast also plays a pivotal role during cardiac repair. Under appropriate stimulation, relatively quiescent fibroblasts can acquire an active synthetic, contractile phenotype and express several smooth muscle cell markers not typical of fibroblasts [50]. These cells express contractile proteins, are more mobile than undifferentiated fibroblasts, can effectively contract collagen gels, and are believed to be important for wound closure and structural integrity of healing scars [51]. Moreover, myofibroblasts are associated with hypertrophic fibrotic scars in various injury models. Apoptosis of the myofibroblast has been shown to be associated with the progression of granulomatous tissue to a mature scar. On the other hand, failure of apoptosis has been suggested to drive the progression to fibrosis. However, upon injury, myofibroblasts appear in the myocardium and are believed to arise from resident interstitial and adventitial fibroblasts. They may also originate from progenitor stem cells in the heart or from the circulation. Whatever their origin, the dynamic balance between fibroblast and myofibroblast phenotype is a critical factor in the wound healing outcome.

Cardiac Endothelial Cells

The complex cavitary surface of the cardiac wall is completely lined by the endocardial endothelium (EE). The morphology of the EE has been well described [42]. It is recognizable as a sheet of endothelial cells with a central nuclear bulge and distinct, extensive intercellular junctions. EE cells are somewhat larger than endothelial cells in most other portions of the circulatory system. The luminal surface of most of these cells possesses a scattered variety of microappendages, or

microvilli, projecting into the cardiac cavity. It can be estimated that the labyrinth of trabeculae and furrows, together with the numerous microvilli on the luminal surface of the EE cells, may augment the surface area by a factor of 100 or more. This surprisingly large contact surface area of the EE offers an astonishingly high ratio of cavitary surface area to ventricular volume, particularly in the right ventricle. This suggests an important sensor role for the EE [75]. One of the key factors for myocardial regeneration is revascularization of damaged tissue. In the normal heart, there is a capillary next to almost every CM, and endothelial cells (ECs) outnumber cardiomyocytes by 3:1 [76]. Developmental biology experiments reveal that myocardial cell maturation and function depend on the presence of endocardial endothelium at an early stage [76]. Experiments with inactivation or overexpression of vascular endothelial growth factor (VEGF) demonstrated that at later stages, either an excess or a deficit in blood vessel formation results in lethality due to cardiac dysfunction [76,77]. Both endocardium and myocardial capillaries have been shown to modulate cardiac performance, rhythmicity, and growth [78]. In addition, a recent study showed the critical importance of CM-derived VEGF in paracrine regulation of cardiac morphogenesis [79]. These findings and others highlight the significance of interactions between CMs and endothelium for normal cardiac function. However, little is known about the specific mechanisms for these interactions, as well as the role of a complex, 3-dimensional organization of myocytes, ECs, and fibroblasts in the maintenance of healthy cardiac muscle. The critical relation of CMs and the microvasculature suggests that successful cardiac regeneration will require a strategy that promotes survival of both ECs and CMs.

2.5 Properties and Functions of Cardiac Extracellular Matrix

The cardiac ECM is composed mainly of collagen and plays a vital role in myocardial structure and function. It serves to direct contractile force generated by cardiac myocytes and contributes to the passive stretch characteristics of the ventricle. Thus deformation of the extracellular matrix may help restore ventricular myocytes to precontraction length [52,53]. This expansion phase is thought to create suction that pulls blood into the ventricles from the atria. Collagen subtypes and fibronectin

compose the majority of cardiac ECM proteins. The majority of ECM components in the heart are secreted by cardiofibroblasts and myofibroblasts [53,54,55]. Cardiac myocytes and cells of many organs adhere to the extracellular matrix via matrix-specific receptors including those for fibronectin [56]. Moreover, these ECM components are able to transmit external stimuli to the myocytes and fibroblast and trigger intracellular signaling that can alter cellularfunctions. Recent work by Ott et al. demonstrated that decellularized native cardiac tissue can be repopulated with CM and CF and give rise to functional tissue that possesses the microarchitecture of native tissue [57]. This suggests that both the composition and organization of ECM molecules are important for proper tissue development. Collagen types I and III are the most abundant forms of collagen within the myocardium [58,59], and cornprise the bulk of replacement scar tissue following myocardial infarction [60]. The cardiac ECM is a complex array of different molecular components, and that it plays an important role for the transfer of mechanical force in both contraction and relaxation phases in the cardiac cycle. As impaired lusitropic cardiac performance may contribute to the overall pathogenesis of heart failure, investigations of the regulation of cardiac ECM component gene transcription and ECM protein deposition in various disease states and growth are essential.

Collagen

Collagen type I is the major collagenous product of cardiac fibroblasts as this molecular species represents about 80% of the total cardiac collagen content [59, 62]. This observation is in agreement with the results of a recent study of collagen isoforms synthesized by cultured cardiac fibroblasts [63]. Two different collagen subunits, the products of a unique gene, coalesce to form the trimeric protein. Two thirds of the trimer is formed by two α-1 (I) chains (molecular weight = 95 kDa); the remainder of the trimer is an α-2 (1) chain (95 kDa). Procollagen polypeptides undergo cleavage before becoming mature cellular proteins wherein peptide sequences are removed from both the amino and carboxyl ends by specific proteases. Collagen type I is characterized by a unique lack of disulfide bonds between adjacent chains. Collagen type III is relatively abundant in the myocardium, accounting for approximately 12% of myocardial collagen content. Collagen type III,

composed of three identical chains of α-1 (III) subunit polypeptides, forms aggregates (struts) which are thinner than those containing collagen type I in heart. The procollagen a-1 (III) subunits have a molecular weight of 140 kDa which is reduced to 95 kDa after posttranslational modification. Jointly, collagen types I and III represent more than 90 % of all myocardial collagen.

2.6 Biomaterials for Cardiac Tissue Engineering

Scaffolds may be formed from natural, synthetic or hybrid materials and fashioned to provide appropriate pore size, connectivity and strength, adhesion, or even to furnish growth factors 'on demand' by incorporating these into surface coatings, in so called 'smart surfaces'. The traditional paradigm of tissue engineering is to seed-shaped scaffolds with cells ex vivo and implants the composite structure into the living animal, where the tissue is vascularized and the scaffold incorporated into surrounding tissues or progressively replaced by the host tissue [4]. Scaffold used in cardiac tissue engineering must reflect the natural cardiac scaffold and will depend on the type of construct desired. For example, scaffolds used to develop a graft overlaying an infarct site may require different elasticity characteristics to a composite tissue used to replace a full-thickness myocardium, which is subjected to full systolic pressure. Material tested for cardiac tissue engineering include polyurethrane [65], 1,3-trimethylene carbonate [66] and D,L-lactide and copolymers, poly(1-caprolactone) [67], polyglycolic acid (PGA), poly(lactic acid) (PLA) and polyglycolic acid [68]. While CM growth has been reported on these polymers, significant problems associated with the elastic properties of polymeric materials (inability to stretch synchronously with native heart), acidic (PGA, PLA) or toxic (PU) degradation products remain to be resolved before these products can be fully utilized in cardiac tissue engineering. While some natural materials such as gelatine [69] and alginate [70,71] can be incorporated into three-dimensional structures in order to encourage better cell interaction with the scaffold, other materials, such as collagen I and Matrigel, are most similar to mammalian cellular ECM-like environments and most supportive of implanted CM survival by facilitating cell adhesion, proliferation and differentiation. However, because of their rapid degradation by local tissue proteases, the potential immunogenicity and as a

potential reservoir of infectious agents [72] these natural materials are currently being modified and are more often used in conjunction with solid scaffolds [73]. Other technologies for cardiac tissue engineering are being developed in very creative ways, such as using mechanical strain to condition tissue [81] or decellularization of a rat heart as a natural scaffold [57]. New technologies that generate scaffold-less cell dense CM sheets have also been reported and have shown potential in vivo. More importantly, this technology bypasses the issues associated with the use of exogenously added natural and synthesized scaffold [80].

2.7 Bioreactors

The 3D cell constructs that are development *ex vivo* usually lack the vascular network that exists in normal vascularised tissue. Thus, the gas and nutrient supply to the scaffold-seeded cells depends merely on mass diffusion. In static cultivation, with no fluid mixing, large diffusion gradient are performed between the cell constructs and their surroundings so that the cells in the centre of the construct do not get sufficient nutrients; the waste removal from the centre is poor; and thus the cells eventually die. Oxygen transport is typically considered as the main limiting factor for nutrient exchange [91]. To improve mass transport, researchers have designed several bioreactors, which exemplify different patterns of fluid dynamics and vessel geometry. A basic fluid-dynamic cultivation vessel is the spinner flask, which is a flask usually agitated at 50 rpm [92,93]. In these vessels, the cells constructs are subjected to turbulently mixed fluid that provides a well-mixed environment around the cell constructs and minimized the stagnant layer at their surface. It has been shown that cultivation of cardiac cell constructs in spinner flasks produces engineered tissues that are superior, in almost every aspect (aerobic cell metabolism, metabolic activity, morphological appearance, etc.) to tissues cultivated under static condition [92,93,94]. Bioreactors combined with mechanical signal stimuli improved the proliferation and distribution of the seeded cardiac cell throughout the scaffold volume and further stimulated the formation and organization of ECM, which contributed to the improvement in the mechanical strength of the cardiac graft [61,95]. Future bioreactors for CTE should combined both perfusion and mechanical stimuli; for example, by allowing for adjustable pulsate flow and varying

levels of pressure. Such bioreactors are currently under development for engineering heart valves *ex vivo* [96,97]. One of the most important difficulties in CTE is to grow 3D structures that contain more than a few layers of muscle cells. Most bioreactors simply cannot supply enough nutrients and oxygen to the growing tissue. Whereas human heart muscle is approximately 100 micrometers, or less than 10 cell layers, thick. Beyond this thickness, the innermost cells are too far from the supply of fresh growth medium to thrive. After transplantation, rapid vascularisation, adequate perfusion, survival, integration, and function of the engineered cardiac patch remain critical steps in the translation of *in vivo* achievements into an effective therapeutic tool [98].

2.8 Fabrication and Culture Platforms for Cardiac Tissue Engineering

One of the key challenges is certainly the choice and the interaction between cells and biomaterials on a molecular level to contribute to the functionality of the engineered tissue. To maximize the regenerative potential of the cells, one must consider the impact of tissue fabrication and culture strategies on the cells' behaviour. Schematic diagrams of these cardiac tissue engineering strategies can be found in figure 5 [90]. The classical tissue engineering approach is the creation of a tissue construct by seeding desired cells into a scaffold in vitro with or without manipulation (e.g. special conditioned culture system [82]) followed by implantation in vivo. The disadvantage of this method is that poor survival of implanted cells; also these can only be seeded on the surface and must actively migrate into the core of the scaffolds. This may lead to uneven cell seeding throughout the depth of the scaffold. The said observations indicate that cell survival in the construct is one of the major obstacles to engraftment of engineered constructs in vivo. To improve cell survival, several strategies have been investigated including the use of various cell-supporting scaffold materials [67,68]. Incorporation of pro-angiogenic factors and pro-survival factors into scaffolds [73,64] co-culturing of CMs with other cell types to form vasculature [83,84] in vitro or a combination of these strategies, with some success in improving engrafted cell survival.

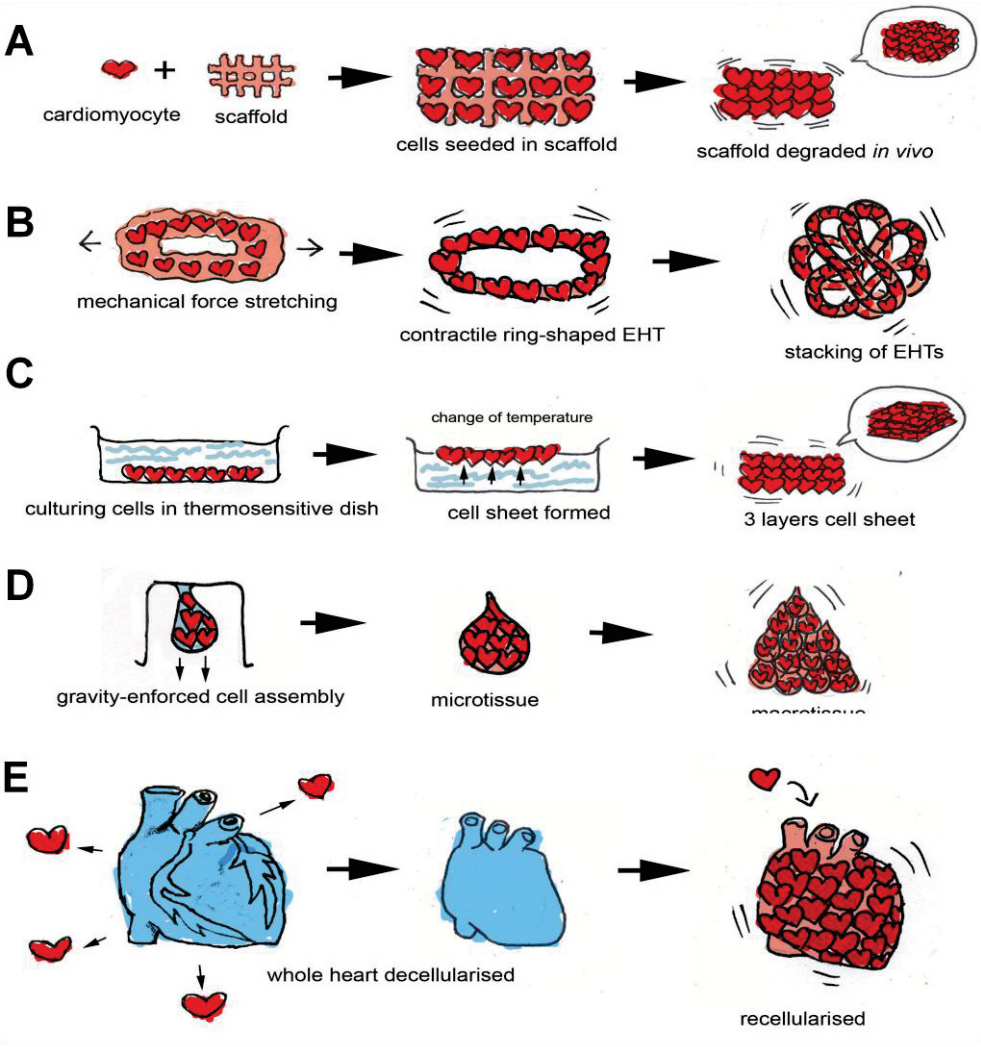

Fig. 5: Diagram showing the main strategies used in cardiac tissue engineering: (a) the 'classical' tissue engineering approach with cells seeded into scaffold then implanted *in vivo*; (b) engineered heart tissue (EHT) approach; (c) cell sheet approach; (d) gravity enforced cell assembly approach; and (e) decellularized heart scaffold approach [90].

One way to improve the uniformity of seeding cells within the scaffold is to combine cells with compatible hydrogel prior to casting and gelation. One of the most creative and promising approaches to cardiac tissue engineering uses neonatal rat CMs, collagen I matrix, Matrigel and a mechanical stretching device to create a spontaneously contractile construct called engineered heart tissue (EHT) [9,81,85]. Nonetheless, cells embedded near the core of the gel still suffer from diffusion limitation and tend to have low viability. Therefore, recent studies have shifted their focus towards the fabrications of micro-tissue such as the approach described within this thesis. By shrinking the size of the scaffold down to several hundred micrometers in diameter, cells embedded in the core may receive sufficient nutrients to survive.

Instead of seeding cells into a three-dimensional porous scaffold, Kelm et al. have created micro-tissues (~100 μm) containing CM capable of supporting core tissue viability, in fact provide an environment for them to migrate and assemble into contractile tissues in vitro using gravity-enforced techniques to form spheroid-like micro-tissues [14,86]. Another CTE approach that holds clinical application potential is the cell sheet technique [87]. The centerpiece of this approach is the invention of a thermo-sensitive cell culture surface, coated with a polymer – poly (N-isopropylacrylamide, PIPAAm), which is cell adhesive at 37°C and changes its properties in reverse below 32°C. Once CMs have aggregated and formed gap junctions, changing the temperature allows the beating cells to be lifted off the culture dish as a cell sheet without disruption to their gap junctions or architecture [88]. Further in vivo work demonstrated the potential for stacking individual CM cell sheets into a three-dimensional contractile cardiac tissue [80], which survived subcutaneous implantation for 1 year and application to rat hearts showed functional integration of the cell sheet with the host heart [89]. The benefit of this approach is a scaffold-free, cell dense tissue similar to compact myocardium. This approach, however, is limited by the diffusion of nutrients to sustain the viability of the patch and remains to be adapted for formation of thick myocardial tissue constructs where specific vascularization strategies will need to be employed.

2.9 Biomaterials for cell delivery into the heart

The past decade's burst in cell therapy clinical trials has resulted in a multitude of reports ranging from high expectations to modest questioning of the efficacy of cell therapy for cardiovascular disease. The perception is, however, that cell therapy would greatly benefit from improvements in the techniques of cell delivery and an improved understanding of mechanisms driving heart repair. A lack of standardization across clinical trials, because of variable cell sources and preparations, delivery methods, target patient population, time points, and functional readouts, has only added to the inherent variability, making a comprehensive cross study comparison rather difficult. To move forward, we need to develop new technologies that can address the challenges and limitations of the current preclinical trials. Cell injection: The administration of cell suspensions in fluid via a syringe or

catheter results in massive attrition after injection, by processes that occur almost immediately, and irrespective of cell type or formulation, delivery mode, and disease state. These processes occur before the vascular supply can be established and integration with the host cells can occur. The capacity of the heart to retain implanted cells for even 24 h is rather small [99], and local barriers of cell survival and function beyond this time point are substantial. Survival of ‘‘naked’’ cells in the hostile environment of an acute or chronic infarct is a major challenge. Several possibilities have been identified for improving cell engraftment and maximizing the potential of cells to mediate heart regeneration. One reported approach identified the addition of prosurvival factors into the cell suspension as a method to substantially improve the survival of transplanted cardiomyocytes [100]. A solution for improved cell retention, just starting to be explored in the lab settings, is the use of catheter-based hydrogel cell delivery, which builds on existing delivery methods used in the clinic. Hydrogels, delivered as a fluid phase suspension via catheter, slowly polymerize during the trajectory to the target tissue, polymerizing and entrapping cells in situ. With already extensive use and demonstrated safety of catheter-based cell delivery [101], the addition of a delivery vehicle offers a possibility for improving engraftment. Hydrogels offer additional distinct advantages of customization using growth factors to specifically enhance the vasculogenic or myogenic capacity of encapsulated cells [102], while physically shielding cells from the harsh ischemic environment, as they mediate repair. Although this option provides for a near-term solution to improve cell retention in the heart, it can be further advanced to mediate the effects of the microenvironment in situ.

Reference

[1] L. Bu, X. Jiang, S. Martin-Puig, L. Caron, S. Zhu, Y. Shao, D. J. Roberts, P. L. Huang, I J. Domian, and K. R. Chien, Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature* 460, 113-117 (2009).

[2] H. Kubo, N. Jaleel, A. Kumarapeli, R. M. Berretta, G. Bratinov, X. Shan, H. Wang, S. R. Houser, and K. B. Margulies, Increased cardiac myocyte progenitors in failing human hearts. *Circulation* 118, 649-657 (2008).

[3] Boyle A. Current status of cardiac transplantation and mechanical circulatory support. *Curr. Heart Fail. Rep.* 2009; 6: 28–33.

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

[5] Wolfram-Hubertus Zimmermann1,6, Ivan Melnychenko1,6, Gerald Wasmeier2, Michael Didie´1, Hiroshi Naito1, Uwe Nixdorff2, Andreas Hess3, Lubos Budinsky3, Kay Brune3, Bjela Michaelis4, Stefan Dhein4, Alexander Schwoerer5, Heimo Ehmke5 & Thomas Eschenhagen1] Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts VOLUME 12 [NUMBER 4 [APRIL 2006 NATURE MEDICINE.

[6] Hillsley MV, Frangos JA. Bone tissue engineering: the role of interstitial fluid flow. Biotechnol Bioeng. 1994 Mar 25;43(7): 573-81.

[7] Martin P. Wound healing–aiming for perfect skin regeneration. Science. 1997 Apr 4;276(5309):75-81.

[8] Bello YM, Falabella AF, Eaglstein WH. Tissue-engineered bskin. Current status in wound healing. Am J Clin Dermatol. 2001;2(5):305-13.

[9] Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, et al. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nat Med. 2006 Apr;12(4):452-8.

[10] Caplan AI, Elyaderani M, Mochizuki Y, Wakitani S, Goldberg VM. Principles of cartilage repair and regeneration. Clin Orthop Relat Res. 1997 Sep;(342); 254-69.

[11] Martin I. Wendt D, Heberer M. The role of bioreactors in tissue engineering. Trends Biotechnol. 2004 Feb;22(2):80-6.

[12] Du Y, Lo E, Ali S, Khademhosseini A. Directed assembly of cell-laden microgels for fabrication of 3-D tissue constructs. Proc Natl Acad Sci U S A. 2008 Jul 15;105(28):9522-7.

[13] McGuigan AP, Sefton MV. Vascularized organoid engineered by modular assembly enables blood perfusion. Proc Natl Acad Sci U S A. 2006 Aug 1;103(31):11461-6.

[14] Kelm JM, Djonov V, Ittner LM, Fluri D, Born W, Hoerstrup SP, et al. Design of custom-shaped vascularized tissues using microtissue spheroids as minimal building units. Tissue Eng. 2006 Aug;12(8):2151–60.

[15] Jakab K, Norotte C, Damon B, Marga F, Neagu A, Besch- Williford CL, et al. Tissue engineering by self-assembly of cell printed into topologically defined structures. Tissue Eng Part A. 2008 Mar;14(3):413-21.

[17] Yang J, Yamato M, Shimizu T, Sekine H, Ohashi K, Kanzaki M, et al. Reconstruction of functional tissues with cell sheet engineering. Biomaterials. 2007 Dec;28(34):5033-43.

[18] Palmiero C, Imparato G, Urciuolo F, Netti P. Engineered dermal equivalent tissue in vitro by assembly of microtissue precursors. Acta Biomater. 2010 Jul;6(7):2548-53.

[19] Urciuolo F, Imparato G, Palmiero C, Trilli A, Netti PA. Effect of process conditions on the growth of three-dimensional dermalequivalent tissue obtained by microtissue precursor assembly. Tissue Eng Part C Methods. 2011 Feb;17(2):155-64.

[20] C. E. Murry and G. Keller, Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132, 661-680 (2008).

[21] K. R. Chien, I. J. Domian, and K. K. Parker, Cardiogenesis and the complex biology of regenerative cardiovascular medicine. *Science* 322, 1494-1497 (2008).

[22] J. Nussbaum, E. Minami, M. A. Laflamme, J. A. Virag, C. B. Ware, A. Masino, V. Muskheli, L. Pabon, H. Reinecke, and C. E. Murry, Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *FASEB J.* 21, 1345-1357 (2007).

[23] V. C. Chen, R. Stull, D. Joo, X. Cheng, and G. Keller, Notch signaling respecifies the hemangioblast to a cardiac fate. *Nat. Biotechnol.* 26, 1169-1178 (2008).

[24] D. C. Kirouac and P. W. Zandstra, The systematic production of cells for cell therapies. *Cell Stem Cell* 3, 369-381 (2008).

[25] M. A. Laflamme, J. Gold, C. Xu, M. Hassanipour, E. Rosler, S. Police, V. Muskheli, and C. E. Murry, Formation of human myocardium in the rat heart from human embryonic stem cells. *Am. J. Pathol.* 167, 663-671 (2005).

[26] K. R. Stevens, L. Pabon, V. Muskheli, and C. E. Murry, Scaffold-free human cardiac tissue patch created from embryonic stem cells. *Tissue Eng Part A* 15, 1211-1222 (2009).

[27] K. Takahashi and S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676 (2006).

[28] G. Vunjak-Novakovic, N. Tandon, A. Godier, R. Maidhof, A. Marsano, T. P. Martens, and M. Radisic, Challenges in cardiac tissue engineering. *Tissue Eng Part B Rev.* 16, 169-187 (2010).

[29] R. Madonna and R. De Caterina, Adipose tissue: a new source for cardiovascular repair. *J. Cardiovasc. Med. (Hagerstown.)* 11, 71-80 (2010).

[30] A. J. Sloan and R. J. Waddington, Dental pulp stem cells: what, where, how? *Int. J. Paediatr. Dent.* 19, 61-70 (2009).

[31] X. Guan, D. M. Delo, A. Atala, and S. Soker, In vitro cardiomyogenic potential of human amniotic fluid stem cells. *J. Tissue Eng Regen. Med.* (2010).

[32] E. Galende, I. Karakikes, L. Edelmann, R. J. Desnick, T. Kerenyi, G. Khoueiry, J. Lafferty, J. T. McGinn, M. Brodman, V. Fuster, R. J. Hajjar, and K. Polgar, Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. *Cell Reprogram.* 12, 117-125 (2010).

[33] S. J. Gwak, S. H. Bhang, H. S. Yang, S. S. Kim, D. H. Lee, S. H. Lee, and B. S. Kim, In vitro cardiomyogenic differentiation of adipose-derived stromal cells using transforming growth factor-beta1. *Cell Biochem. Funct.* 27, 148-154 (2009).

[34] L. Zhang and C. Chan, Isolation and enrichment of rat mesenchymal stem cells (MSCs) and separation of single-colony derived MSCs. *J. Vis. Exp.* (2010).

[35] S. H. Bhang, S. J. Gwak, T. J. Lee, S. S. Kim, H. H. Park, M. H. Park, D. H. Lee, S. H. Lee, and B. S. Kim, Cyclic mechanical strain promotes transforming-growth-factorbeta1- mediated cardiomyogenic marker expression in bone-marrow-derived mesenchymal stem cells in vitro. *Biotechnol. Appl. Biochem.* 55, 191-197 (2010).

[36] A. Cselenyak, E. Pankotai, E. M. Horvath, L. Kiss, and Z. Lacza, Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cellto- cell connections. *BMC. Cell Biol.* 11, 29 (2010).

[37] V. Schachinger, S. Erbs, A. Elsasser, W. Haberbosch, R. Hambrecht, H. Holschermann, J. Yu, R. Corti, D. G. Mathey, C. W. Hamm, T. Suselbeck, B. Assmus, T. Tonn, S. Dimmeler, and A. M. Zeiher, Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N. Engl. J. Med.* 355, 1210-1221 (2006).

[38] S. R. Li, X. Y. Qi, F. L. Hu, J. Q. Zhang, T. H. Wang, Y. Dang, C. L. Meng, H. L. Liu, Y. X. Li, D. Wu, J. Dong, L. Y. Xun, L. H. Gao, and F. C. Jin, Mechanisms of improvement of left ventricle remodeling by trans-planting two kinds of autologous bone marrow stem cells in pigs. *Chin Med. J. (Engl.)* 121, 2403-2409 (2008).

[39] C. H. Yoon, M. Koyanagi, K. Iekushi, F. Seeger, C. Urbich, A. M. Zeiher, and S. Dimmeler, Mechanism of Improved Cardiac Function After Bone Marrow Mononuclear Cell Therapy. Role of Cardiovascular Lineage Commitment. *Circulation* (2010).

[40] S. Zhang, P. Zhang, J. Guo, Z. Jia, K. Ma, Y. Liu, C. Zhou, and L. Li, Enhanced cytoprotection and angiogenesis by bone marrow cell transplantation may contribute to improved ischemic myocardial function. *Eur. J. Cardiothorac. Surg.* 25, 188-195 (2004).

[41] M. A. Laflamme, S. Zbinden, S. E. Epstein, and C. E. Murry, Cell-based therapy for myocardial ischemia and infarction: pathophysiological mechanisms. *Annu. Rev. Pathol.* 2, 307-339 (2007).

[42] ANDRIES LJ. *Endocardial Endothelium: Functional Morphology*. Austin, TX: Landes, 1994, p. 1–143.

[43] J. B. Delcarpio, W. C. Claycomb, and R. L. Moses, Ultrastructural morphometric analysis of cultured neonatal and adult rat ventricular cardiac muscle cells. *Am. J. Anat.* 186, 335-345 (1989).

[44] C. A. Souders, S. L. Bowers, and T. A. Baudino, Cardiac fibroblast: the renaissance cell. *Circ. Res.* 105, 1164-1176 (2009).

[45] Eghbali-Webb M. *Molecular Biology Intelligence Unit Molecular Biology of Collagen Matrix in the Heart.* Austin, TX: Landes, 1994.

[46] Kanekar S, Hirozanne T, Terracio L, and Borg TK. Cardiac fibroblasts: form and function. *Cardiovasc Pathol* 7: 127–133, 1998.

[47] S. W. van den Borne, J. Diez, W. M. Blankesteijn, J. Verjans, L. Hofstra, and J. Narula, Myocardial remodeling after infarction: the role of myofibroblasts. *Nat. Rev. Cardiol.* 7, 30-37 (2010).

[48] P. Kohl, Heterogeneous cell coupling in the heart: an electrophysiological role for fibroblasts. *Circ. Res.* 93, 381-383 (2003).

[49] C. Louault, N. Benamer, J. F. Faivre, D. Potreau, and J. Bescond, Implication of connexins 40 and 43 in functional coupling between mouse cardiac fibroblasts in primary culture. *Biochim. Biophys. Acta* 1778, 2097-2104 (2008).

[50] Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, and Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3: 349–363, 2002.

[51] Sun Y and Weber KT. RAS and connective tissue in the heart. *Int J Biochem Cell Biol* 35: 919–931, 2003

[52] Robinson TF, Factor SM, Sonnenblick EH: The Heart as a suction pump. Scientific American 254: 84-91, 1986

[53] Robinson TF, Cohen-Gould L, Factor SM: The skeletal framework of mammalian heart muscle: Arrangement of inter- and pericellular connective tissue structures. Lab invest 49: 482-498, 1983

[54] Eghbali M, Blumenfeld OO, Seifter S, Buttrick PM, Leinwand LA, Robinson TF, Zern MA, Gambrione MA: Localization of types I, III, and IV collagen mRNAs in rat heart cells by in situ hybridization. J Mol Cell Cardiol 21: 103-113, 1989

[55] Eghbali M, Czaja MJ, Zeydel M, Weiner FR, Zern MA, Siefter S, Blumenfeld OO: Collagen mRNAs in isolated adult heart cells. J Mol Cell Cardiol 20: 267-276, 1988

[56] Wayner E, Carter W, Piotrowitz R, Kunicki T: The function of multiple extracellular matrix receptors mediating cell adhesion to extracellular matrix: Preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins. J Cell Bio1107: 1881-1891,1988

[57] H. C. Ott, T. S. Matthiesen, S. K. Goh, L. D. Black, S. M. Kren, T. I. Netoff, and D. A. Taylor, Perfusion-decellularized matrix:using nature's platform to engineer a bioartificial heart. Nat. Med. 14, 213-221 (2008).

[58] Speiser B, Riess CF, Schaper J: The extracellular matrix in human myocardium: Part 1: Collagens I, II, IV, and VI. Cardioscience 2: 225-232, 1991

[59] Bashey RI, Martinez-Hernandez A, Jiminez SA: Isolation, characterization, and localization of cardiac collagen type VI. Associations with other extracellular matrix components. Circ Res 70: 1006-1017, 1992

[60] Knowlton AA, Connelly CM, Romo GM, Manuya W, Apstein CS, Brecher P: Rapid expression of fibronectin in the rabbit

heart after myocardial infarction with and without reperfusion. J Clin Invest 89: 1060-1068,1992

[62] Weber KT: Cardiac interstitium in health and disease: The fibrillar collagen network. J Am Coll Cardio113: 1637-1652, 1989

[63] Bashey RI, Donnelly M, Insinga F, Jiminez SA: Growth properties and biochemical characterization of collagens synthesized by adult rat heart fibroblasts in culture. J Mol Cell Cardiol 24: 691-700~ 1992

[64] Dvir T, Kedem A, Ruvinov E *et al*. Prevascularization of cardiac patch on the omentum improves its therapeutic outcome. *Proc. Natl. Acad. Sci. U S A* 2009; 106: 14990–5.

[65] McDevitt TC, Woodhouse KA, Hauschka SD, Murry CE, Stayton PS. Spatially organized layers of cardiomyocytes on biodegradable polyurethane films for myocardial repair. *J. Biomed. Mater. Res. A.* 2003; 66: 586–95.

[66] Pego AP, Van Luyn MJ, Brouwer LA *et al*. In vivo behavior of poly(1,3-trimethylene carbonate) and copolymers of 1,3-trimethylene carbonate with D,L-lactide or epsilon-caprolactone: degradation and tissue response. *J. Biomed. Mater. Res. A.* 2003; 67: 1044–54.

[67] Ishii O, Shin M, Sueda T, Vacanti JP. In vitro tissue engineering of a cardiac graft using a degradable scaffold with an extracellularmatrix-like topography. *J Thorac. Cardiovasc. Surg.* 2005; 130: 1358– 63

[68] Ozawa T, Mickle DA, Weisel RD, Koyama N, Ozawa S, Li RK. Optimal biomaterial for creation of autologous cardiac grafts. *Circulation* 2002; 106: I176–82.

[69] Li RK, Jia ZQ, Weisel RD, Mickle DA, Choi A, Yau TM. Survival and function of bioengineered cardiac grafts. *Circulation* 1999; 100: II63–9.

[70] Kao KT, Hilker DM, McGavack TH: Connective tissue. V. Comparison of synthesis and turnover of collagen and elastin synthesis in tissues of rats at several ages. Proc Soc Exp Biol Med 106: 335-338,1961

[71] Amir G, Miller L, Shachar M *et al*. Evaluation of a peritonealgenerated cardiac patch in a rat model of heterotopic heart transplantation. *Cell Transplant.* 2009; 18: 275–82.

[72] Schmidt CE, Baier JM. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. *Biomaterials* 2000; 21: 2215– 31.

[73] Kutschka I, Chen IY, Kofidis T *et al*. Collagen matrices enhance survival of transplanted cardiomyoblasts and contribute to functional improvement of ischemic rat hearts. *Circulation* 2006; 114: I167–73.

[74] Urciuolo F, Imparato G, Totaro A, Netti PA. Building a Tissue In Vitro from the Bottom Up: Implications in Regenerative Medicine. Methodist Debakey Cardiovasc J. 2013 Oct;9(4):213-217. Review.

[75] BRUTSAERT DL. The endocardium. *Annu Rev Physiol* 51: 263–273, 1989.

[76] Brutsaert DL. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiol Rev*. 2003;83:59–115.

[77] Miquerol L, Langille BL, Nagy A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development*. 2000;127:3941–3946.

[78] Miquerol L, Langille BL, Nagy A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development*. 2000;127:3941–3946.

[79] Giordano FJ, Gerber H-P, Williams S-P, et al. A cardiac myocyte vascular endothelial growth factor paracrine pathway is required to maintain cardiac function. *Proc Natl Acad Sci U S A* 2001;98:5780–5785.

[80] Shimizu T, Sekine H, Yang J *et al*. Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. *FASEB J.* 2006; 20: 708–10.

[81] Zimmermann WH, Schneiderbanger K, Schubert P *et al*. Tissue engineering of a differentiated cardiac muscle construct. *Circ. Res.* 2002; 90: 223–30.

[82] Radisic M, Park H, Gerecht S, Cannizzaro C, Langer R, Vunjak- Novakovic G. Biomimetic approach to cardiac tissue engineering. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 2007; 362: 1357–68.

[83] Lesman A, Habib M, Caspi O *et al*. Transplantation of a tissueengineered human vascularized cardiac muscle. *Tissue Eng. Part A.* 2009; 16: 115–25.

[84] Caspi O, Lesman A, Basevitch Y *et al*. Tissue engineering of vascularised cardiac muscle from human embryonic stem cells. *Circ. Res.* 2007; 100: 263–72.

[85] Zimmermann WH, Didie M, Doker S *et al*. Heart muscle engineering: an update on cardiac muscle replacement therapy. *Cardiovasc. Res.* 2006; 71: 419–29.

[86] Kelm JM, Djonov V, Hoerstrup SP *et al*. Tissue-transplant fusion and vascularization of myocardial microtissues and macrotissues implanted into chicken embryos and rats. *Tissue Eng.* 2006; 12: 2541– 53.

[87] Shimizu T, Yamato M, Kikuchi A, Okano T. Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. *Tissue Eng.* 2001; 7: 141–51.

[88] Shimizu T, Yamato M, Isoi Y *et al*. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ. Res.* 2002; 90: e40.

[89] Furuta A, Miyoshi S, Itabashi Y *et al*. Pulsatile cardiac tissue grafts using a novel three-dimensional cell sheet manipulation technique functionally integrates with the host heart, in vivo. *Circ. Res.* 2006; 98: 705–12.

[90] Richard Tee,* Zerina Lokmic,*† Wayne A. Morrison*†‡ and Rodney J. Dilley. Strategies in cardiac tissue engineering ANZ Journal of Surgery Volume 80, Issue 10, pages 683–693, October 2010

[91] Colton,C.K. 1995. Implantable biohybrid artificial organs. Cell Transplant. 4:415-436.

[92] Carrier, R.L.,M. Papadaki, M. Rupnick, et al. 1999 Cardiac tissueengineering:cell seeding, cultivation parameters and tissue construct characterization. Biotechnol.Bioeng. 64:580-589.

[93] Carrier,R.L., M. Rupnick, R. Langer, et al. 2002. Perfusion improves tissue architecture of engineered cardiac muscle. Tissue Eng. 8: 175-188.

[94] Papadaki, M., N. Bursac, L. Langer, et al. 2001. Tissue Enginnering of functional cardiac muscle:molecular, structural, and electrophysiological studies. Am.J. Physiol. Heart Circ. Physiol. 280:H168-H178.

[95] Akhyari, P., Fedak, R.D., Weisel, et al. 2002. Mechanical stretch regimen enhances the formation of bioengineered autolugus cardiac muscle grafts. Circulation 106: I137-I142.

[96] Sodian, R., J.S. Sperling, D.P. Martin, et al. 2000. Fabrication of a trileaflet heart valve scaffold from a polyhydroxyalkanoate biopolyester for use in tissue engineering. Tissue Eng. 6:183-188.

[97] Dohomen, P.M., S. Ozaki, E. Verbeken, et al. 2002. Tissue engineering of an auto xenograft pulmonary heart valve. Asian Cardiovasc. Thorac. Ann 10:25-30

[98] Jonathan Leor and Smadar Cohen. 2004 Myocardial tissue engineering: creating a muscle patch for a wounded heart.. Ann. N.Y. Acad.Sci. 1025:312-319

[99] Sherman, W., and Martens, T.P. Catheter-based delivery of cells to the heart. Nat Clin Pract Cardiovasc Med 3, S57,2006.

[100] Laflamme, M.A., Chen, K.Y., Naumova, A.V., Muskheli, V., Fugate, J.A., Dupras, S.K., et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25, 1015, 2007.

[101] Siminiak, T., Burchardt, P., and Kurpisz, M. Postinfarction heart failure: surgical and trans-coronary-venous transplantation of autologous myoblasts. Nat Clin Pract Cardiovasc Med 3 Suppl 1, S46, 2006.

[102] Ferreira, L.S., Gerecht, S., Fuller, J., Shieh, H.F., Vunjak- Novakovic, G., and Langer, R. Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells. Biomaterials 28, 2706, 2007.

[103] *Yukiko T. Matsunaga , Yuya Morimoto , and Shoji Takeuchi*Molding Cell Beads for Rapid Construction of Macroscopic 3D Tissue Architecture *Adv. Mater.* 2011, *23*, H90–H94

[104] Y. T. Matsunaga, Y. Morimoto and S. Takeuchi, Molding cell beads for rapid construction of macroscopic 3D tissue architecture, Adv. Mater., 2011, 23(12), H90–H94.

[105] V. Mironov, R. P. Visconti, V. Kasyanov, G. Forgacs, C. J. Drake and R. R. Markwald, Organ printing: tissue spheroids as building blocks, Biomaterials, 2009, 30(12), 2164–2174

[106] C. Norotte, F. S. Marga, L. E. Niklason and G. Forgacs, Scaffold-free vascular tissue engineering using bioprinting, Biomaterials, 2009, 30(30), 5910–5917.

[107] H. Onoe, R. Gojo, Y. Tsuda, D. Kiriya, M. Kato-Negishi and S. Takeuchi, Cell fibers: construction of centimeter-scale 3D tissues by weaving, 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences, 2010, pp. 629–631.

[108] Yates C, Shepard CR, Papworth G, Dash A, Beer Stolz D, Tannenbaum S, et al. Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. Adv Cancer Res 2007;97: 225–46.

[109] Figallo E, Cannizzaro C, Gerecht S, Burdick JA, Langer R, Elvassore N, et al. Micro-bioreactor array for controlling cellular microenvironments. Lab Chip 2007;7(6):710–9.

[110] Wong AP, Perez-Castillejos R, Christopher Love J, Whitesides GM. Partitioning microfluidic channels with hydrogel to construct tunable 3-D cellular microenvironments. Biomaterials 2008;29(12):1853–61.

[111] Yu H, Meyvantsson I, Shkel IA, Beebe DJ. Diffusion dependent cell behavior in microenvironments. Lab Chip 2005;5(10):1089–95.

[112] Salazar-Ciudad I, Jernvall J, Newman SA. Mechanisms of pattern formation in development and evolution. Development 2003;130(10):2027–37.

[113] Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. Bull Math Biol 2006;22:287–309.

[114] Lecuit T, Le Goff L. Orchestrating size and shape during morphogenesis. Nature 2007;450(7167):189–92

Chapter 3

A BOTTOM-UP APPROACH TO CARDIAC TISSUE ENGINEERING: HOW TO BUILD-UP A TISSUE EQUIVALENT *IN VITRO*

Bottom up strategy

In this study, we exploited a bottom-up approach to build a functional cardiac µTPs *in vitro*. Initially, we focused on generating µ-sized tissue modules with specific microarchitectural features that can be used alone as living fillers in damaged areas or serve as building blocks to engineer large biological tissues through a bottom-up approach. The process philosophy is schematized in figure 1. The optimized dynamic culture conditions used, coupled with the sub-millimetric nature of the µTP, allowed to overcome the transport limitations and enabled the massive production of viable and functional micrometric building blocks. Moreover, we devoted particular attention to the role of micro-scaffolds in addressing µ-tissue reorganization. Indeed, despite many works regarding scaffold free tissue regeneration [19,20,22], we demonstrated that micro-scaffolds are crucial in the early stages of the culture because they triggers the cell to synthesize precursors of ECM that act as a natural surrounding environment for cardiac cells. In this work, we observed that the scaffold does not hinder the electromechanical properties of the cardiac µ-modules, which showed spontaneous beating and synchronization properties. This suggests that the cardiac µ-TP as an implantable living micro-tissue for infracted zone regeneration or can be used as representative tissue modules for TOC applications.

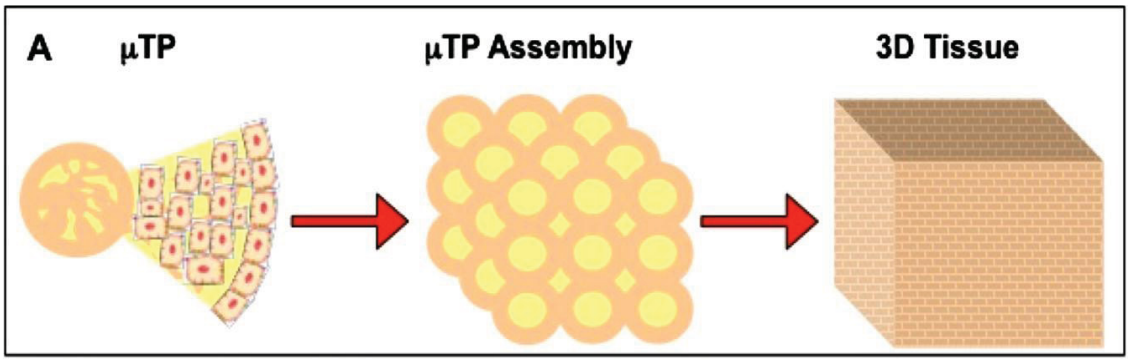

Figure 1: Scheme of the bottom-up process

3.1 Introduction

The heart is the first organ formed in the embryo and all subsequent activities in life depend on its functioning. The past decade has witnessed decisive advances in understanding cardiac functions and dysfunctions, both a genetical and molecular level. Although such insights into the mechanisms of heart development and disease have stimulated new therapeutic opportunities for the prevention and palliation of cardiac pathogenesis, mortality rates associated with heart related pathologies remain at the top of disease statistics in industrialized countries [10]. Since cardiac myocytes lose their ability to divide after birth, the regenerative capacity of adult heart tissue is limited and substantial cell loss or dysfunction, such as what occurs during myocardial infarction, is largely irreversible and may lead to progressive heart failure [11]. Recently, cell transplantation to repair or supplement impaired heart tissue has been pursued by various approaches as an alternative therapy for heart transplantation [12]. Several possible cell types, including myoblasts, cardiomyogenic cells derived from bone marrow stroma, Lin^- c-kit^{pos} bone marrow cells, multipotent endothelial cells, and embryonic stem cells, have been reported as human implantable cell sources [13,14]. Myocardial injection of autologous myoblasts has been clinically performed and shown to produce some limited recovery from heart dysfunction [15]. In these therapies using direct delivery of isolated cells, each cell differentiates and remodels in response to its surrounding environment, leading to tissue regeneration and functional repair. In contrast to isolated cells, research on

further advanced therapies to transplant tissue-engineered functional heart grafts has also now begun [16]. As originally proposed by Langer and Vacanti [17] the most popular in tissue engineering approach is based on the premise that preparations of cells, extracellular matrix (ECM), and growth factors together lead to tissue reconstruction, and that 3-dimensional (3-D) biodegradable scaffolds are useful as alternatives for ECM. The scaffold temporarily provides the biomechanical support for the cells until they produce their own extracellular matrix. Because tissue-engineering constructs contain living cells, they may have the potential for growth and cellular self-repair and remodelling. This context has also been applied to cardiac tissue engineering. A crucial factor in creating viable 3-dimensional tissue *in vitro* is the achievement of adequate perfusion throughout the tissue. This is especially true for cardiac tissue due to its high metabolic rate and oxygen demand. Radisic *et al.* [18] incorporated oxygen carriers and flow channels into neonatal rat cardiomyocyte seeded scaffolds to overcome the transport limitations. A 3-dimensional model was used by Zimmermann et *al.* [19], where a neonatal ventricles rat cell population was embedded into a Matrigel/collagen gel. Other 3-dimensional culture systems rely on cell secreted extracellular matrix (ECM) as the major matrix component [23]. Other techniques for the recreation of cardiac muscle *in vitro* are based on the creation of cardiac micro-muscle that can be used alone or serve a building block for the creation of macroscopic tissue. Kelm et *al.* [20, 21] created tissue spheroids containing CM in a hanging drop culture system. The scale of these micro-tissues (~100 μm) has been shown to support core tissue viability. Sefton et al. made cardiac micro-modules by cell laden technology where cardiac cells were embedded in biopolymer micro-modules. Cardiac cells were able to survive and to respond to external stimuli, but they could neither self-beat nor self-aggregate into superior scales. Finally cardiac muscle can also be fabricated by means of cell sheet technology. Shinizu et *al.* [22] demonstrated that sheets of cardiac cells can be harvested as an intact micrometric layer and stacked with other cardiac sheets to obtain a macroscopic tissue of about a hundred of microns. In the works discussed so far there is a lack of information regarding the possibility of recreating a cardiac surrogate *in vitro* ensuring that cardiomyocytes are embedded in their own ECM. Indeed, even if such micro-tissues possessed many of the functions of cardiac muscle at micron scale they were composed mainly by cell aggregates. We argue that the presence of an endogenous ECM plays a relevant role for the tissue

engineered constructs. Cell / ECM cross-talk is a key-factor in the native tissue and should be maintained *in vitro*. This leads to the creation of more representative tissue models improving the performance of both *in vitro*, such tissue on a chip system (TOC), and in *vivo* applications.

3.2 Materials and Methods

3.2.1 Micro-scaffold production

Preparation of gelatin porous micro-beads

Gelatin porous micro-beads (GPMs) have been prepared according to a modified double emulsion technique (O/W/O) [1]. Gelatin (type B Sigma Aldrich Chemical Company, Bloom 225, Mw=l 76654 Dalton) was dissolved into 10 ml of water containing TWEEN 85 (6% w/v) (Sigma Aldrich Chemical Company). The solution was kept at 60°C. Toluene containing SPAN 85 (3% w/v) (Sigma Aldrich Chemical Company) was continuously added to the aqueous gelatin solution (8 % w/v) to obtain primary oil in water emulsion. The added toluene formed droplets in the gelatin solution until saturation. Beads of gelatin containing droplets of toluene were produced through the addition of excess toluene (30 ml). The overload of toluene allowed the obtaining of a double emulsion (O/W/O). After cooling below 5°C, 20 ml of ethanol was added to extract toluene and stabilize GPMs. The resulting microspheres were filtered and washed with acetone and then dried at room temperature. Microspheres were separated selectively by using commercial sieves (Sieves IG/3-EXP, Retsch, Germany). GPMs with 75-150 μm size range were recovered and further processed. After sieving the number of micro-carriers per milligram was determined by counting micro-beads in cell culture dish (w/2 mm grid Nunc). Finally the micro-beads morphology has been examined by means of Scanning Electron Microscopy (SEM).

Crosslinking of GPM

Crosslinking of GPMs have been stabilized by means of chemical treatment with glyceraldehyde (GAL), in order to make them stable in aqueous environment at body temperature. In particular, GPMs were dispersed into acetone/water solution containing GAL and mixed at 4°C for 24 h. Then microspheres were filtered and washed with acetone and dried at room temperature. The percentage of the crosslinking agent (GAL) introduced during the stabilization step was 2% and 5% w/w (GAL / micro-beads).

Scanning electron microscopy (SEM)

SEM was performed to analyze the morphology of naked micro-beads. The former didn't need any dehydratation process so micro-beads were mounted onto metal stubs using double-sided adhesive tape and then gold-coated using a sputter coater at 15 mA for 20 min. Coated samples were then examined by scanning electron microscopy (FE-SEM, Ultraplus Zeiss).

3.2.2 Cardiomyocyte isolation

Hearts were removed aseptically from neonatal (2–3 days old) Wistar rats immediately after euthanasia with CO_2. The atria were removed and the ventricles placed in cold CBFHH buffer(NaCl 58.44g/mol, KCl 74.56g/mol, $MgSO_4x2H_2O$ 246.48 g/mol, KH_2PO_4 136.09 g/mol, $Na_2HPO_4x2H_2O$ 177.99 g/mol, Glucose dehydrated 180.16 g/mol, HEPES 238.3 g/mol, PH 7.4) containing antibiotics [streptomycin and penicillin (50 U/ml; GIBCO BRL, Carlsbad CA); ampicillin (100 mg/ml; Sigma, St. Louis, MO)] and antimycotic amphotericin B (5 _g/ml; Mediatech, Herndon, VA). The ventricles were rinsed in CBFHH three times to remove leftover blood and minced into pieces<1mm^3. Then the ventricles were dissociated into single cells by proteolytic enzymes during repeated digestions with gentle stirring.

Pre-digestion

- Add 8ml of Collagenase (180U/ml Collagenase type II Worthington co#CLS-2) solution to the tissue.

- Digest for 10 min on the shaker to 37°C.
- Let tissue sediment and discard supernatant.

Digestion cycles

- Add 8ml of Collagenase solution to the tissue.
- Digest for 10 min on the shaker to 37°C.
- Let tissue sediment and transfer supernatant to the first “tube collection” (The enzyme solution containing the digested tissue was neutralized with an equal volume of culture medium (DMEM, 1g/l Glucose), supplemented with 10% fetal bovine serum, 1% penicillin (100U/mL) and streptomycin (100 mg/mL).
- Add 7ml of DNase solution (4µg/ml DNase I, type V from bovine spleen, Sigma co#D8764) to the tissue.
- Pipette up and down for 20 to 25 times to ensure adequate mixing.
- Let sediment deposit again and transfer the supernatant to same “collection tube”.
- Start again by adding collagenase solution and follow the same cycle only changing in the time the amount of solution and the digestion time until the tissue has completely been dissolved.

The cell suspension in the collection tubes was sifted by the sieve (pore size 100µm) with 10-15 ml of culture medium and then centrifuged at 1200 rpm for 15 min at 4°C. Isolated cells were pre-plated onto tissue culture polystyrene to reduce the initial non-myocardial cells (NMC) contamination by exploiting the differential attachment time between myocardial and non-myocardial cells. By pre-plating the whole cell population for one hour it was obtained a cell population named cardiomyocite-rich (CM-R). By pre-plating for three step of one hour it was obtained a cell population named cardiomyocytes-extra rich (CM-ER). The population CM-R will be named along the paper CM-R(+) if used in combination with 5-bromo-2deoxyuridine (BrDu) or CM-R(-) it used in absence of BrDu.

3.2.3 Micro-tissue realization and characterization

Process overview

The realization of cardiac micro-tissues (c-μTPs) consists of a dynamic cell culture on gelatin porous microspheres. The whole process is divided in three phases: the seeding phase (SP, duration three days) and evolution phase (EP, duration nine days) allow cell attachment and micro-tissue maturation in the spinner flask bioreactor; the aggregation phase (AP, duration seven days) allows micro-tissue bio-fusion and building-up of the macroscopic tissue in a maturation chamber kept under dynamic culture conditions.

Seeding Phase (SP)

Dry GPM were sterilized before using by absolute ethanol sub-immersion for 24h. Successively several washings in calcium-free and magnesium-free phosphate-buffered saline (PBS) were performed to remove completely the ethanol. Before cell seeding PBS was removed and replaced with the culture medium. Cardiac-μTPs cultivation was initiated by inoculating CM-R and gelatin porous micro-spheres at a ratio of 1000 cells*bead^{-1} in a spinner flask bioreactor (Integra Bioscience, cod#182 023) filled with 150 ml of culture medium (DMEM, 1g/l Glucose supplemented with 10% foetal bovine serum, 1% penicillin 100U/mL and streptomycin 100 mg/mL) and inserted in an incubator with controlled atmosphere (37°C in humidified atmosphere containing 5% CO_2). To optimize the seeding parameters, the spinners were operated up to three days under two different conditions: a) 5 min stirring at 30 rpm and 30 min resting for six h followed by continuous stirring at 30 rpm up to three days; b) 5 min stirring at 30 rpm and 30 min resting up for three days.

MTT assay

Aliquots containing micro-tissues at S3 were collected from the spinner operating under conditions (a) and (b) and, transferred in a Petri dish, then 900μl of culture medium and 100μl of MTT (3-(4, 5_dimethyltiazol-2-yl)-2, 5-diphenyltetrazolium bromide - Sigma) solution were added. The MTT assay is a colorimetric assay for

assessing cell viability. Enzyme-based methods using MTT rely on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method was easy-to-use, safe, has a high reproducibility, and was widely used in both cell viability and cytotoxicity tests. Among the enzyme-based assays, the MTT assay was the best known method for determining mitochondrial dehydrogenase activities in the living cells. MTT was reduced to a purple formazan by NADH. After incubation for 45 min at 37°C the micro-tissues were observed and imaged under optical microscope (BX53; Olympus).

Evolution Phase (EP)

The realization of the micro-tissues was carried out by using CM-R (-), CM-R (+) and CM-ER cells in different experiments. For all spinner culture conditions the seeding phase was performed under intermittent stirring (5 min stirring at 30 rpm and 30 min resting up to three days – corresponding to SP condition (b), see seeding phase). After the SP phase, the dynamic conditions were switched from intermittent to continuous stirring at 30 rpm to allow µTP evolution (EP phase) for a total duration of nine days. In the experiments with CM-R(+), culture medium encircled with BrDu 0.2 mM was used from the third day till the ninth day. The culture medium was changed every two days. Aliquots of 5 ml of culture medium containing micro-tissues were collected for subsequent analyses at two time intervals: short time, ranging from 3 - 5 days (EP3 - EP5); long time, ranging from 6 – 9 days (EP6 - EP9).

Alamar Blue assay

After the seeding phase, three aliquots from spinner flasks containing CM-R (-) and three aliquots from CM-R (+) were collected into cell culture dish (w/2 mm grid Nunc) for µ-TP counting and after was transferred in multiwell low attachment (Corning Costar Ultra-Low Attachment Multiwell Plates 24 well). The multiwell containing µ-TPs was kept in incubator under continuous stirring at 30 rpm to recapitulate the spinner flask conditions. At time point corresponding to EP1, EP3, EP5, EP7, EP9, EP11, the µ-TPs were incubated for 4 h at 37°C in humidified atmosphere containing 5% CO_2 with Alamar Blue (Invitrogen) and the number of cells as indicated by the

was evaluated by spectrophotometric reading as indicated by the manufacturer . It was thus possible to have an index of the number of cells per micro-beads during the culture time. Alamar Blue is a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the resorufin. The active ingredient of Alamar Blue (resazurin) was a nontoxic, cell permeable compound that was blue in colour. Upon entering cells, resazurin was reduced to resorufin. Viable cells continuously converted resazurin to resorufin, thereby generating a quantitative measure of viability cellular. After incubation with Alamar Blue, the samples can readily be measured on spectrophotometer (on a UV-Vis at 570 nm and 595nm). Finally, results were analyzed by plotting absorbance intensity *vs* compound concentration.

3.3.4 Cell viability and morphology on the micro-beads

Histology

Over culture time (EP1 to EP9) 1 ml of µTP suspension was collected from the spinner culture every two days and fixed in a solution of 10% neutral buffered formaline for 24 h, dehydrated in an incremental series of alcohol (75%, 85%, 95% and 100%, and 100% again, each step 20 min at room temperature) treated with xylene and then embedded in paraffin. Successively, the samples were sectioned at a thickness of 7µm, and stained using hematoxylin-eosin (Bio Optica) solutions and Picro Sirius Red (PSR) (Sigma Aldrich) following standard procedure, finally the sections were mounted with Histomount Mounting Solution (invitrogen) on coverslips and the morphological features of constructs were observed with a light microscope (Olympus, BX53). Moreover, histological sections stained with PSR were further observed by using polarized microscope. Polarized light images of samples stained with PSR alone were acquired with an inverted microscope (BX53; Olympus) with a digital camera (Olympus DP 21). A linear polarizer was placed between the light source and the specimen, while the analyzer was installed in the light path between the specimen and the camera. It is known that the colour of collagen fibers stained with PSR and viewed with polarized light depends upon fiber thickness; as fiber thickness increases, the colour changes from green to r

ECM composition

To quantitatively determine the proportion of different colored collagen fibers, we resolved each image into its hue, saturation and value (HSV) components by applying the software's "color threshold" function. Only the hue component was retained and a histogram of hue frequency was obtained from the resolved 8-bit hue images, which contain 256 colors. We used the following hue definitions; red 0-51, green 52-120 [4,5]. The number of pixels within each hue range was determined and expressed as a percentage of the total number of collagen pixels, which in turn was expressed as a percentage of the total number of pixels in the image. The analysis was performed on 10 Picro Sirius Red stained sections of each time points and different region of interests (ROIs) were examined for each section. Since the aim of imaging analysis was to investigate the evolution of endogenous ECM composition, it was necessary to exclude by ROI the areas occupied by the micro-scaffold when it was still present. This condition was verified only for the short time μTP.

Immunofluorescence and Multiphoton anlaysys

Over culture time (EP1 to EP9) 1 ml of μTP suspension was collected from the spinner culture every two days in order to be imaged by Confocal Leica TCS SP5 II combined with a Multiphoton Microscope where the NIR femtosecond laser beam was derived from a tunable compact mode-locked titanium:sapphire laser (Chamaleon Compact OPO-Vis ,Coherent). μ-TPs were fixed with 4% paraformaldehyde for 20 min at room temperature, rinsed twice with PBS buffer, and incubated with PBS-BSA 0.5% to block unspecific binding. After fixation, the samples were stained with phalloidin tetramethylrhodamine B isothiocyanate (phalloidin, Sigma-Aldrich) and SYTOX Green (Invitrogen), for actin microfilaments and nucleus detections, respectively. In particular the samples were incubated with SYTOX Green stock solution (10 mg/mL in dimethyl sulfoxide) diluted in PBS (1/500 v/v) for 10 min at 37°C, and after rinsing in PBS, they were stained with phalloidin for 30 min at room temperature. Moreover two-photon excited fluorescence has been used to induce second harmonic generation (SHG) and obtain high-resolution images of unstained collagen structures in μ-TPs' ECM. The samples were observed under the simultaneous excitation of the three different lasers: λex =488 nm, λem = 500-530

nm for cell nuclei; λex = 543 nm, λem=560-650nm for cell cytoskeleton) and λex = 840nm (two photons), λem =415-425 under for unstained collagen detection generated by Second Harmonic Generation (SHG). Other samples where stained to detect specific cardiac markers (α- actinin sarcomeric and Cx43 gap junction). The immunofluorescences were performed on sample slices 5-8 μm thick obtained by cryomicrotome (Leica CM 1850). Anti-α actinin sarcomeric monoclonal antibody was used at dilution 1:800 (Sigma); Anti-Cx43 polyclonal antibody was used at dilution 1:2000 (Sigma). The secondary antibodies for α-actinin sarcomeric and Cx43 were: Alexa Fluor 488 mouse anti-mouse IgG (H+L) (dilution 1:500; Life technologies) , Alexa Fluor 546 mouse anti-rabbit IgG (H+L) (dilution 1:500; life technologies). Finally, the sample slices were imaged under Confocal Leica TCS SP5 with: λex =488 nm, λem = 500-530 nm for α-actinin sarcomeric; λex = 543 nm, λem=560-650nm for Cx43.

Ultrastructural analysys (TEM)

TEM was performed to observe cells and the typical functional structure of cardiac tissue. μ-TPs were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in sodium cacodylate buffer 0.1 M (pH 7.2), washed with sodium cacodylate buffer and then fixed with 1% aqueous osmium tetroxide (Electron microscopy sciences, USA). Afterwards, the samples were dehydrated in a graded series of ethanol (Sigma-Aldrich), block contrasted with 1% uranyl acetate (Merck, Germany) and embedded in EMbed 812 (Electron microscopy sciences, USA). The prepared ultra-thin sections (65 nm) were contrasted with 0.3% lead citrate (Merck, Germany) and imaged with a Philips CM12 transmission electron microscope at an accelerating voltage of 80 kV.

Self assembly: spontaneous aggregation of μ-tissues

At time point EP3, a small amount of micro-tissues were collected from the spinner and placed in a round bottom multiwell low attachment (Corning Costar Ultra-Low Attachment Multiwell Plates 96 well) in order to promote their fusion. Images from the multiwell were taken every other day up to 11 days. From the images was evaluated the total area of the micro-tissues and reports as ration of the area between total

area at EP (t) and EP3 (where t varied from t = 1, to t = 11 days).

μ-tissue self beating measurements

The investigation of spontaneously beating of μ-TPs was carried out by placing a small amount of micro-tissue collected from the spinner in a Petri low attachment placed under an inverted microscope (OLYMPUS - X81), coupled with a CMOS-camera (Hamamatsu Photonics – C11440-22CU). The beating micro-tissues were imaged at 30 fps. The movies were inspected both visually and by image processing tool. For visual inspection, the number of beating was counted and divided by the movie duration time (approximately 35 sec) and converted in beat per minutes. The image analysis was performed by measuring the change of micro-tissue morphology along the time. To obtain the change of morphology as function of time the image sequences were analyzed with Stack Differences plug in Image J (NIH, version 1.43m). The peaks of the curves obtained corresponded to a single beat.

Self assembly and synchronization: Membrane depolarization measurements

For analysis of electrical communication we used a membrane voltage sensitive, di-4-ANEPPS (molecular probes), to monitor the action potential propagation and the electrical connection between the μ-TPs. Di-4-ANEPPS stock solution (2mM) was freshly prepared with DMSO (sigma) solution and added to the culture medium to give a final concentration of 10μM of D-4-ANEPPS. The samples were exposed to the dye at 37°C for 30 min, the samples were then washed in Tyroide's solution consisting (in mM) 140 NaCl, 4 Cl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 55 D-glucose(PH 7.4). We recorded the samples by Confocal Leica TCS SP5 II and the signals were monitored through a high resolution by Leica TCS SP5 Confocal microscope (70ms/frame, pixel size 713.7nm, objective HCX IRAPO L 25X/0.95 water immersion, resonant scanner 8000Hz, pinhole 600μm). Fluorescence ratio imaging measurements are typically performed by recording fluorescence intensities excited at about 450 and 510 nm, detecting emission at >570 nm. The ratio of these intensities (F450/F510) decreases upon membrane hyperpolarization.

External stimuli responsiveness

μ-TP were equilibrated in 1x Tyrode's solution at 37°C for 1 hour prior to measurement. Electrical responses of μ-TPs were assessed using custom made testing chambers. μ-TP were paced between a pair of graphite electrodes kept 1 cm apart by a silicon bracket glued to a glass slide. Using this device made in laboratory we collected the μ-TP among the electrodes were placed with Tyrode's solution, which was enough to partially submerge the electrodes. The small chamber volume ensured that most of the μ-TP was located between the electrodes. A signal generator (Thurlby Thandar Instruments, TGA 1230) connected to the electrodes provided the desired electrical stimulation (Pulse features: Ampl. : 4.5V/cm, Freq. : 1Hz / 0.5Hz). Contractions and response to external stimuli of μ-modules were recorded with a CMOS-camera (HAMAMATSU PHOTONICS – C11440-22CU) on an Inverted microscope (OLYMPUS - X81) with an acquisition of 30 fps. The image sequences were analyzed using ImageJ (NIH, version 1.43m). The contractility of μ-TP was defined as the fractional en face area change during one contraction cycle using frame-by-frame video analysis in ImageJ.

3.3 Results and discussion

3.3.1 Micro-scaffold production and characterization

The morphology of GPMs is shown in figure 2 (A, B). The modified double emulsion technique allows the fabrication of micro particles having good sphericity, high superficial and bulk porosity with a high degree of interconnection. The average pore size was about 20 μm and the microspheres diameters distribution falls in the range of 75-150 μm. As described above, the GPMs were prepared in two formulations by varying their crosslink degree, 2% and 5% respectively. We showed [2] the higher the crosslink extent, the higher the degradation time. Lastly, the number of dry micro-carries per milligram was evaluated to be 5000 beads per milligram by video-microscopy analysis. This was done in order to control the cell/microspheres ratio at the inoculums step of the seeding phase, as described below.

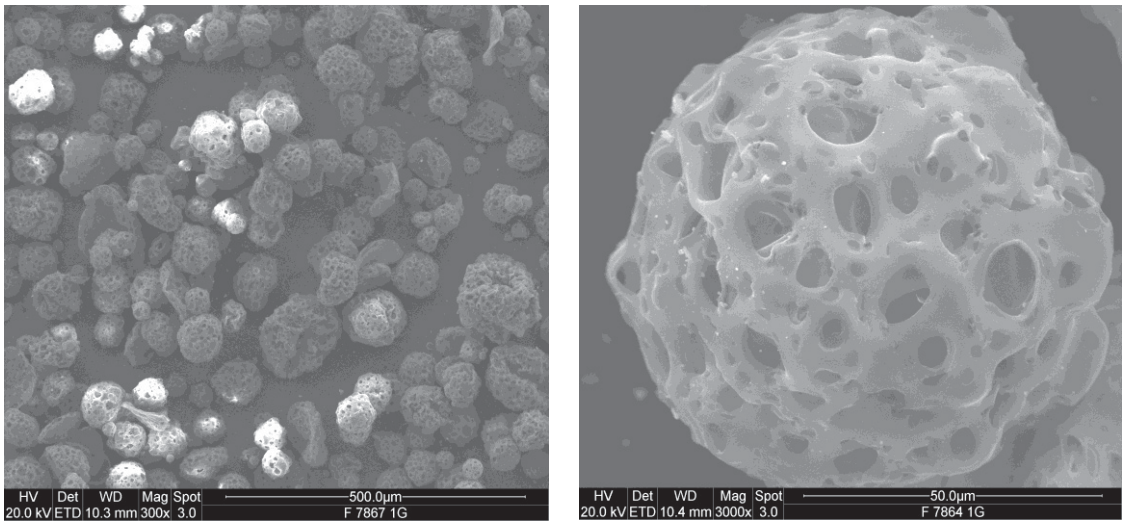

Figure 2: GPM morphology by SEM micrographs.

3.3.2 Cardiac-µTP evolution and characterization

Cell adhesion and proliferation: viability and morphology of Cardiac-µTP

As explained in chapter 2, the cardiac function is determined by the coordinated and dynamic interaction of several cell types together with components of the extracellular matrix (ECM). This interaction is regulated by mechanical, chemical, and electrical signals between the cellular and non-cellular components of the heart. Recent studies using fluorescence-activated cell sorting indicate that the number of myocytes remains relatively constant during development and disease, whereas the number of fibroblasts and other cell types can change dramatically. Cardiac fibroblasts appear to have different origins at different stages of development and fluctuate in response to a variety of physiological signals. Fibroblasts form a network of cells that are connected to each other via specific cadherins and connexins, to the ECM via integrins, and to myocytes by a variety of receptors, including connexins Quantitative changes in mechanical, chemical, and electrical signals can alter the overall cardiac form and function [4]. In the heart, the cellular components primarily consist of myocytes, fibroblasts, and the vascular system. In addition, transient cells, such as mast cells, macrophages, and lymphocytes, can be found under certain conditions, such as in response to pathophysiological stimuli (Fig. 3). Finally, in

cultures with more NMCs, the cardiomyocytes may have slower beating rates, less negative membrane potential, and slower action potential upstroke velocities [24,25].

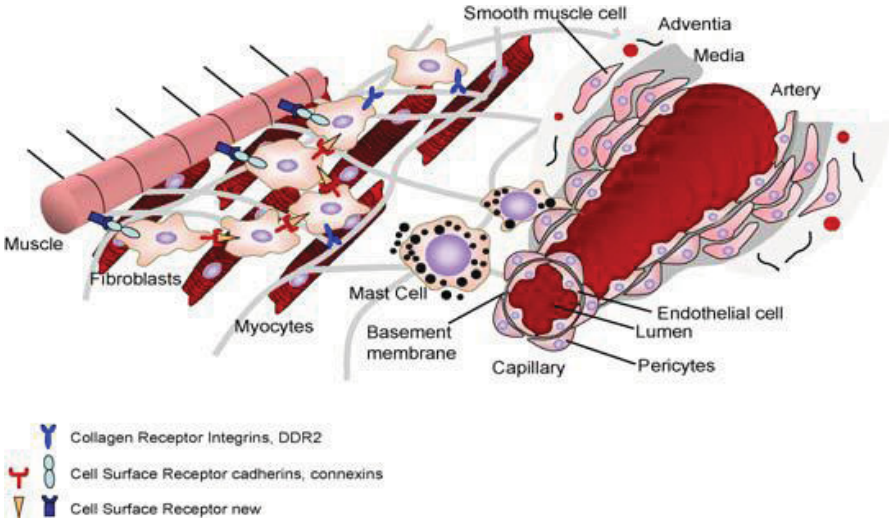

FIGURE 3: Model of interactions between cells and extracellular matrix in the heart.

Seeding Phase (SP)

Seeding phase parameters were optimized by inoculating CM-R and porous gelatine micro-spheres at the ratio of 1000 cells*bead^{-1} in a spinner flask. Cell adhesion and distribution on gelatine micro-spheres, was strongly related to dynamic culture parameters. The MMT images in figure 4, show that cell cell-to-bead distribution and cell seeding efficiency, were improved by using the dynamic condition “b” (5 min stirring at 30 rpm and 30 min resting up to three days) compared with dynamic condition “a” (5 min stirring at 30 rpm and 30 min resting for six h followed by continuous stirring at 30 rpm up to three days).

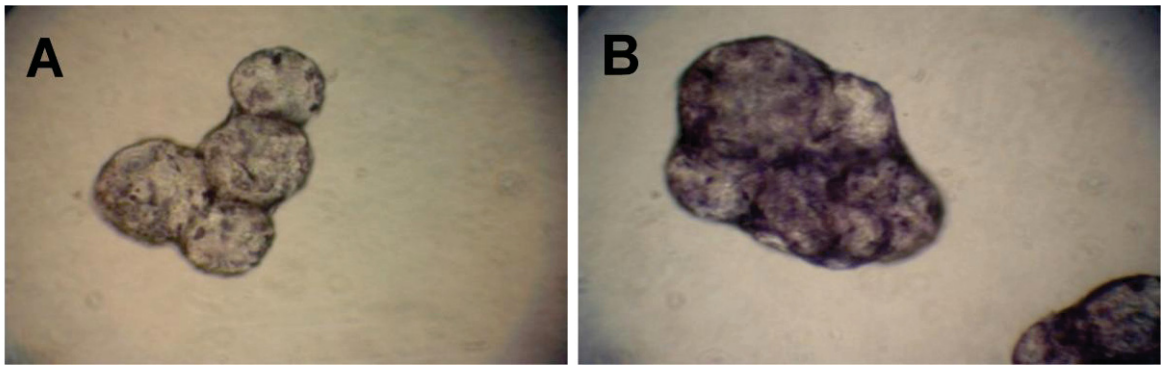

FIGURE 4: MTT on µ-TP after 3 days of culture, 4A: condition "a". 4B: condition "b" showed the increased cell seeding efficiency.

Evolution Phase (EP)

The evolution phase was carried out by using three different formulations with cardiac cells: CM-R (+), CM-R (-) and CM-ER. In figure 5 the results of Alamar blue assay are shown and a comparison between CM-R (+) and CM-R (-) was made in terms of the number of cells per micro-beads during culture time. For both micro-muscle types the cell number increased with culture time. During the first 3 days of culture the cell number was quite similar (600 cell / beads, figure 5), but, as culture time increased, the micro-tissue created with CM-R (-) showed an hyper proliferation of cells compared with C-MR (+). This was due to the effect of BrDu addition that hinders fibroblast proliferation. The cell population named CM-ER, did not proliferate over culture time due to the high cardiomyocytes content (data not shown) according to literature data [26,27].

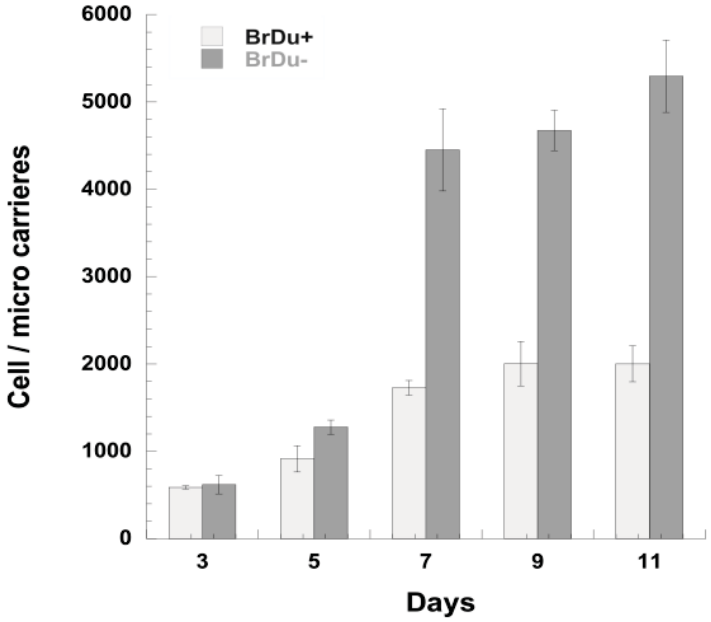

Figure 5: Alamar blue assay showed that During the first 3 days of culture the cell number was quite similar (600 cell / beads, figure 5), but, as culture time increased, the micro-tissue created with CM-R (-) showed an hyper proliferation of cells compared with C-MR(+). This was due to the effect of BrDu addition that hinders fibroblast proliferation.

Histology

Histological analysis in Figure 6 shown the morphological difference of the micro-tissues realized with the three kind of cell population at time point EP3 and EP9. CM-ERs were no not able to colonize the micro-scaffold and they shown to grow and to aggregate around the scaffold (figure 6 A). As the culture time increased the cells was able to form spherical clusters resembling a cardiac spheroids made-up mainly by cell aggregate (figure 6B). CM-R (-)s shown a good integration with micro-scaffold just after the seeding phase. Indeed, figure 6C (time point EP3) shows that cells are embedded within the micro scaffold and a small amount of extracellular matrix has been produced. At the time EP9 the scaffold was completely disappeared and the micro-muscle is mainly composed by extracellular matrix. Moreover the centre of micro-muscle was observed to be quite necrotic (figure 6D). By using CM-R (+) cells it was observed a similar behaviour as CM-R (-) at the time point EP3 (figure 6E). Interestingly, at time point EP9 the micro-muscle shown a better morphology in terms of cell distribution and ECM synthesis and no necrotic core was observed (Figure 6F).

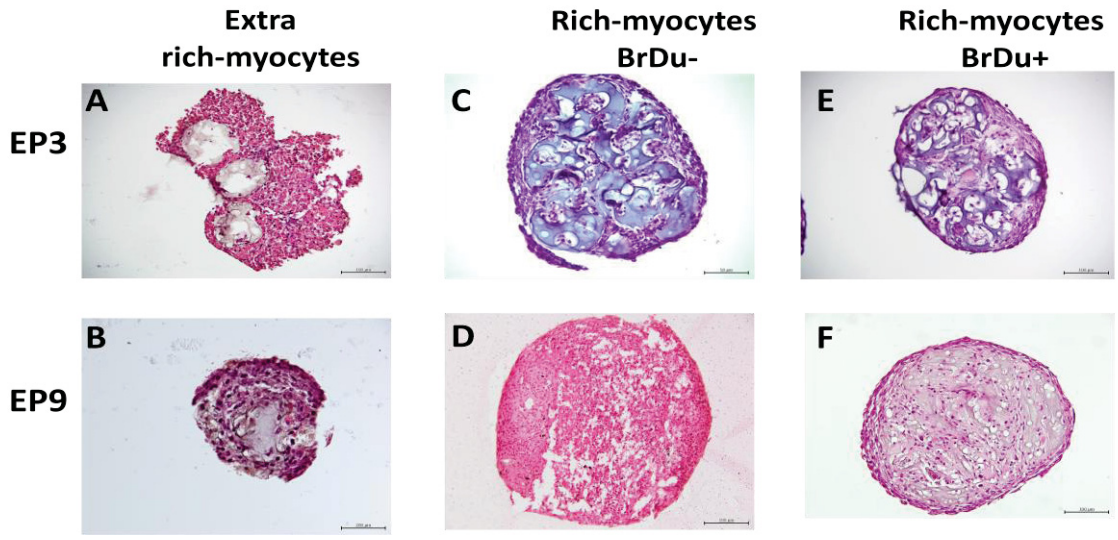

Figure 6: Hematoxylin and eosin staining images of cardiac µ-TP. Images showed the time evolution of µTP in terms of cells number increase, the ability to colonize the pores of the GMP and cell-seeded GPM aggregate formation. Scale bar is 100µm.

In summary, we showed that morphological and functional cardiac µ-TP characteristics are different, depending on the presence or absence of proliferating NMCs. As described above, the number of cardiac fibroblasts appears to be crucial in the evolution and function of the heart tissue. Whether similar cell interactions occur *in vivo*, for example pathological states were frequently associated with myocardial remodelling involving fibrosis. This was observed in ischemic and rheumatic heart disease, inflammation, hypertrophy, and infarction. The growth in fibrous tissue content was based on the maintained proliferative potential of fibroblasts, and the synthesis of extra-cellular matrix (ECM) proteins, predominantly by fibroblasts [6,7].

Immunofluorescence and Multiphoton analysis

For the following investigation only data concerning to CM-R (+)-µTPs are presented because it was considered the gold standard formulation. Spatial distribution of cells and ECM within the CM-R(+)-µTPs has been analyzed by means of multichannel fluorescence and multiphoton imaging (figure 7 A,C), as well as polarized microscopy images from PRS stained µTP sections (figure 7 B,D). The images refer to “short

time" (EP3-EP5) and "long time" (EP6-EP9) of spinner culture. The multichannel images highlight cell's nuclei in green, cell's cytoskeleton in red, unstained collagen in gray. In the polarized images the de-novo synthesized collagen is shown in a colour scale ranging from green to red. The images showed the time evolution of μTPs in terms of number of cells, collagen deposition, μTPs aggregation and degradation of micro-scaffold. At short time of culture (figure 7A) the cells are mainly present on the surfaces of the GPM and colonize a small fraction of the inner pores; no signal of mature collagen was detected by SHG. At "long time" (Fig.7C) the μTPs morphology changed, the GPM porosity appeared collapsed and it was impossible to distinguish individual GPM. The number of cells increased with culture time and some neo-ECM deposition was observed (gray).

As explained in section 2.5 *(Properties and Functions of Cardiac Extracellular Matrix)*, the role of extracellular matrix proteins in the heart during development and in several pathologic situations has received considerable attention in recent years. Recently developed biochemical and morphologic methodologies have revealed that extracellular matrix components are important in the organization of cellular structures as well as in the developing heart, and were partially responsible for contractile function and dysfunction in healthy and diseased hearts, respectively. Furthermore, in figure 7 B, D the PRS staining allowed monitoring collagen maturation and evolution with time. According to histology and SHG images, from short time culture to long time culture there is an increase in collagen deposition (red and green pixel) but it is interestingly to highlight that the proportion of green fibers decrease while the proportion of red fibers increases (Figure 7 E,F).

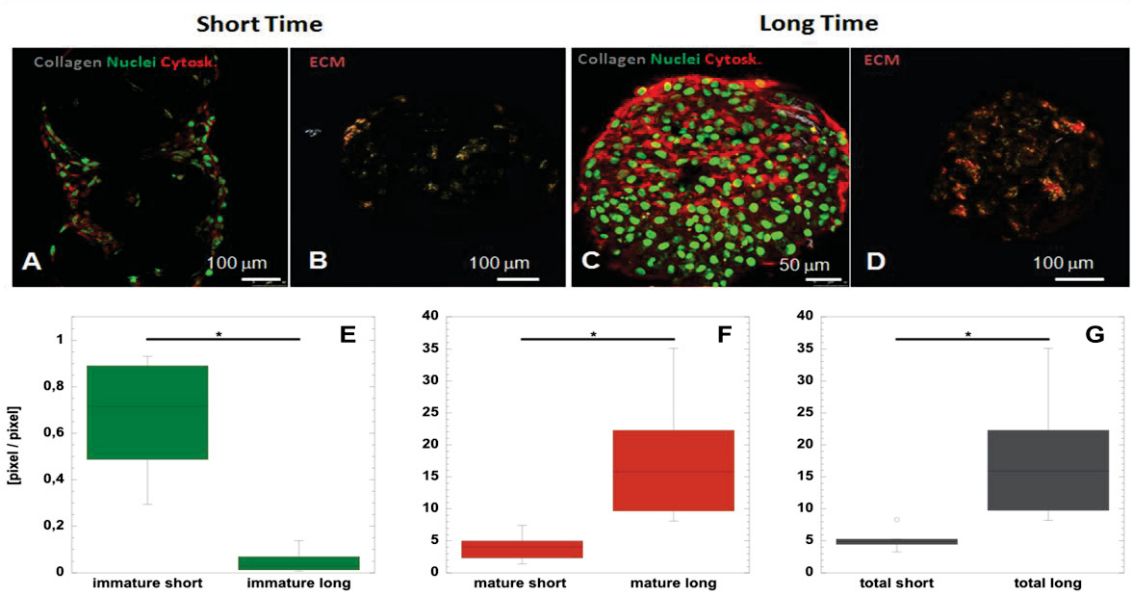

Figure 7: time evolution of morphology and ECM composition

Ultrastructural analysis (TEM)

TEM micrographs demonstrated that from the fourth day onwards the cardiac-µTPs showed development of typical functional structures of cardiac tissue (Figure 8). Ultrastructural hallmarks of cardiomyocyte were the development of T tubules with sarcoplasmic reticulum, specialized cell-cell junctions as an intercalanted disk and desmosomes. Moreover in figure 8D the cell well stretched on micro-scaffold was observed and in Figure 8E the transmission electron micrographs of the constructs demonstrated the presence of well-organized myofilaments with equally spaced Z lines.

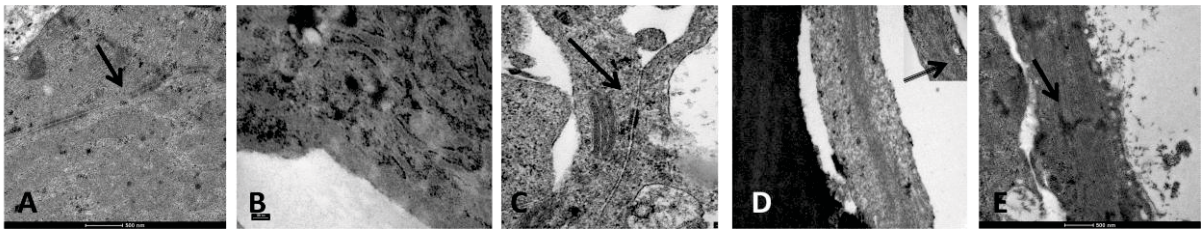

Figure 8: TEM micrograph of µTP showed development of typical functional structures of cardiac tissue after 4 days of culture. A) Intercalanted disk, arrow B) Glycogen, C) Desmosomes , arrow D) the cell stretch on micro-beads and tuble t with the sarcoplasmic reticulum (SR), arrow E) Well-organized myofilaments with clearly defined Z-lines (arrow).

Self assembly: spontaneous aggregation of μ-tissues

Cardiac μ-tissue precursors obtained by previous steps were collected in a low attachment multiwell and the ability of biological fusion was studied by image analysis. In the graph (Fig. 9) it was possible to observe a reduction in μm^2 in the time of the micro-tissues.

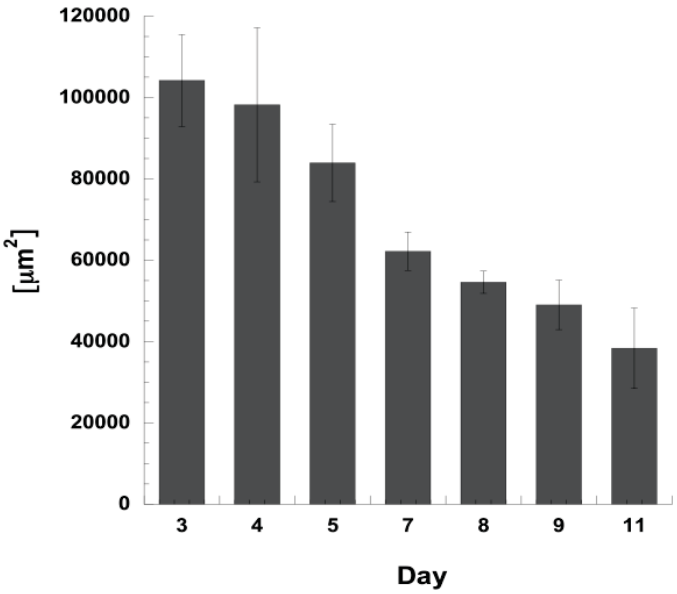

Figure 9: Reduction in μm2 in the time of the micro-tissues.

Time evolution of functional properties

At first day of evolution phase (EP1) spontaneous beating was recorded. The beating rate, evaluated by ImageJ (figure 10 A,B) analysis, revealed a decrease in the beating activity of μTPs from short time (20 b.p.m) to long time (12 b.p.m) as shown in figure 10 C. This observation was confirmed by further analysis: the expression of Cx-43 [5]. In fact the beating properties are believed to be related to GJ communications and finally linked to expression of Cx-43. In figure 10 D,E immunofluorescence images showing the distribution of α-actinin sarcomeric (green) and Cx-43 (red) during culture time are reported. Cardiomyocytes were tightly

interconnected with gap junctions and pulsate simultaneously in native heart tissue [9]. It is also well-known that confluent cultured cardiomyocytes on culture surfaces connect via gap junctions and beat simultaneously. Therefore whether electrical and morphological communications were established between μTPs was a crucial point. In fact, we examined by immunofluorescent analysis whether GJ communication was established between the μTPs during a “short” and “long time”. Figure 10 shows the monoclonal antibody against Cx43-specific peptides revealed distinct punctuate staining at myocyte cell-cell contacts. The number and size of Cx43-positive spots decreased with the culture period, at "short time" abundant Cx43 was detected not only at cell-to-cell interfaces, but also on the free cell membrane, while at "long time" there were a small number of Cx43-positive spots at the zones of contact between myocytes and the size of each spot was also small. Finally, by means of image analysis it was possible to extrapolate the time evolution of the Cx-43 density expressed as pixel / mm^2, shown in figure 10, indicating a decrease of junction density in the tissue with culture time.

These results demonstrated that the cardiac micro-tissues during the “short time” preserve intact Cx43 on their surfaces. These results suggest that, in the time that coincides with “short time”, the capability of self aggregation with a rapid and intact GJ formation in addition to deposited the right amount ECM, provide a functional communication between μTPs, creating a cardiac micro modules equipped with self-beating and synchronization. Instead, the “long time” micro-modules could be considered pathological μTP; decrease in the beating and cx-43 is probably due to the increase in the cardiac fibroblasts and the factors they produce.

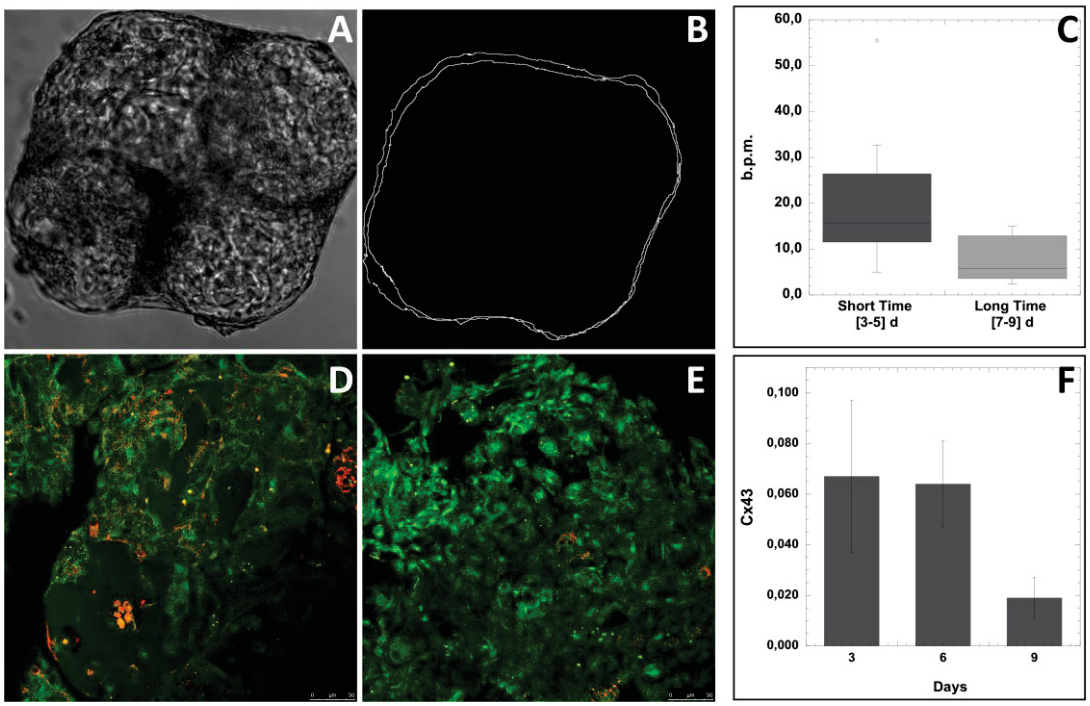

Figure 10: Expression of Cx43 between µTPs during "short" and "long time". The constructs were fixed and the frozen sections were obtained. The frozen sections stained with anti-Cx43 antibody (red) and α-actinin sarcomeric (green) revealed that Cx43 was abundant during the "short time" not only at cell-to-cell interfaces, but also on the free cell membrane, while in the "long time" the quantity of cx-43 decreases dramatically. The graph showed the morphometrical analysis of Cx43-positive spots during "short" and "long time".

Self-Assembly and synchronization: Membrane depolarization measurements

The conduction and propagation of action potential within and between the µTPs was critical for successful cardiac tissue engineering. Biological communication between the µTPs, was proved by use of voltage sensitive probes in order to evaluate time evolution of fluorescence directly linked to the membrane depolarization. The depolarization rate in localized areas in the µTPs was compared with their global beating rate marked as "a", "b", "c" and "global", respectively (figure 11 A,B). By reporting the variation of the fluorescence intensity with the time (figure 11 C-F), it is possible to see that there is signal synchronization within and between the µTPs. Indeed, the beating frequency evaluated in all points was similar (30 b.p.m.) and the values are very close those one evaluated by means of bright field imaging.

Of particular importance was the ability to assess local differences in contractility and whether action potentials propagated throughout the thickness of the construct or merely on the surface. This data demonstrates the ability of cells to migrate into the

porous micro-scaffold, not remaining on the surface, and the skill to communicate with each other generating the synchronizing of the beat. It was found that three days were necessary to establish a sufficient electrical connection between the μTPs without any conduction disturbance. Thus, we thought that these analyses would provide important basic data for the transplantation of myocardial μTPs in future studies.

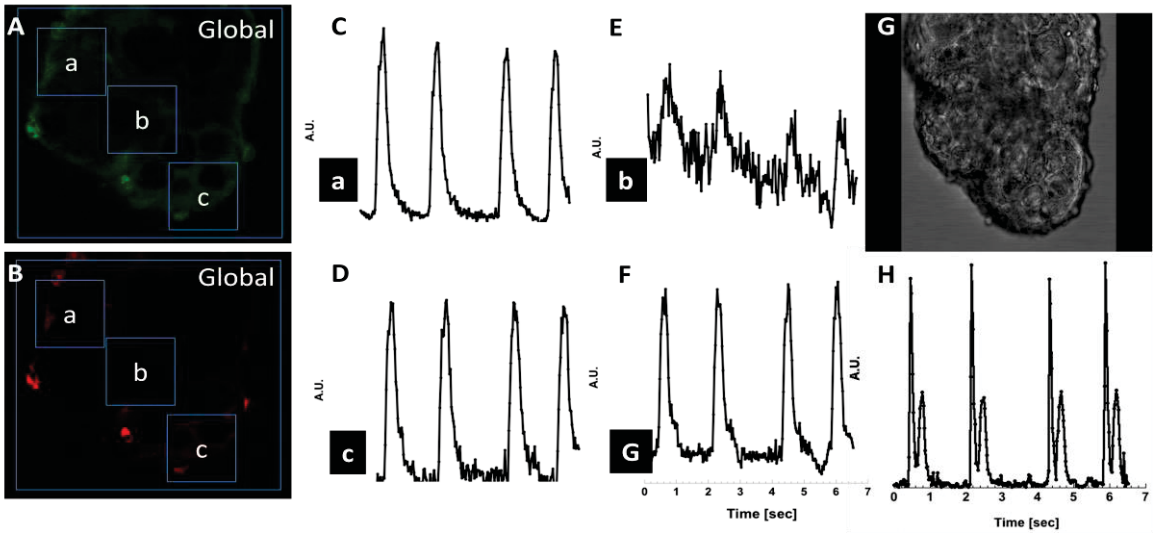

Figure 11: Image analysis performed on the same samples, in bright field and with voltage sensitive probes, demonstrated that there is synchronized spontaneous beating. We compared the beating of three different zones in the micro-tissue: a, b, c with a beating of the global micro-tissue.

Electrical response assessment

A certain amount of the μTPs contracted spontaneously in the absence of external electrical stimulation. Other than such active properties, the passive beating was investigated by placing non-beating μTPs under pulsating electrical stimulations by means of the device described above and showed in figure 12 A. Two train pulse signals having different frequency were imposed: 1Hz and 0.5Hz respectively. By recording the shape change by bright field images of the μTPs, it was possible to establish that μTPs contract with the same frequency (figure 12 B,C).

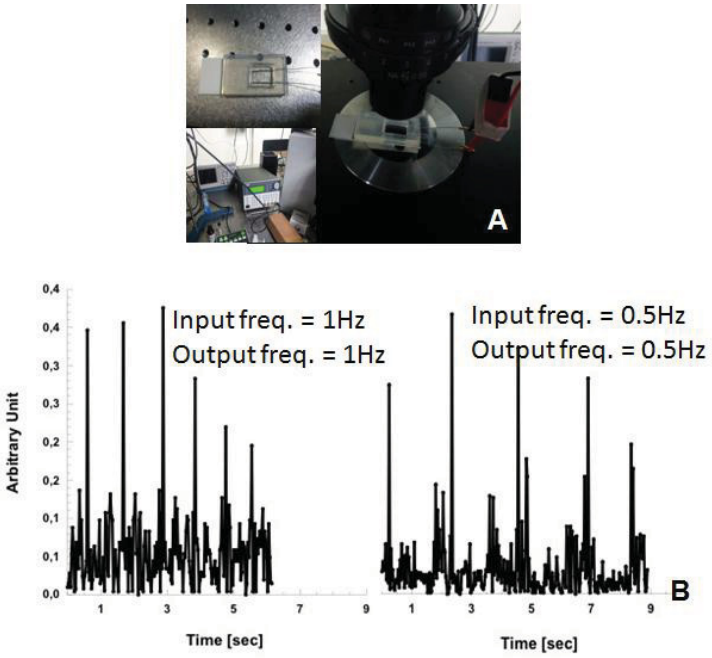

Figure 12: A) Device made in lab to stimulate the micro-tissue. B) The graphs show the response at the different frequency stimuli.

3.4 Conclusions

The *in vitro* fabrication of dense native heart-like tissue holds great promise not only for myocardial infarction treatment but also as an *in vitro* experimental model for the study. Due to limited cell migration in 3D preformed scaffold and restriction of nutrient transportation, traditional top down scaffolds-based approach had not proven to be suitable for the fabrication of dense engineered heart tissues, unless external stimulation and bioreactor are used. Other techniques, such as scaffold free tissue engineering or cell laden microgel, even if lead to the realization of functional cardiac muscles does not allow the development of a cardiac ECM and are formed mainly by cell aggregates that because of correctly arranged give rise to macroscopic functions such self-beating.

Here we focused on the possibility to build-up a functional cardiac micro-muscle where the ECM growth and assembly is taken in to account. It has been used the gelatine because cell are able attaches and migrates, and is thus suitable for cultivating the adherent cells. Moreover it has been demonstrated that the interaction

between pore surface and fibroblasts lead to a deposition of endogenous ECM in the pore space [5,28]. This study demonstrates the feasibility of forming dense CTE grafts *in vitro* by means of porous gelatine composite micro-beads, exploiting to main advantages:

- The small size of the micro-beads facilitates rapid transport of nutrients by diffusion to cells at any location in the beads. Indeed using the correct cell composition the micro-tissues are viable up to nine days of culture.
- Characterizing sub-millimeter sized micro-tissues containing cardiomyocytes and cardiac fibroblasts cells we found that fibroblast interacting with gelatine micro-scaffold was able to lid down ECM entrapping cardiomyoocytes, generating a tissue equivalent formed by cell embedded in their own ECM.

It has been demonstrated that to do this, cellular composition and choice should be optimized. We demonstrated that cardiac micro-tissues containing intact GJ precursors in addition to deposited ECM allow for rapid and complete electrical communications. Finally, characterizing the individual μTP and we found that there are able to self assemble and by exploiting such properties we realized a 3D cardiac patch as described in the next chapter.

Reference

[1] Nilsson K, Buzsaky F, Mosbach K. Growth of anchorage-dependent cells on macroporous microcarriers. Nature Biotech. 1986;4:989-990

[2] Rich L, Whittaker P. Collagen and picrosirius red staining: a polarized light assessment of fibrillar hue and spatial distribution. Braz. J. morphol. Sci. 2005;22:97-104.

[3] Nadkarni SK, Mark CP, Park BH, De Boer JF, Whittaker P, Bouma, BE et al.Measurement of Collagen and Smooth Muscle Cell Content in Atherosclerotic Plaques Using Polarization-Sensitive Optical Coherence Tomography. Journal of the American College of Cardiology 2007;49: 1475-1481.

[4] INDRONEAL BANERJEE, KRISHNA YEKKALA, THOMAS K. BORG,AND TROY A. BAUDINO. Dynamic Interactions between Myocytes, Fibroblasts, and Extracellular Matrix Ann. N.Y. Acad. Sci. 1080: 76–84 (2006). C _ 2006 New York Academy of Sciences. doi: 10.1196/annals.1380.007

[5] Palmiero C, Imparato G, Urciuolo F, Netti P. Engineered dermal equivalent tissue in vitro by assembly of microtissue precursors. Acta Biomater. 2010 Jul;6(7):2548-53. 20.

[6] MacKenna D, Summerour SR, Villarreal FJ. Role of mechanical factors in modulating cardiac fibroblast function and extracellular matrix synthesis. Cardiovasc Res 2000;46:257– 63.

[7] Patrizia Camellitia, Thomas K. Borgb, Peter Kohl Structural and functional characterisation of cardiac fibroblasts. Cardiovascular Research 65 (2005) 40– 51

[8] Yuji Haraguchi1, Tatsuya Shimizu1, Masayuki Yamato, Akihiko Kikuchi2, Teruo Okano. Electrical coupling of cardiomyocyte sheets occurs rapidly via functional gap junction formation. Biomaterials 27 (2006) 4765–4774.

[9] Oyamada M, Kimura H, Oyamada Y, Miyamoto A, Ohshika H, Mori M. The expression, phosphorylation, and localization of connexin 43 and gap-junctional intercellular communication during the establishment of a synchronized contraction of cultured neonatal rat cardiac myocytes. Exp Cell Res 1994;212:351–8.

[10] Olson, E. N. (2004). "A decade of discoveries in cardiac biology." Nat Med **10**, 467-474.

[11] Pasumarthi, K. B. and L. J. Field (2002). "Cardiomyocyte cell cycle regulation." Circ Res **90**, 1044-1054.

[12] Reinlib L, Field L. Cell transplantation as future therapy for cardiovascular disease? A workshop of the National Heart, Lung, and Blood Institute. *Circulation*. 2000;101:e182–e187.

[13] Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. *J Clin Invest*. 1996;98:2512–2523.

[14] Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest*. 2001;108:407–414.

[15] Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP. Myoblast transplantation for heart failure. *Lancet*. 2001;357:279–280.

[16] Fuchs JR, Nasseri BA, Vacanti JP. Tissue engineering: a 21st century solution to surgical reconstruction. *Ann Thorac Surg*. 2001;72:577–591.

[17] Langer R, Vacanti JP. Tissue engineering. *Science*. 1993;260:920–926.

[18] M. Radisic, H. Park, F. Chen, J. E. Salazar-Lazzaro, Y. Wang, R. Dennis, R. Langer, L. E. Freed, and G. Vunjak-Novakovic, Biomimetic approach to cardiac tissue engineering: oxygen carriers and channeled scaffolds. *Tissue Eng* **12**, 2077-2091 (2006).

[19] W. H. Zimmermann, C. Fink, D. Kralisch, U. Remmers, J. Weil, and T. Eschenhagen, Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. *Biotechnol. Bioeng.* **68**, 106-114 (2000).

[20] J. M. Kelm, E. Ehler, L. K. Nielsen, S. Schlatter, J. C. Perriard, and M. Fussenegger, Design of artificial myocardial microtissues. *Tissue Eng* 10, 201-214 (2004).

[21] J. M. Kelm, V. Djonov, L. M. Ittner, D. Fluri, W. Born, S. P. Hoerstrup, and M. Fussenegger, Design of custom-shaped vascularized tissues using microtissus spheroids as minimal building units. *Tissue Eng* 12, 2151-2160 (2006).

[22] T. Shimizu, M. Yamato, Y. Isoi, T. Akutsu, T. Setomaru, K. Abe, A. Kikuchi, M. Umezu, and T. Okano, Fabrication of pulsatile cardiac tissue grafts using a novel 3- dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ. Res.* 90, e40 (2002).

[23] H. C. Ott, T. S. Matthiesen, S. K. Goh, L. D. Black, S. M. Kren, T. I. Netoff, and D. A. Taylor, Perfusion-decellularized matrix:using nature's platform to engineer a bioartificial heart. Nat. Med. 14, 213-221 (2008).

[24] Hyde A, Blondel B, Matter A, Cheneval JP, Filloux B, Girardier L (1969) Homo- and heterocellular junctions in cell cultures: An electrophysiological and morphological study. Prog Brain Res **31:** 283-311.

[25] Jourdon P (1980) Ultrastructure and electrical activity of newborn rat heart aggregates. Biol Cell 37: 149-154

[26] Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse Indroneal Banerjee , John W. Fuseler , Robert L. Price , Thomas K. Borg , Troy A.

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[27] Cardiac Tissue Engineering: Implications for Pediatric Heart Surgery Wolfram-Hubertus Zimmermann Æ Robert Cesnjevar Pediatr Cardiol (2009) 30:716–723 DOI 10.1007/s00246-009-9405-6.

[28] Giorgia Imparato, Francesco Urciuolo, Costantino Casale, Paolo A. Netti The role of micro-scaffold properties in controlling the collagen assembly in 3D dermis equivalent using modular tissue engineering, Biomaterials 34 (2013) 7851e7861.

CHAPTER 4

3D CARDIC-TISSUE EQUIVALENT *IN VITRO*

4.1 Introduction

One of the most challenging issues of cell-based tissue engineering is the implementation of strategies to successfully culture large construct *in vitro* mimicking the natural tissue organization. To reach this aim recent efforts have been concentrated on bottom-up tissue fabrication methods [6] using both scaffold-free [7,8] and scaffold-based micro-modules as building blocks [9,10] to generate a larger 3D tissue construct. However, while many studies focused their attention on the molding capability of building blocks [8,9,11,12], at best of our knowledge few efforts have been spent to reach a deep understanding of their role in ECM organization as well as the cellular organization. To address this challenge cells have been mixed with natural or artificial hydrogel or seeded on micro-carriers to form modular tissues of specific geometries and mechanical properties. In this context, cell-laden hydrogel resembled the architecture of the target tissue at micron-scale, but scaling up the production to tissue level dimensions has not been proven yet [6,9]. On the contrary, a long time culture of 3D engineered tissue rich in endogenous ECM have been performed by molding cell-seeded micro-beads [12,10]. This kind of scaffold-based building block have the ability to temporarily support the new tissue, giving the possibility to the cells to organize and in order to be provided by cell-derived ECM layer essential for the cells to grow and differentiate in a tissue like environment, biologically fused in a 3D tissue equivalent under appropriate culture conditions. In this fashion, our has developed [13] a strategy yielding 3D cardiac tissue constructs of defined size and geometry by means of the biological sintering of cell seeded micro-scaffold so-called micro-tissue precursors (μTPs). Since μTPs were shown to spontaneously aggregate in a stable manner, a 3D viable tissue formation strategy based on the assembly of μTPs was proved feasible (Chapter 3). Furthermore, we

assessed that the optimal time and dynamic process conditions optimized in order to promote and to control the cellular organization and the assembly of de novo deposited ECM lead to the realization of a 3D cardiac equivalent.
In this chapter the optimal time for taking the μTPs for aggregation in 3D tissue equivalent and the role of micro-scaffold properties in providing guidance to direct tissue morphogenesis has been investigated. Engineered constructs then were compared to native cardiac tissue to assess their suitability to serve as an *in vitro* model for scientific studies and potentially as tissue equivalents for *in vivo* tissue repair.

4.2 Materials and methods

4.2.1 Cardiac-μTP molding

As previously described [1] cardiac-μTPs suspension was transferred from the spinner flask, to a 50 ml Falcon centrifuge tube and, after settling, transferred by pipetting into the maturation chamber to allow their molding in disc-shaped construct (1 mm in thickness, 5 mm in diameter). After 3 days in spinner flask it was collected the μTP into the chamber maturation. During the filling procedure, the maturation chamber was accommodated on a device connected with a vacuum pump to make the process faster and to assure that any bubble was in the maturation space. Finally the assembling chamber was placed on the bottom of a spinner flask and completely surrounded by culture medium. The spinner was operated at 60 rpm and the medium was exchanged every 2 days. The maturation space was disk shaped (1 mm in thickness, 1 mm in diameter) inserted in a dynamic environment allowing tangential flow that guarantee optimal nutrient supply and waste removal through the maturation space during the culture time. After 7 days in the assembling chamber obtained a 3D cardiac tissue equivalent.

4.2.2 Self-beating

The 3D cardiac tissue after 7 days into maturation chamber was characterized of self- beating. The investigation of spontaneously beating of μ-TP was undertaken

using a CMOS-camera (hamamatsu photonics – C11440-22CU) on an inverted microscope (OLYMPUS - X81).

4.2.3 Cell and ECM organization

Cell and ECM organization along the biohybrid's thickness were assessed by performing histological analysis on transverse sections of biohybrid. Transverse sections of samples were stained using hematoxylin-eosin (Bio Optica solutions, Masson's trichrome (Sigma Aldrich) and Picro Sirius Red (PSR) (Sigma Aldrich) following standard procedure and analyzed by an optical microscope (BX53; Olympus). Moreover histological sections from neonatal ventricle rat stained with the same histological staining were observed and compared. Polarized light images of samples stained with PSR alone were acquired with an inverted microscope (BX53; Olympus) with a digital camera (Olympus DP 21). A linear polarizer was placed between the light source and the specimen, while the analyzer was installed in the light path between the specimen and the camera. It was known that the color of collagen fibers stained with PSR and viewed with polarized light depends upon fiber thickness; as fiber thickness increases, the color changes from green to red [14,15].

4.3 Results and discussion

4.3.1 Self-beating

To realize a 3D macroscopic cardiac-like tissue, it was choose to use as building block µTPs-CM-R(+) in the short time phase (EP-4) because their major functional properties. To do this, µTPs were loaded in a micro pipette and casted in the maturation chamber that was then placed in a spinner flask bioreactor to guarantee dynamic culture conditions. The Assembly process was stopped after 7 days obtaining a 3D cardiac-like tissue 5 mm in diameter and 1 mm thick. The beating frequency of the patch was evaluated by means of video-microscopy (see supplementary info patch.avi) and it was evaluated a beating frequency of approximately 150 b.p.m. Moreover, we interpret these data to reflect a "conditioning" effect: greater initial CM number promote CM survival, in fact CM-CM interactions

may also be important. It has been reported that the MCs release into the culture medium complex proteins that promote their own survival and beating [23]. It was therefore possible that MCs condition their microenvironment in culture and that a great number of NMCs somehow interfere with this process, perhaps by degradation or binding of a macromolecule necessary for MC communication. However, a great beat showed that the biological fusion was complete into 3D and that with this strategy cardiac cells retain the ability to communicate between them.

4.3.2 Histology

Figure 1 shows the morphological analyses performed on cross section of cardiac patch and comparison with native cardiac muscle from neonatal rat is also reported. Figure 1 A, D shows Masson Trichrome staining of cardiac patch and native tissue, respectively. From the images it is possible to see that collagen (in blue) was present in small amounts, along the whole thickness and was distributed between the cells as well as in native tissue. Images of H/E histology of the cross section of biohybrid (Figure 1 B) shown complexes of multicellular aggregates and longitudinally oriented cell bundles as in the native tissue (Fig.1 E). Moreover, by PRS analyses, Fig 1C and 1F, it is possible to observe a network of fibrillar collagen filling the interstitial space and bridging the gap between the cells and the porous gelatine scaffold.

Mechanical stress plays an important role in the regulation of myocardial structure and function, a process well recognized by cardiac surgeons [16]. Increased mechanical stress can stimulate cells to hypertrophy, orient, and increase their contractile state [17,18,19]. Increased mechanical load can also stimulate the secretion and reorganization of extracellular matrix components [20]. The extracellular matrix was the substrate for cell adhesion, growth, and differentiation, and it provided the mechanical support necessary for effective cardiac contraction [21,22]. Given the critical role of mechanical stimuli in maintaining effective myocardial structure and function, the goal of this study was that without any mechanical stimulation there was a balanced cell proliferation, without necrotic areas, and highly organized formation of the fibrillar collagen network that results with an organization of tissue in which cells and ECM were not arranged randomly but there is an orientation along an axis.

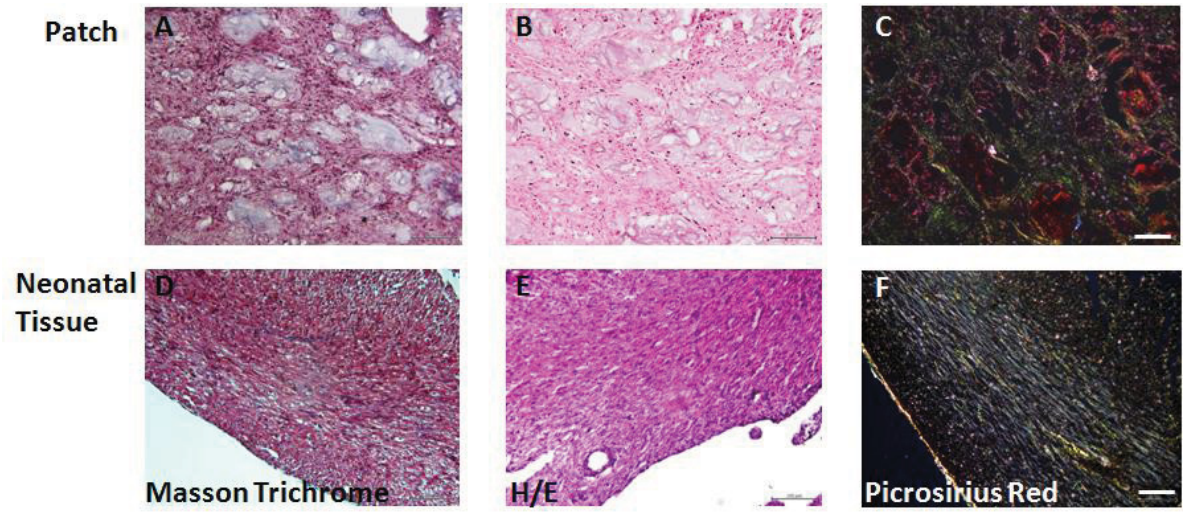

Figure 1: Histological analyses. A, D) Masson trichrome stained cross section of cardiac patch and neonatal ventricle rat, respectively. B, E) Hemtoxylin and eosin staining of cardiac tissue equivalent and neonatal tissue, respectively. C, F) PRS staining on cardiac patch made in lab and neonatal tissue, respectively. Scale bar is 100µm.

4.4 Conclusions

In this study we have demonstrated that modular cardiac tissues could be used to produce a 3D cardiac tissue equivalent. The matrix scaffold provided a transient but crucial structural support for the cells until they can secrete and maintain their own extracellular matrix. In order to obtain a macroscopic viable 3D tissue equivalent we needed firstly to engineer the µTPs in terms of their ECM composition. Cardiac cells were able to adhere and to growth on micro-scaffold and by means of cell / material cross talk, the key components of the ECM were secreted and assembled. Such process leads to an up-regulation of the ECM assembly on the µTPs in suspension, leading to the death of the µTPs. Indeed, as spinner culture increased we observed that even if the fibroblast growth was blocked, the mature fraction increased. At the early time (short time culture) of the spinner culture, the immature fraction of the cardiac ECM was higher and we used such micro tissues as building block for the realization of the 3D cardiac muscle. After one week of assembly and maturation we observed a tissue characterized by an increased self-beating and the ECM was quite similar to that one of the native tissues. We observed that this behavior was the opposite of the micro-tissue evolution where the cells were engaged in assembly the existing ECM, leading to a formation of a scar-like tissue. In the assembly phase it seems that cells were preferentially engaged in leading down new ECM in order to

reach a configuration characterized by a completely connected and synchronized 3D structure. The development of a functional tissue construct was dependent on the capacity of the seeded cells to re-establish an architecture that resembles native myocardium. Our observations indicate that with this approach, the heart cells possess an innate capacity to re-establish complex 3D myocardial organization if provided with appropriate environmental cues.

References

[1] Urciuolo F, Imparato G, Palmiero C, Trilli A, Netti PA. Effect of Process Conditions on the Growth of Three-Dimensional Dermal-Equivalent Tissue Obtained by Microtissue Precursor Assembly. Tissue Eng. 2011;17:155-64.

[2] Akins RE, Boyce RA, Madonna ML, et al. Cardiac organogenesis in vitro: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. Tissue Eng. 1999;5:103–118.

[3] Carrier RL, Papadaki M, Rupnick M, et al. Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization. Biotechnol Bioeng. 1999;64:580–589.

[4] Li RK, Yau TM, Weisel RD, et al. Construction of a bioengineered cardiac graft. J Thorac Cardiovasc Surg. 2000;119:368–375.

[5] Li RK, Mickle DA, Weisel RD, et al. Human pediatric and adult ventricular cardiomyocytes in culture: assessment of phenotypic changes with passaging. Cardiovasc Res. 1996;32:362–373.

[6] Lydia Sorokin.The impact of the Extraacellular matrix on inflammation. Nature review Immunol. 2010 Vol. 10

[7] Hugo Fernandes,Lorenzo Moroni,Extracellular matrix and tissue engineering applications. Journal of Material Chemistry.

[8] M. J. Buehler, Proc. Natl. Acad. Sci. U. S. A., 2006, 103, 12285– 12290

[9] F. W. Keeley, C. M. Bellingham and K. A. Woodhouse, Philos. Trans. R. Soc. London, Ser. B, 2002, 357, 185–189.

[10] Braverman IM, Fonferko E (1882) Studies in cutaneous aging: I.the elastic fibre network. JInvest dermatol 78:434-443

[11] A. Lamure, M. F. Harmand and C. Lacabanne, J. Biomed. Mater. Res., 1990, 24, 735–747

[12] G. C. Gurtner, S. Werner, Y. Barrandon and M. T. Longaker, Nature, 2008, 453, 314–321.

[13] Lavker RM. Cutaneous aging: chronologic versus photoaging. In: Gilchrest BA, ed. Photoaging. Cambridge, Mass.: Blackwell Science, 1995: 123-35.

[14] Rich L, Whittaker P. Collagen and picrosirius red staining: a polarized light assessment of fibrillar hue and spatial distribution. Braz. J. morphol. Sci. 2005;22:97-104.

[15] Nadkarni SK, Mark CP, Park BH, De Boer JF, Whittaker P, Bouma, BE et al.Measurement of Collagen and Smooth Muscle Cell Content in Atherosclerotic Plaques Using Polarization-Sensitive Optical Coherence Tomography. Journal of the American College of Cardiology 2007;49: 1475-1481.

[16] Payam Akhyari, Paul W. M. Fedak, Richard D. Weisel, Tsu-Yee Joseph Lee, Subodh Verma, Donald A. G. Mickle and Ren-Ke Li Mechanical Stretch Regimen Enhances the Formation of Bioengineered Autologous Cardiac Muscle Grafts, *Circulation.* 2002;106:I-137-I-142.

[17] Ruwhof C, van der LA. Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. *Cardiovasc Res.* 2000;47:23–37.

[18] Sadoshima J, Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol.* 1997;59:551–571.

[19] Fink C, Ergun S, Kralisch D, et al. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J.* 2000;14: 669–679.

[20] Chiquet M. Regulation of extracellular matrix gene expression by mechanical stress. *Matrix Biol.* 1999;18:417–426.

[21] Ross RS, Borg TK. Integrins and the myocardium. *Circ Res.* 2001;88: 1112–1119.

[22] Weber KT. A whisper on the wind spawns a storm. *Cardiovasc Res.* 2000; 46:211–213.

[23] Gordon HP, Brice MC (1974) Intrinsic factors influencing the maintenance of contractile embryonic heart cells in vitro. I. The heart muscle conditioned medium effect. Exp Cell Res 85: 303-310.