Engineering 3D thick porcine extracellular matrix for cardiac regeneration

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NANYANG
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**ENGINEERING 3D THICK PORCINE
EXTRACELLULAR MATRIX
FOR CARDIAC REGENERATION**

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ENGINEERING 3D THICK PORCINE EXTRACELLULAR MATRIX FOR CARDIAC REGENERATION

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1 Abstract

This project focused on engineering a thick cardiac patch using an acellular scaffold derived from a porcine left ventricle as a possible replacement therapy for the scar tissue formed following acute heart damage. Whereas most studies demonstrated cardiac constructs with thicknesses up to a few hundred micrometers, this project aims to recellularize an acellular scaffold with comparable thickness to the human native heart (1-1.2 cm).

The first part of this study focused on the interactions between the chosen cardiomyocyte model cell type, human bone marrow mesenchymal stem cells (bmMSCs), and the porcine cardiac extracellular matrix (pcECM). Before recellularizing 1 cm-thick large pcECM slabs, initial studies were assessed in an experimental model using 8mm-round pcECM discs in static conditions (no media perfusion). PcECMs were treated with different biochemical treatments including addition of functional groups, adhesion proteins and polymers to evaluate possible improvement in cell support ability. Dissolved nitrocellulose treatment demonstrated the most significant enhancement (up to 3 fold, $p < 0.05$) in terms of ultimate cell growth.

The second part of this study focused on pcECM functional effects on the seeded cell types. Whereas bmMSCs harvested from pcECMs were found positive for common positive bmMSC markers (CD29, CD44, CD90, CD73 and CD105) by flow cytometry, they surprisingly started to express CD31 with 10% and 55% CD31-positive bmMSCs

populations after 2 and 4 weeks respectively. Further qPCR studies confirmed the conclusions obtained from immunostaining that genes specific for endothelial cells (e.g. PECAM1, VWF, FLT1, FLK1) were upregulated in bmMSCs seeded on pcECMs. In addition, RNA expression of cardiac specific genes and ECM-related genes obtained from bmMSCs and cvFBs respectively seeded on pcECMs were also conducted and demonstrated some interesting gene upregulation.

Finally, the third part of this study aimed to recellularize physiologically relevant pcECM slabs. To guarantee oxygen and nutrient supply in such thick constructs, a customized bioreactor system was used. Dynamic cultures were based on the concepts and optimal parameters obtained from the static studies determined in previous parts. While different seeding methods were attempted to increase recellularization penetration into the tissue construct, a method combining a static pre-cultivation period prior to dynamic culturing demonstrated to allow up to 12 fold deeper cell infiltration than achieved under static cultures.

Taken together, those results support the feasibility of obtaining physiologically relevant cardiac constructs for potential scar replacement in the infarcted heart and the suitability of the pcECM for such application. By combining a vasculature-preserved thick pcECM and a customized bioreactor system for sustainable cell viability in such thick construct, significant cell infiltration depth (1,2 mm) was achieved and gives favorable prospects into recellularizing physiologically relevant tissue constructs. It also validates the bioreactor capabilities to sustain cell survival and growth in 3 dimensional environment. In addition, pcECM may provide particular cues towards endothelial transdifferentiation, which could be a substantial advantage in term of angiogenesis.

2 Nomenclature and Abbreviation

3D	Three Dimensional
α -actin	Cardiac alpha actinin (sarcomeric)
α -MEM	Minimum Essential Medium Eagle Alpha modification
α -SMA	Alpha Smooth Muscle Actin
A*Star	Agency for Science, Technology and Research
ABAM	Anti Bacterial Anti Mycotic antibiotic mix for cell culture
ACE	Angiotensin-Converting-Enzyme
AMLPL	Advanced Molecular Pathology laboratory
bFGF	Basic Fibroblast Growth Factor Human Recombinant
bmMSC	Bone Marrow Mesenchymal Stem Cell
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CH	Confoederatio Helvetica (Switzerland)
CM	Cardiomyocyte
CO ₂	Carbon Dioxide
CSC	Cardiac Stem Cell
CTE	Cardiac Tissue Engineering
CVD	Cardiovascular Diseases
cvFB	Cardiac Ventricular Fibroblast
Cx43	Connexin43
DAPI	4'6- diamodino-2-phenylindole dihydrochloride Hydrate
DDR2	Discoidin Domain Receptor 2
DDW	Double distilled Water
DE	Germany
DNA	Deoxyribonucleic Acid
fFB	Human Foreskin Fibroblast
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EHT	Engineered Heart Tissue

eSC	Embryonic Stem Cell
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting machine
FB	Fibroblast
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FN	Fibronectin
FR	Fold Regulation
GDC	Genomic DNA Control
GEL	Gelatin
GFP	Green Fluorescence Protein
HCl	Hydrochloric Acid
H&E	Hematoxylin & Eosin
HKG	Housekeeping Gene
HUVEC	Human Umbilical Vein Endothelial Cell
IMCB	Institute of Molecular and Cell Biology
iPS	Induced Pluripotent Stem Cell
KCl	Potassium chloride
KH ₂ PO ₄	Potassium phosphate monobasic
LAM	Laminin
LS&E	Life Science and Engineering
LV	Left Ventricle
MeOH	Methanol
MI	Myocardial Infarction
MPP	Matrix Metalloproteinases
NA	Numerical Aperture
Na ₂ HPO ₄	Sodium phosphate dibasic
NaCl	Sodium chloride
NC	Nitrocellulose
OCT	Optimal Cutting Temperature compound for hisological cryo-embedding
OD	Optical Density
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffer Saline
PE	Phycoerythrin

pFB	Porcine cardiac Fibroblast
pcECM	Porcine Cardiac Extracellular Matrix
PCL	Polycaprolactone
PiPAAm	poly(N-isopropylacrylamide)
PFA	Paraformaldehyde
PPC	Positive PCR Control
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
rpm	round per minute
RT	Room Temperature
RTC	Reverse Transcriptase Control
RT-PCR	Real-Time Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl)aminomethane
TRS	Time-Resolved Fluorescence
USA	United States of America
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor
WD	Working Distance

3 Introduction

3.1 General introduction

The heart is the central component of the cardiovascular system¹ and is situated in the center of the thoracic cavity. This muscle organ acts as a pump to deliver blood throughout the body. It collects blood from the biological tissues, sends it to the lungs for renewal of oxygen supply and finally pumps it back to all tissues.

Cardiovascular disease (CVD), which involves all medical conditions related to the heart and blood vessels, is a major health problem that affects not only the well-advanced countries but also the developing ones. According to Gaziano *et al.*², the rapid rise in the low and middle income countries is due to socio-economic changes. The World Health Organization³ has estimated that 17 million people die every year of CVD and this number is expected to increase to 24 million people in 2030 (30% of all deaths). CVD includes heart and blood vessel disorders like coronary heart disease (heart attacks), cerebrovascular disease (stroke), peripheral artery disease, congenital heart disease and heart failure. The risk factors to develop CVD are related to inappropriate lifestyle (tobacco use, absence of physical activity and an unhealthy diet) but also include poverty, stress and hereditary causes⁴. In consequence, fatty material slowly builds up in the vascular vessels forming plaques (phenomenon known as atherosclerosis) and narrowing the blood arteries. Upon plaque rupture, the most common forms of cardiovascular disease manifest as acute coronary syndrome (ACS), myocardial infarction or stroke⁵.

In the particular case of a heart attack, the clot forms in one of the coronary arteries that supply the heart. Subsequently, the nutrient/oxygen supply decreases and if the blood flow is not rapidly restored, death of heart muscle cells ensues within a short time (myocardial infarction - MI)⁶. The adult heart is unable to repair the damaged tissue because of the very limited proliferative capacity of the contracting cells (cardiomyocytes)^{7 8}. Instead, a rapid inflammatory response takes over, bringing the formation of a fibrotic scar tissue. The resulting damage within this scar tissue is irreversible and greatly affects heart function as the scar lacks the contractile, mechanical and electrical properties of the healthy heart muscle⁹. Consequently, the efficiency of the heart to pump and circulate blood is greatly reduced.

Various treatments to improve existing heart condition, including healthier lifestyle and pharmaceutical treatments¹⁰, provide only a compensation for the reduced pumping efficiency aiming to prevent as much as possible recurrences and further deterioration. Accordingly, in end-stage heart failure cases, these treatments reveal themselves insufficient and the only options are heart transplantation and mechanical ventricular assist devices that could postpone the requirement of heart transplantation¹¹. While heart transplantation is still regarded as the golden standard treatment, shortage of organ donors is a major limitation. Additional limitations include the risk of organ rejection and a possible requirement for long-term immune-suppression treatments, which exposes the host to higher chance of life threatening infections¹². As for the ventricular assist devices, these are highly expensive and suffer from low patient adherence thus they are usually regarded as a provisional solution serving only as a bridge to transplantation.

Undeniably, none of the above mentioned treatment modalities offer a genuine regeneration of the infarcted scar tissue manifesting the urgent need to find an alternative

solution. For this reason, cardiac tissue engineering (CTE) has received much attention aiming to provide new panacea for cardiac regeneration and repair.

3.2 Cardiac Tissue Engineering: background and theoretical considerations

Langer and Vacanti defined tissue engineering as “*an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue or organ function*”¹³. It has the potential to address transplantation shortage issue, as there are not enough organ donors. Natural tissues comprise cells and extracellular matrix (ECM) where complex signaling systems assure tissue homeostasis. Thus, it is essential to understand the basic biology of the tissue concerned, its development and its function. In the scope of this project, I will outline the current strategies in CTE and relevant aspects of the human heart biology and function. The main components (i.e. cell source, scaffold and signaling) of the myocardium will be explained, as well as the causes and consequences of a diseased cardiac tissue.

3.2.1 The human heart

3.2.1.1 Physiology of the normal heart

In brief, the human heart is composed of four chambers: the two upper chambers (atria) serve as blood-collecting chambers and the two lower chambers, the right (4-5 mm thick) and left (8-15 mm thick) ventricles pump the blood to the lungs and to the body respectively¹⁴ (see Fig. 1). To assure a one-way flow, the heart also includes four valves that force blood circulation from atria to ventricle (atrioventricular) and from ventricle to out of the heart (semilunar)¹⁴.

Fig. 1 Schematics of the blood circulatory system

The oxygenated blood (red) leaves the lungs and is sent throughout the body by the left ventricle. The deoxygenated blood (blue) comes back to the right ventricle and is pumped to the lungs for re-oxygenation.

In summary, the efficiency of heart function depends on many important factors and requires: i) the contraction of individual muscle cell to be coordinated and synchronized to give regular intervals, ii) the valve to be fully opened and with no leak when closed and finally iii) firm ventricular contractions to be able to eject blood throughout the body. The next section will emphasize on the myocardium physiology and structure to have a better understanding on the consequences in heart failure.

a) Histology and cellular structure of the myocardium

The myocardium is the muscular tissue of the heart, which contracts after an electrical propagation. It is composed of cardiac muscle cells, called cardiomyocytes (CMs) that are arranged into circular networks through the heart wall¹⁵. These cells are roughly cylindrical and branch to connect with the neighboring cells via a junction system called intercalated disks. The junction system ensures a strong mechanical link and also electrical connections between CMs to promote an orderly electrical wave propagation in order to obtain an synchronized heart contraction¹⁶.

Whereas CMs occupy around 75% of the myocardial tissue volume, they only consist of 30-40% of total cell number¹⁷. The remaining and majority of the cells are fibroblasts (FBs) and can be found in the entire cardiac tissue. They fill the gaps between CMs and thus, each myocyte is linked to at least one FB¹⁸. The CM-FB coupling supports synchronization of distant CMs, which are indirectly connected by FBs only¹⁹. But FB main function is to maintain ECM homeostasis by secreting protein related to synthesis or degradation of connective tissue, producing ECM proteins, growth factors and matrix metalloproteinases (MMP)²⁰. Because FBs maintain the ECM, they logically play a critical role in cardiac remodeling in cardiovascular diseases. For instance, excessive proliferation of FBs and increase of ECM protein synthesis (fibrosis) lead to myocardial stiffening in many cardiac dysfunctions²¹.

Cardiac ECM provides a support for the different cell types and influences intercellular communications. ECM contributes to cellular interactions and activities (proliferation, adhesion, migration and differentiation) by facilitating mechanical, electrical and chemicals signals during homeostasis and during injury response²². The ECM composition includes an extensive collection of compounds: structural proteins (collagen and elastin), adhesive proteins (laminin and fibronectin) and proteoglycans²². Collagen is the main component of ECM backbone structure. There are at least twenty-eight different types of collagen that have been identified in vertebrates²³ but collagen I and III represent 90% of the total amount of collagen in the cardiac ECM²⁴. They provide the visco-elastic properties of the myocardium by being located at different regions of the heart depending on the stiffness and contractility required²⁵. As major components of basal membrane, laminins (LAMs) are important cell adhesion receptors and bind to ECM components like collagen²⁶. They are not only found in muscle and vasculature, but

also in nerve, kidney, lung and skin²⁷. Similarly, fibronectins (FNs) act as an adhesive protein and are mainly produced by fibroblasts²⁸.

The myocardium is a complex tissue, which is not only composed of contractile cells but also includes an extensive amount of supporting cells and proteins to maintain a suitable environment for a healthy myocardium. To cure diseases related to the cardiac tissue, there is a strong need to understand the ECM-cells interactions, which are essential to CTE.

b) The heart muscle functional unit

The contractile proteins reside in the entire cytoplasmic space of the CMs²⁹. They are arranged into networks called myofibrils. Each myofibril contains smaller contractile units called sarcomeres, which are separated from each other by a matrix of α -actinin and actin proteins, called Z-disk²⁹ (see Fig. 2). In each sarcomere, thick filaments of myosin extend from one Z-disk to the opposite Z-disk (around 1.6 μm long) and thin filaments of actin are connected to the Z-disks (approximately 1 μm long)³⁰. Myosin protein is the molecular motor of the muscle tissue. It is composed by a long “tail” and two “head” domains at one of its extremity. These two “head” domains include an ATP hydrolysis site and an actin binding site to attach thin filaments of myosin²⁹. In brief, myocyte contraction is driven by the interactions between myosin “heads” and thin actin filaments. During an ATP hydrolysis cycle, the myosin “heads” bind to the actin filaments and pull these filaments relative to the thick filaments producing the force to contract.

Fig. 2 Schematics of a sarcomere - the smallest functional unit of muscle tissue

It is mainly composed of myosin thick filaments, which are the motor of the muscle tissue. ATP hydrolysis drives myosin heads to move along actin thin filaments, bringing the Z-disks closer to each other during contraction.

c) Heart vasculature

Even though the heart is filled with blood, nutrient and oxygen diffusion alone from ventricles cannot support the entire thick cardiac tissue. The heart possesses its own blood supply that arises directly from the base of the aorta³¹. This system divides into two arteries called left and right coronary arteries that branch into smaller arteries and capillaries to supply the entire cardiac tissue. Both left and right ventricles are drained by coronary veins, which empty into the right atrium³².

To supply oxygen basal demand, the myocardium extracts the maximum available oxygen ratio from the coronary blood supply (>70%). Thus, any increase in oxygen demand, for instance during exercise, requires a proportional increase in blood flow rate³³. Therefore, heart vasculature has the crucial role of supplying the heart with the

sufficient quantity of oxygenated blood and valuable nutrients. It is easily understandable that many heart pathologies arise from initial issues associated with the cardiac vasculature.

3.2.1.2 Heart failure condition

Heart failure is clinically defined as “*the inability of the heart to pump enough blood to meet the body metabolic demands which results in death*”³⁴. Progression towards heart failure is a slow and gradual process, which is silent at first and is later on characterized through several signs and symptoms appearing prior to the end result. These include shortness of breath, oedema, liver and kidney malfunction³⁵. Diagnosis for heart failure is complex and includes a complete physical examination of blood pressure, blood biochemistry, electrocardiogram, exercise test chest X-ray, echocardiography as well as magnetic resonance imaging (MRI) to evaluate the severity of the condition³⁶. Treatments essentially focus on early stage prevention through adopting healthier lifestyle (diet, exercise, no consumption of alcohol or cigarettes), which was shown to improve some of the symptoms and slow down the progression of heart failure. In late stages however, healthier lifestyle or pharmaceutical drugs have no more effect and surgical interventions (i.e. heart transplantation, bi-ventricular pace makers for cardiac resynchronization therapy or ventricular assist devices as bridge to transplantation) are the last alternatives to improve heart function³⁷.

a) Ischemia and causes of heart failure

In most patients, heart failure due to left ventricular systolic dysfunction is essentially caused by coronary heart disease³⁸, where a shortage of blood occurs due to narrowing of blood vessels supplying the heart. The lack of oxygen supply (ischemia) leads to major damages to the myocardium, which cannot always recover even if there is

reperfusion following an ischemic event. During MI, heart metabolism tries to counter the imbalance caused by oxygen shortage by producing ATP through an anaerobic pathway³⁹. It results with increased tissue acidity (acidosis), which decreases myocardial contractility⁴⁰. If the ischemia event is prolonged, the anaerobic pathway is insufficient to produce enough ATP leading to cellular necrosis.

Different ischemia syndromes have been described⁴¹. The stunned myocardium endures a brief ischemia (minutes) caused usually by a transient coronary occlusion³⁹ characterized by little to no necrosis and the myocardium recovers after reperfusion with some delay. A prolonged partial coronary occlusion (weeks to months) reduces myocardial metabolism and only revascularization may re-establish its initial state. Sustained coronary occlusion will cause an infarct resulting in massive necrosis in the affected area and leading to a reduction of contractile performance. In severe cases, infarction may also lead to cardiac arrest.

b) Physiology of the diseased heart

In response to acute loss of myocardium, the heart is subjected to increased workload, which triggers various cellular and structural changes, a process known commonly as remodeling⁴². At first (within hours after MI), infarction results in wall thinning and ventricular dilatation, which elevate wall stresses⁴³. In response to this stimuli, surviving cardiomyocytes achieve remodeling of gap junction distribution and hypertrophy in the bordering areas of the myocardial infarcts to distribute the increased wall stresses more evenly⁴⁴. In addition, accumulation of tissue fibroblasts leads to increased collagen deposition resulting in a lower distensibility of the ventricle walls⁴⁵. In post-infarction dilation, the end-systolic volume index (related to the volume in ventricle at the end of a contraction) progressively increases and ejection fraction (volumetric

fraction of ejected blood) declines⁴². The timeline of this process depends on the severity of the case and the age of the patient, and can be anywhere from weeks to months⁴³ or even years⁴⁶.

3.2.1.3 Current treatment modalities

It is important to define the different steps of heart failure. Diagnostic of the exact stage of the disease is a key towards the suitable treatments that should be provided. For asymptomatic patients with high risk of heart failure (hypertension, coronary artery disease), the goal is mainly to prevent cardiac remodeling by treating hypertension or by providing angiotensin-converting-enzyme (ACE) inhibitors⁴⁷. ACE inhibitors interfere with the renin–angiotensin–aldosterone system, which results in peripheral vasodilatation. The addition of beta-blockers to ACE inhibitors aims to reduce the symptoms and slow the progression of the disease by inhibiting the sympathetic nervous system as well⁴⁷. Those beta-blockers slow down heart rate, decrease blood pressure and enhance reverse remodeling⁴⁸, a process which describes regression of pathological myocardial conditions⁴⁹. The main issues when using those pharmacological treatments are side effects, unsureness of optimal doses^{50, 51} and unsuitability for a number of patients with airway disease and diabetes. Patients with higher chances of getting recurrent ischemic conditions (depending on the location, extent and number of occluded coronary arteries) may require re-perfusion therapy through surgical intervention which could be either minimally (i.e. catheter-based) or more invasive (i.e. bypass surgery)⁵². The main treatments for end stage heart failure include transplantation of bi-ventricular pace makers⁵³ and ventricular assist devices⁵⁴, yet the golden standard still remains heart transplantation. However, this golden standard still suffers from usually long waiting time (causing further deterioration) due to lack of sufficient donors, and the requirement for chronic immunosuppression therapy causing patients to become more susceptible to

infections and possible complications thereof. In addition, the open surgery involves many risks, and in cases of many patients (mainly elderly) survival chances are fairly low⁵⁵. Therefore the development of an alternative treatment is eminent which serves as the basic driving force behind the vast research on cardiac tissue engineering.

3.2.2 Cardiac tissue engineering

In the early 1970s, WT Green performed the first experiment that was going to emerge as the main concept of tissue engineering by growing chondrocytes on a “scaffold” to generate new cartilage⁵⁶. The concept of tissue engineering was only defined in 1987 as *“the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure, function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function”*⁵⁷. It is finally in the mid-90s that the first cardiac therapies were demonstrated on cardiac necrosis by cell injection followed by therapies using synthetic scaffolds and cells⁵⁸. Since then, the number of investigation in CTE has constantly increased to reach the future demand of a continuous aging population with cardiac conditions. CTE includes multidisciplinary fields (biology, engineering and medicine) to achieve generation of a new cardiac muscle. It requires the understanding of structure-function relationships in healthy and pathological cardiac tissues to be able to develop substitutes that could restore or improve the heart muscle function after myocardial infarction injury. CTE aims to design strategies to repair infarcted heart using (i) cells, which replace CM loss during MI, (ii) biomaterial scaffolds, which provide a sustainable environment for cells to grow into a cardiac tissue and (iii) regulator factors or mechanical stimuli to support and promote cell differentiation into cardiac tissue.

3.2.2.1 Main factors in engineering a cardiac tissue

CTE includes three important parameters, namely cells^{59 60 61}, biomaterial scaffolds^{62 63 64} and the environment^{65 66} (usually supplied through adequate bioreactor technology). Various combinations of these three factors can emerge into an infinite number of strategies. For example, engineered cardiac tissues may consist of one or several autologous or immune-tolerated regenerative cell populations. Those populations could hopefully give rise to the three main parenchymal cell types of the heart (i.e., CMs, FBs and vascular cells). Hence, the choice of initial cell source is crucial for many functional aspects. These cells could be used either alone (i.e. as cell sheets⁶⁷) or within a supporting biomaterial construct^{68 69 70} mimicking the natural ECM of the heart tissue. Alternatively, biomaterial scaffolds could be employed solely, as either a patch or an injectable platform⁷¹ with the aim of providing mechanical support and attracting host progenitor cells to enable tissue regeneration. Furthermore, the native cardiac tissue is approximately 1 to 1.5 cm thick in an adult heart⁷². Hence, an engineered cardiac construct needs to have a clinically and physiologically relevant thickness and size to make a significant impact¹⁴¹. For instance, increasing wall thickness can theoretically reduce myocardial wall stress (as stipulates in Laplace's law where the wall stress is inversely proportional to the wall thickness⁷³). However it is expected that, in order to substantially increase the human wall thickness, thicker than currently employed constructs should be utilized. Therefore, in thick constructs containing cellular components, the development of inherent vascularization is necessary due to the low nutrient and oxygen diffusivity. For these same reasons, cell culture platform-systems need to be developed to maintain the generation of such thick cardiac constructs *ex vivo*³⁴. It is therefore clear that in order to engineer a cardiac tissue, one must have an interdisciplinary understanding of each of these three and crucial parameters as discussed below.

a) Cell source

As there is no sufficient intrinsic repair after MI, a lot of efforts are focused in exploring the reparative capabilities of different types of stem cells. These include embryonic stem cells (eSC)^{74 75 76}, mesenchymal stem cells (MSC)^{77 78 79}, cardiac stem cells (CSC)^{80 81 82}, induced pluripotent stem cells (iPSC)^{83 84 85} and skeletal myoblasts^{86 87 88}. To supply all cardiac cell populations (myocytes, fibroblasts and vascular cells), only eSCs can provide both cardiogenic and vasculogenic cells^{89 90}. However, using omnipotent stem cells can also lead to uncontrolled differentiation and proliferation, resulting in teratoma formation after post-transplantation^{91 92}. In addition, those cells are also considered immunogenic and their utilization for heart failure treatment might require immunosuppressive therapy⁹³.

Promising development has been done on creating autologous embryonic-like cells, called induced pluripotent stem (iPS) cells⁹⁴ from different human tissue using genetic transformation. These iPS cells have the advantages of being autologous while maintaining their capabilities to replenish all three types of cardiac cells. However, there are still obstacles to overcome before using iPSs for clinical purpose such as improving the low reprogramming efficiencies and ensuring the safety of the resulting differentiated cells⁹⁵.

The traditional view of the myocardium lacking any regenerative capacity has been recently questioned since some researchers have reported the presence of CSCs in the adult human myocardium^{96,97}. Human CSCs are believed to maintain myocardial homeostasis by replenishing cardiac population and therefore, hold great promises as cell source for cardiac regeneration. Similarly as for iPSs, reproducible results for routine

patient care are still lacking before allowing clinical testing⁹⁸. Until safety and feasibility of these latter stem cell sources are established in *in vitro* and *in vivo* experimental models, more restricted lineage cell types, such as skeletal muscle-derived myoblasts and MSCs, remain the most studied donor cell types for cardiac regeneration⁹⁹. Found in muscle fibers in quiescent state, some myoblasts have the capacity to divide and differentiate into functional skeletal muscle. Unlike iPSs or CSCs, they are sufficiently lineage restricted to avoid any threat of tumorigenicity¹⁰⁰. Unfortunately, myoblasts demonstrated in some preclinical studies improper electrical coupling with the myocardium¹⁰¹.

MSCs are multipotent cells and can be derived from bone marrow, adipose, umbilical vein or dental pulp³⁴. For instance, bone marrow derived MSCs can be obtained in relatively large quantities through standard clinical procedures (i.e. bone marrow harvest or liposuction)¹⁰² and are easily expendable, which allows potential clinical applications to be practical. They naturally give rise to bone, fat and cartilage, but also have demonstrated capacity to differentiate into CM-like cells *in vitro*¹⁰³ with limited efficiency. They have further shown some *in vitro* capacity to differentiate into endothelial^{104 105} and smooth muscle cells¹⁰⁴, which are very important in cardiac tissue engineering. In addition, they secrete a large range of bioactive molecules that are advantageous for the tissue implant integration in the body¹⁰⁶. In human clinical studies, injection of MSCs derived from bone marrow has demonstrated some increase in ejection fraction (0-5%)^{107 108} but this improvement is likely due to paracrine effect rather than actual cardiac differentiation²⁵⁰ as little formation of new CMs was observed. Even though the potential of MSC in myogenesis is limited, MSCs have significant advantages in terms of availability, expandability and transplantability as compared to the more pluripotent cell lineages¹⁰⁹. They have been indeed considered safe for transplantation and

have been widely tested in clinical trials in cardiovascular disease¹¹⁰. In addition, allogeneic MSC transplants have shown so far little to no immunorejection in human trials, allowing the possibility of third-party donor¹¹⁰.

Another cell source of equally importance is cardiac fibroblasts (FBs). These are the most abundant cell type in the heart and play an important role in regulating normal myocardial function. They are native to cardiac tissue and should possess the proper signalling system corresponding to cardiac ECM environment. Thus, to generate a fully functional cardiac scaffold, the idea of co-culturing FB with the cardiac model cell type chosen has become undisputable. For instance, Radisic *et al.*¹¹¹ have demonstrated enhancement of engineered cardiac assembly by creating an environment supportive for cardiomyocyte attachment by pre-treating synthetic elastomeric scaffolds with cardiac FBs. As reported by several studies^{112 113}, cardiac FBs were further used to produce derived-ECM scaffold *in vitro*, emphasizing the importance of their ECM protein secretion. A more recent study by Twardowski *et al.*¹¹⁴ has suggested that cardiac FBs could support endothelial cell proliferation and capillary sprouting formation in a fibrin gel co-culture model. However, such experiments were demonstrated for scientific purpose and are more complicated to reproduce if human cells need to be harvested from the patients. Autologous human cardiac FBs required for a potential clinical application are indeed only obtained by performing biopsy on the patient, which is usually an invasive procedure. That is why human foreskin FB was also employed as cell control and was obtained previously in our laboratory in Israel¹¹⁵. Some investigations have already reported the use of human dermal and foreskin FBs instead of using cardiac FBs in cardiac tissue engineering (CTE)^{116 117}.

In view of the advantages associated with MSC, human MSC of bone marrow

origin were chosen as CM model cells. Many studies have reported to use this cell type, not only in cardiac tissue engineering^{118 119 120}, but also in bone^{121 122 123} and cartilage tissue engineering^{124 125 126}. MSC were identified in many tissues acting as pericytes and reparative cells¹²⁷. They are thus applicable for tissue regeneration and repair in many organs around the body and are widely studied for these purposes.

b) Scaffolds

Biomaterial scaffolds provide suitable environment with specific mechanical and biochemical properties towards cell migration^{128 129}, growth^{130 131 132}, differentiation^{133 134}¹³⁵ and alignment^{136 137 138}. The scaffolds are mainly used as substitutes of the native ECM in an ex-vivo setting. They try to mimic the structure and stiffness of the native tissue by modulating fibrosity and porosity level.

Different synthetic and/or natural polymers are used¹³⁹. Natural materials have the great benefit of providing cellular signals¹⁴⁰, crucial to trigger cell mechanisms such as cell attachment and migration by providing numerous chemical and physical signals (structure of ECM, protein receptor and collagen). However, their integrity and hierarchical structure may be difficult to preserve during processing while still removing the immunogenic compounds¹⁴¹. On the other hand, synthetic material properties can be easily tailored in term of mechanical, chemical and degradation properties¹⁴² but are limited in term of functional cellular interaction unless they are modified with extra peptides and surface proteins.

In recent years, research has also focused on injectable scaffolds. Even though interactions between the hydrogel and cells are not as controllable as in *in vitro* engineered scaffolds, they remain an attractive method to regenerate infracted

myocardium as they do not require open surgery, and can be delivered in a minimally invasive method through catheters^{143 144}. Those injectable materials are usually polymeric hydrogels (of natural or synthetic origin), which take the shape of the injected area. After injection, gelation occurs by polymer cross-linking (covalent, ionic or physical bonds), triggered by different stimuli such as temperature, UV and pH¹⁴⁵.

c) Vascularization

For clinical and physiological relevancy, a cardiac engineered construct should have at least an area of 10-50 cm² and a thickness of several millimeters¹⁴⁶. Whereas obtaining cardiac constructs with a large surface area is feasible, viable cardiac constructs with reasonable thickness still remains a major challenge. The most important limitation is the low diffusion of nutrients and oxygen throughout the tissue, which is limited up to 100 µm in soft tissues¹⁴⁷. Even if a thick cardiac patch is successfully maintained using an entire *in vitro* setting, the survival of reseeded cells following implantation is low due to the slow time frame (value of capillary growth rate or of time frame per functional blood vessel infiltration) for vasculature infiltration through angiogenesis^{148 149}. By the time functional angiogenesis (i.e. formation of new vessels by growth or sprouting from pre-existing ones) occurs *in vivo*, necrosis would have already reached the core of the construct. It is therefore, necessary to create an intrinsic microvasculature within the cardiac patch before implantation. Commonly, endothelial cells, pericytes and smooth muscle cells are the main compounds used to induce angiogenesis¹⁵⁰. These cells have already demonstrated to generate a mature vascular network when co-implanted in the heart^{151 152}.

d) Bioreactors

A bioreactor platform is necessary when dealing with thick tissue engineered

constructs¹⁵³. A bioreactor provides a very tight control on the environment conditions of the engineered construct by assuring constant high mass transport to provide enough nutrient and oxygen exchange to the cells, even those situated in the inner core of a thick scaffold. Physical factors like mechanical or electrical stimuli can also be implemented to improve cell maturation towards the generation of a fully functional cardiac tissue^{154 155}. These parameters can influence cell proliferation, migration and cell differentiation, which are necessary to form a 3D construct. It was also shown that mechanical forces could be involved into the formation of new blood vessels by producing shear stress, luminal pressure and circumferential and longitudinal stretch¹⁵⁶.

3.2.2.2 Current achievements and strategies in cardiac tissue engineering

Strategies in CTE can be divided into two different approaches:

- i) *In situ* CTE, where most of the studies focused on injectable biomaterial and cell combination to the infarcted heart. The infarcted area is believed to provide the right environment and signals for the injectable constructs to differentiate or develop into a functional new cardiac tissue. The main strength of this approach is that the delivery method could be minimally invasive.
- ii) *In vitro* CTE, where the main approach focuses on developing a cardiac tissue mimetic prior to implantation. Developing *in vitro* allows much more control on the cell environmental conditions and biomaterial. Addition of growth factors, mechanical or electrical stimuli and use of a bioreactor to assure nutrient diffusion are the main assets in *in vitro* CTE.

a) Cellular cardiomyoplasty

Before CTE development, researchers hypothesized that stem cell injection into the infarcted heart will provide new source of cells that would be able to differentiate into

CMs and integrate in the heart tissue. This method was commonly called cellular cardiomyoplasty. It was indeed demonstrated in animal models that stem cell injection reduced scar tissue formation and improved the contractile function of the heart^{157 158 159}. But these reports also demonstrated that only a small fraction of these injected stem cells differentiated into CMs in the damaged heart, meaning that the improvement unlikely came from the differentiation of the injected stem cells. It was suggested that stem cells might secrete growth factors and cytokines in the infarcted heart, which stimulated angiogenesis and differentiation of local cardiac stem cells¹⁶⁰. Despite the small cardiac function improvement after stem cell injection, this *in situ* CTE approach has several disadvantages. First of all, the low survival rate of injected stem cells is explained by the absence of supportive matrix to which they can attach (leading to apoptosis). The infarcted tissue area is indeed not supportive for cell engraftment and nutrition supply is poor¹⁶¹. It was demonstrated later on, that only 10% of bone marrow cells were detected in the infarcted heart within 2 hours after injection^{162 163}. Evaluation studies of cardiomyoplasty in animal models or clinical trials exhibited either little improvement¹⁶⁴ to no improvement^{166 167} of heart function between treated and non treated groups suggesting the effect of this treatment modality is uncertain and pointing to the necessity of different approaches which led to the developments of CTE.

b) Injectable hydrogels

In situ CTE is similar to cellular cardiomyoplasty except that it combines injectable biomaterials either alone or as a supporting milieu to adjuvant cells, delivered to the infarcted myocardium. This approach is less invasive than implanting *in vitro*-engineered patch tissue, and is therefore more clinically appealing. Acellular injectable biomaterials serve to support left ventricle (LV) wall and prevent the negative remodelling after an MI according to the law of Laplace¹⁶⁸, and can additionally deliver therapeutic genes and

proteins to ischemic myocardium⁶⁴. Similarly to conventional scaffold-based constructs, the biomaterials for injectable matrices include natural (i.e. collagen¹⁶⁹, fibrin¹⁷⁰ or Matrigel¹⁷¹) and synthetic (PLA¹⁷², PLGA¹⁷³, PCL¹⁷⁴ or polyethyleneglycol (PEG)¹⁷⁵) polymers.

Christman *et al.*¹⁷⁶ were the first to demonstrate improvement in cell survival when they transplanted skeletal myoblasts with fibrin hydrogel compared to cellular cardiomyoplasty technique (where only cells are delivered to the damaged myocardium). They demonstrated neovascularization within the infarcted area and reduction of infarcted expansion. Others groups^{177,178} have then confirmed similar beneficial effects of fibrin glue by using various cell sources. Another study by Kofidis *et al.*¹⁷⁹ reported delivery of mouse embryonic stem cells using matrigel and demonstrated improved LV function compared to either injection of biomaterial alone or cells alone. Harboursing unique cardiac ECM composition, decellularized cardiac tissues have also been used as source material for hydrogels. Singelyn *et al.*¹⁴³ have decellularized and processed porcine myocardial tissue so it has the ability to gel at 37°C. Cells were shown to migrate towards the myocardial matrix *in vivo* with a significant increase in arteriole formation at 11 days post-injection. The safety and efficacy of such injectable hydrogel was further examined in small and large animal model¹⁸⁰. One of the earliest studies using synthetic hydrogels was reported by Jiang *et al.*¹⁸¹. Four weeks after treatment, the injection of PEG- and PCL-based hydrogels showed to prevent scar expansion and wall thinning compared with the control, but unlike natural-based hydrogels, there was no significant improvement in neovascularization in the infarcted area. Gepstein *et al.*¹⁸² have combined a delivery of CMs with an *in-situ* polymerizable PEGylated-fibrinogen hydrogel into a post-MI rat heart and demonstrated higher improvements than performing each intervention alone.

c) Conventional scaffold-cells constructs

In vitro CTE involves the use of 3D tissue constructs and allows better control over the biomaterial (e.g. mechanical and physical properties) and cells (e.g. differentiation, effect of growth factors etc.). The predominant approach is to seed a scaffold with the desired cells *in vitro* and consequently implant the whole tissue construct on the surface of the infarcted myocardium. Abundant studies have examined different scaffolds combined with various culture conditions. Synthetic and natural polymers have both been used as patch-based platforms. Commonly used synthetic materials¹⁸³ for cardiac regeneration include polylactic acid (PLA)¹⁸⁴, polyglycolic acid (PGA) (or their copolymer (PLGA))^{185 186} and poly-ε-caprolactone (PCL)¹⁸⁷ mainly because their degradation products are considered biocompatible (even though they still may cause undesirable effects like local acid increase or sudden bulk release¹⁸⁸). On the other hand, natural polymers, like gelatin¹⁸⁹, collagen¹⁹⁰ or alginate¹⁹¹, generally offer better biocompatibility and biodegradability¹⁹², but suffer from batch-to-batch variability and poor mechanical strength¹⁹³.

Li *et al.*¹⁹⁴ were the first to demonstrate the transplantation of cell on a biomaterial scaffold for cardiac regeneration. They reported spontaneous contracting of CMs derived from fetal rat ventricular muscle seeded into a commercial gelatin mesh *in vitro* and implanted the scaffold on the myocardial scar in a rat model. Despite the formations of blood vessels and cell junction between the seeded cells and recipient heart cells five weeks after implantation, the cardiac construct did not improve cardiac function. Similarly, Leor *et al.*¹⁹⁵ grew rat fetal cardiac cells but on a 3D porous alginate scaffold which displayed multicellular contracting aggregates after a few day of *in vitro* culturing. After implantation in a rat MI model, they reported no change in LV contractility but significant attenuation in LV dilation. Kellar *et al.*¹⁹¹ also implanted on infarcted LV in

mouse model a pre-formed scaffold, the commercially available Dermagraft, which contains human dermal fibroblasts cultured on a poly(glycolide)/poly(lactide) mesh. Infarcted mice with transplanted Dermagraft demonstrated significant higher ejection fractions compared to control models, which received no treatment. A creative technique was presented by Zimmermann *et al.*¹⁹⁶ (but first demonstrated by Eschenhagen *et al.*¹⁹⁷) to form a spontaneously beating 3D engineered heart tissue (EHT) *in vitro* with neonatal rat cardiac myocytes mixed with collagen I and soluble extracellular matrix extract produced from Engelbreth-Holm-Swarm tumor cells *in vitro* (referred to as Matrigel). The cell-collagen-matrigel mix was cast in circular molds and was subjected to mechanical stretch resulting with high morphological resemblance and contractile properties of the EHTs to the adult rat cardiac tissue. The graft implantation of EHTs on rat infarcted myocardial hearts proved to prevent further dilation and to improve systolic wall thickening of infarcted myocardial segments¹⁹⁸. To induce vascularization, Levenberg's group¹⁹⁹ has investigated endothelial cell co-cultures in engineered constructs. They demonstrated the induction of endothelial vessel networks using a 3D multiculture system consisting of myoblasts, embryonic fibroblasts and endothelial cells co-seeded on highly porous and biodegradable polymer scaffolds. The embryonic fibroblasts were suggested to increase the levels of vascular endothelial growth factor expression in the construct and promoted formation of the endothelial vessels²⁰⁰.

d) Scaffold-free constructs

Another approach, developed by Yamada *et al.*²⁰¹ and Okano *et al.*²⁰², involved the construction of a scaffold-free cell-sheet based cardiac patch. Cell sheets (including MSCs and endothelial cells) are produced by allowing cells to reach confluency on a thermo-sensitive cell culture surface (N-isopropylacrylamide, PIPAAm). Once confluent the cells secrete their own ECM in an attempt to adapt to their new environment and

create inter connective gap junctions. As PIPAAm acts as cell adhesive at 37°C and has a reverse property below 32°C, a mild reduction of temperature results in an intact cell sheet floating from the plate base into the cultivation medium. Using proper tools those cell sheets could be collected and handled further for transplantation. The main strengths in this strategy are the following: i) inflammation that could be caused by the degradation of a scaffold is avoided in a scaffold-free construct ii) there are more cell-cell connections due to the absence of external scaffolding material (better conductivity) and iii) cell density and inter-communication is similar to the compact myocardium tissue.

Using this methodology, Shimizu *et al.*²⁰³ demonstrated electrically communicative pulsatile 3-D cardiac constructs of neonatal rat CMs sheets (4-layer construct). In a further *in vivo* study, the same group²⁰⁴ implanted CMs sheets on rat infarcted hearts. The echocardiography demonstrated that cardiac performance was ameliorated and that the cardiomyocyte sheets were tightly attached to the infarcted myocardium. However, the thickness for such layered cell sheets was limited at approximately 80µm due to poor vascularization. Their attempts to overcome this limitation included repeated transplantation of triple-layer grafts²⁰⁵ (named as poly-surgery) to allow the grafts to survive and co-culturing endothelial cells into the cell sheets to induce *in vitro* pre-vascular network²⁰⁶. Steven *et al.*²⁰⁷ also developed a scaffold-free cardiac construct by placing human CMs, endothelial cells and FBs into suspension on a rotating orbital shake. The patches survived significantly after implantation *in vivo* in a rat model because of the pre-vascularization formed by the cell co-culturing (entire CMs patches did not survive after implantation). However, this main shortcoming of this methodology is the ultimate construct thickness, which is unable to reach relevant physiological cardiac dimensions. Researchers have addressed this problem with multiple surgery approach (polysurgery) but it is unlikely applicable on patients.

e) Decellularized matrices

Another recently studied scaffolding strategy is to decellularize organs from an animal source. The organ is deprived of its cellular content and only the native 3D scaffold remains. The main strengths of this method are that the remaining acellular scaffold provides an environment with proper 3D architecture, functional protein and glycosaminoglycan contents. Furthermore, the inherent vasculature should be conserved and provide the essential platform to induce angiogenesis and support nutrient/oxygen diffusion throughout the scaffold. Decellularization methods include physical (agitation, sonication, mechanical massage), chemical (alkalis, acids, detergents, organic solvents) and enzymatic treatments to remove cell content²⁰⁸. Those different treatments are combined to maximize the decellularization process but minimizing the adverse effects of the ECM bioactive and mechanical properties. Studies have extensively reported decellularized ECM scaffolds from a wide range of tissues: small intestinal submucosa²⁰⁹^{210 211}, lung^{212 213}, trachea²¹⁴, skin^{215 216}, nerve^{217 218 219}, cornea^{220 221 222}, oesophagus²²³, liver^{224 225}, kidney^{226 227}, bladder²²⁸, cartilage²²⁹, ligament²³⁰, blood vessels^{231 232}, heart valves^{233 234} and heart^{235-243 251}. The composition and distribution of constituents are tissue-specific and thus, the bioactivity varies depending on the ECM source. Therefore, cardiac ECM may be advantageous in term of cardiac regeneration and differentiation.

In a seminal work, Ott *et al.*²³⁵ decellularized entire rat hearts by coronary perfusion with detergents. The acellular scaffold obtained could preserve the vasculature skeleton making the acellular scaffold perfusable. These constructs were seeded with cardiac or endothelial cells and exhibited macroscopic contractions after 4 days only. Since, others groups have also demonstrated similar work on rat hearts but using various cell sources²³⁶ or decellularization protocol^{237 238}. Another group led by Wainwright²³⁹ succeeded into

decellularizing entire adult porcine hearts. The acellular ECM conserved the collagen and glycosaminoglycan content and preserved its mechanical integrity. But they only demonstrated viability of chicken CMs on sheets of acellular porcine heart. Eitan *et al.*²⁴⁰ have also demonstrated a decellularization process for ECM from native porcine cardiac tissue. The resulted acellular scaffolds (sheet) were seeded with rat cardiomyocytes and began to beat spontaneously with expression of functional cardiac markers such as alpha-actinin, troponin I, and connexin43. These findings using the decellularization approach have demonstrated the potential use of acellular ECM as a biomaterial for heart regeneration. Though the isolation of physiologically relevant thick human²⁴¹ and porcine heart ECM^{242 243} have already been reported by several groups, including our own²⁸⁶, the recellularization of those scaffolds has still proven to be very challenging to obtain a functional cardiac construct with physiologically relevant dimension. This includes aspects of cell delivery into their hierarchical tissue localizations, cell differentiation towards the required cell types and proper support throughout the construct thickness through an adequate bioreactor system. In addition, the effects induced by the ECM on the seeded stem cells on the RNA and protein level are not fully characterized and understood.

f) “Thick” cardiac constructs

Many approaches have been reported to obtain thicker cardiac constructs (as opposed to monolayer cardiac constructs) by either dealing directly with the diffusional limitation of oxygen transport or by circumventing the problem with various strategies. Some of the following references were already mentioned earlier but are discussed in here with further comments. Shimizu *et al.*²⁰³ have demonstrated by using cell-sheet technology that their constructs were limited to 6 layered sheets (100 µm) without resulting in a necrotic core. To overcome this limitation, they performed repeated

implantations²⁰⁵ (called poly-surgery) subcutaneously to induce vascularization. But this strategy may probably be impractical for human application. The same group has further attempted to induce *in vitro* pre-vascular network using co-culturing systems with endothelial cells into the cell sheets²⁰⁶ but did not report any successful attempt into increasing cell-sheet construct thickness by using such co-culturing strategy. Zimmermann *et al.*¹⁹⁸ have demonstrated 1–4 mm thick cardiac constructs by fusing five loop-shaped ETHs to form a single synchronously contracting multi-loop construct *in vitro*. In addition, they have shown that such large constructs have survived after implantation and supported contractile function of infarcted rat hearts. In a study performed by Radisic *et al.*²⁴⁴, channeled scaffolds were perfused with culture medium enriched with oxygen carriers and resulted with contractile cardiac constructs, yet the extent of cell infiltration, cell density within the core and the effect of such construct on animals was not reported, suggesting no significant beneficial effect in term of cell penetration. Other groups^{245 246} have reported enhanced cell viability in thick constructs using medium perfusion but only in the vicinity of the core perfusion conduits. A pre-vascularization platform is probably necessary at some point when engineering thick tissue constructs *in vitro*. To induce angiogenesis and growth of a new vasculature in cardiac constructs, there have been extensive studies in co-culturing vascular and cardiac cells, adding angiogenic growth factors or using highly porous structures to promote the growth of vascular conduits^{247 248 249}, but to our knowledge, those constructs with enhanced neovascularization were not yet used as a main support for thick engineered constructs.

3.3 Problem statement

Despite major advance in the field of CTE in recent years, to the best of our knowledge, there is still no publication to date demonstrating proper recellularization of a

human relevant sized tissue engineered cardiac construct (~1.2 cm thick), nor has there been publication on the achievement of any *ex vivo* tissue engineered construct repopulated with cells to a depth beyond a few hundreds of micrometer (>600-700 μm). A tissue construct of the reported thicknesses obtained using current state-of-the-art in myocardial tissue engineering, even if well integrated onto the infarcted area, is by far not sufficient to make a crucial improvement in clinically relevant progressive states towards heart failure. In fact, the minimal thickness required should reach at least several millimeters²⁵⁰. To recellularize thick constructs, nutrient and oxygen diffusion is the main limitation as culture static conditions only maintain cell viability within a thin layer in immediate contact with the culture medium (up to 100 μm thick only)^{251 252}.

In addition, little is known on the ECM support in term of cell capacity, cell proliferation and cell differentiation. To the best of our knowledge, there is no publication reporting the effects of the cardiac ECM (or any type of ECM) on stem cells focusing on the cell proliferative behaviour and the mRNA level. Understanding whether if and how the cardiac ECM could influence stem cells towards cardiac differentiation is crucial for the advancement in CTE field.

Overall, there are still extensive investigations required before succeeding in obtaining a functional cardiac construct with similar dimensions compared to the native human heart. A suitable combination of cell source and biomaterial remains the main core in CTE, but additional strategies are essential to overcome limitation of the oxygen diffusion to develop cardiac constructs of clinically relevant thickness.

4 Hypothesis and Objectives

4.1 Hypothesis

We hypothesized that thick decellularized porcine cardiac extracellular matrix (pcECM) preserves natural bioactive composition, ultrastructural 3D-architecture, and inherent vasculature, that can jointly enable human bone marrow mesenchymal stem cell (bmMSC) and fibroblast (FBs) cell support and differentiation towards the achievement of viable physiological-like thick constructs (>700 μm thick).

4.2 Research objectives

The overall goal of this project is to engineer a thick clinically relevant cardiac constructs using an acellular scaffold derived from porcine left ventricle wall cardiac tissue. The main challenge of this study is to repopulate the decellularized thick pcECM and develop the required methodologies, under both static and dynamic culture conditions, that enable maximal cell penetration and support. In this study, bmMSCs and FBs were used as model cells to optimize cell seeding and culture methods. BmMSCs have significant advantages in terms of availability, expandability and transplantability (for potential clinical applications). In addition, they have demonstrated capacity to differentiate into CM-like cells *in vitro*¹⁰³. FBs possess intrinsic proper signalling system supporting electrical signal propagation and corresponding to ECM remodelling for cell supportive environment. Therefore, cardiac ventricular FBs were used to provide cell support in a co-culture system with bmMCSs, to simulate ECM remodeling effects, and study possible electrical support by cell-cell and cell-ECM interactions.

To achieve the stated research goal, three aims were pursued (see Fig. 3). The first two aims focused on studying cell supportive capabilities of the pcECM scaffolds, and its effects on phenotypic profile and RNA expression, respectively, both studied in static culture conditions. To circumvent dependence on nutrient and oxygen diffusion limits, pcECM slabs were adjusted and cut into smaller pieces to be tested in static conditions. Finally, the third aim concentrated on the recellularization process of thick pcECM slabs using a customized bioreactor, which enables pulsatile perfusion of oxygenated culture media throughout the entire scaffold in a physiological mimicking fashion. The three aims of this study were therefore formulated as follows:

Aim 1: Studying and characterizing the porcine extracellular matrix in terms of its cell support ability under static conditions

Aim 1 focused on assessing the suitability of pcECMs as a preferable biomaterial scaffold for cardiac regeneration. Before recellularizing entire pcECM slabs, it was important to assess cell behaviors on the acellular scaffold and determine the optimal seeding and culturing parameters that were subsequently used in the other two aims. First of all, cell attachment and cell growth were optimized using the cell type chosen as cardiomyocyte model, bmMSCs. Various strategies were further attempted to enhance cell attachment and growth by bio-chemically modifying pcECM surfaces. In addition, this aim also focused in the determination of suitable co-culturing system between bmMSC and FBs. Because FBs play a critical role in maintaining normal cardiac function and cardiac remodeling, it was important to study FBs-bmMSCs co-culturing platforms on pcECMs. Several FB types were compared as single and co-culturing approaches with bmMSCs to determine effect in cell viability of such co-culturing platforms. To demonstrate that native cardiac FBs enhance also the electrical signal transduction properties of our

cardiomyocyte model cells (bmMSCs), bmMSC-FB co-cultures were also analyzed in terms of their gap-junction protein expression (through immune-quantification of connexin43 levels).

Aim 2: Characterizing the effects of porcine extracellular matrix on reseeded cells and possible differentiation induction under static culture conditions

Aim 2 focused on assessing the effects generated by the pcECM scaffold on cellular phenotypic expression at the RNA and protein levels from either bmMSCs or FBs. Studies on phenotypic profile of seeded bmMSCs on pcECMs were conducted to determine potential differentiation processes (to endothelial or cardiac-like cells) triggered by pcECM environment using fluorescence-activated cell sorting (FACS) and quantitative polymerase chain reaction (qPCR). In addition, remodeling effects at a RNA and protein levels were similarly performed with FBs seeded on pcECMs for an extensive panel of genes related to ECM and adhesion molecules, and by collagen synthesis measurements respectively. The results obtained have shed new light on the complicated relations at the cell-pcECM interphase.

Aim 3: Achieving dense recellularization under dynamic conditions

Aim 3 attempted to achieve dense recellularization within the thick pECMs slabs under dynamic culture conditions. This would serve as a proof of concept to the applicability of such pcECM scaffold for future studies on cardiac tissue *ex vivo* construction using other more designated cells (e.g. iPSC derived CM, cardiac ventricular fibroblasts and endothelial cells in a tri-culture conditions). In order to reach this goal, a bioreactor, previously developed, is necessary to support nutrient and oxygen diffusion within the thick pcECM slabs. Different cell seeding approaches were attempted. The optimal parameters obtained from static conditions were applied in dynamic conditions as a

starting point. Using our customized bioreactor system, cell penetration depth was increased by 12-fold, demonstrating the capabilities of such bioreactor to sustain cell viability in thick constructs.

Fig. 3 Schematics of all three aims towards recellularization of thick pcECM

First and second aims focused on studying cell supportive capabilities of the pcECM scaffolds and effects on phenotypic profile and RNA expression in static conditions on pcECMs. These studies resulted with better understanding of the pcECM cell holding capacity and support ability as well as with optimal parameters for cell interactions using FBs and bmMSC in co-culturing conditions and observing cell behavior on pcECM scaffolds. Finally, the third aim concentrated on the recellularization process on thick pcECM slabs using a customized bioreactor, which enables pulsatile perfusion of oxygenated culture media throughout the entire scaffold in a physiological mimicking fashion.

5 Research significance

Substantial prior art

Decellularization processes have been extensively reported for tissue engineering in a variety of tissues, including blood vessels^{253 254 255 256}, heart valves^{257 258 259 260 261}, skin^{262 263} and small intestine submucosal^{264 265 266}. Decellularization aims to efficiently remove all cellular and nuclear material with minimal adverse effect on the composition, biological activity, and mechanical integrity of the remaining extracellular matrix (ECM). Because such decellularized scaffolds offer similarity in composition and structure properties to native tissue, they offer a powerful platform to promote the regeneration functional tissues. In the past few years, interest has grown into the isolation of cardiac-derived decellularized ECM^{240 267 268 286}. Ott *et al.*²⁶⁷ were the first to demonstrate perfused decellularization and recellularization of a whole rat heart scaffold using rat cells and demonstrating intact 3D geometry and vasculature. Since, several research groups have successfully demonstrated decellularized whole-organ cardiac models physiologically relevant to human heart size^{269 270}. However, the success of the whole heart strategy may be threatened by the limited technologies available for large-scale cell expansion needed to repopulate an entire heart, particularly for the cardiomyocytes²⁸⁶. Thus, using thick slabs originating from the whole heart may be advantageous, as this would enable scaling down the cell quantity required for repopulation while still providing proper functional support. Our group has previously demonstrated a decellularization process for thin ECM patches by Eitan *et al.*²⁴⁰. When reseeded with neonatal rat cardiomyocytes, those 2 mm thin constructs were shown to synchronously beat a few days after seeding and up to 30 days. This decellularization process was further

modified to accommodate thicker ECM slabs (10-15 mm thickness²⁸⁶) where the native main vasculature was preserved.

Novel aspects of the suggested research

1. The use of inherent vasculature of the ECM as perfusion conduits in a perfusion bioreactor to support cell seeding, viability and growth in such thick scaffolds.
2. The use of thick physiologically relevant (up to 1 cm) decellularized ECM for potential applications in cardiac regeneration.
3. Studying the interactions between reseeded cells and this unique ECM as well as the cellular interactions in co-cultures using human primary cells with regenerative potential.

6 Materials

6.1 Materials

6.1.1 General materials and consumables

Material	Manufacturer (cat. no.)
12-well and 96-well low binding plates	NUNC, DK (145385/145397)
25 G x 1'' Sterican needles	B. Braun, DE (9186158)
4',6-diamidino-2-phenylindole (Dapi)	Sigma–Aldrich, USA (D-8417)
Antibiotic-Antimycotic (100X) ABAM	Gibco-Invitrogen, USA (15240-062)
Acetone	Thermo Fishier Scientific, USA
AlamarBlue™	Invitrogen, USA (DAL1100)
Ammonium Persulfate (APS)	Bio-Rad, USA (161-0700)
Beadbug pre-filled tubes (3mm, zirconium)	Benchmark Scientific, USA (D1032-30)
Bovine serum albumin (BSA)	Sigma–Aldrich, USA (A2153)
Collagenase	Worthington, USA (4182)
Ethanol absolute (EtOH)	Merck, DE (1.00983.2500)
Ethylenediaminetetraacetic acid (EDTA)	Sigma–Aldrich, USA (E5134)
Fibronectin from human placenta	Gibco-Invitrogen, USA (33016-015)
Filters, 50 µm	Millipore, DE (SLGVV255F)
Fluoromount-G (+DAPI)	Southern Biotech, USA (0100-20)
Formaldehyde (Formalin, 37% in DDW)	Sigma–Aldrich, USA (533998)
Gelatin from porcine skin	Sigma–Aldrich, USA (G1890)
Hematoxylin, Harris	Sigma–Aldrich, USA (HHS-16)
Hoechst 33258	Life Technologies, USA (H21491)
Hydrochloric acid (HCl)	Sigma–Aldrich, USA (43.557-0)
L-[2,3,4,5-3H]-Proline	Perkin-Elmer, USA (NET483005MC)
Laminin from human placenta	Sigma–Aldrich, USA (L6274)
Masson's tricomica kit	Bio-Optica, IT (04-010802)
Microscope slides Superfrost Plus	Menzel Glaser, DE (J1800AMNZ)

miRNeasy mini kit	Qiagen, NL (217007)
Methanol (MeOH)	Tedia, USA (MA1921)
Nitrocellulose (pure membrane)	Bio-Rad, USA (162-0112)
Polycaprolactone (PCL)	Sigma–Aldrich, USA (440744)
Paraformaldehyde (4% PFA)	Electron Microscopy Science (15710)
Pepsin bovine	Sigma–Aldrich, USA (P7000)
Qiazol lysis reagent	Qiagen, DL (79306)
RT ² PCR array kit	Qiagen, DL
PCR array for endothelial cell biology (PAHS-015Z)	
PCR array for human ECM and adhesion molecules (PAHS-013Z)	
qBiomarker iPSC PCR Array Cardiomyocytes differentiation (IPHS-102E)	
RT ² First strand kit (330401)	
containing buffer GE, RNase-free water, 5x buffer BC3, Control P2, RE3 reverse transcriptase mix	
RT ² PCR array loading reservoir (338162)	
RT ² SYBR Green qPCR mastermix (330500)	
containing 2x RT ² SYBR Green qPCR mastermix	
Sodium bicarbonate	Sigma–Aldrich, USA (S5762)
Temed (for catalyst for polyacrylamide gels)	Bio-Rad, USA (161-0800)
Tissue-Tek OCT compound	Sakura Finetek, JP (4583)
Triton X-100	BioLab Ltd., IL (201805c)
Xylene	Gadot, IL (1330-20-71)

6.1.2 Antibodies

Type	Antibody details	Manufacturer (cat. no.)
Primary	α-SMA (1A4), raised in mouse	Sigma-Aldrich (A-2547)
	CD29 (TS2/16), raised in mouse	Biolegend (303002)
	CD31 (WM59), raised in mouse	Biolegend (303102)
	CD31 (9G11), raised in mouse	R&D Systems (BBA7)
	CD44 (G44-26), raised in mouse	BD Bioscience (555476)
	CD44 (IM7), raised in rat	Biolegend (103001)
	CD73 (IE9), raised mouse	Santa Cruz (32299)
	CD90 (5E10), raised mouse	BD bioscience (555593)
	CD105 (266), raised mouse	BD bioscience (555690)

Connexin43, raised in rabbit	Sigma–Aldrich (C6219)
Connexin43-FITC	Invitrogen (138388)
DDR2, raised in rabbit	Abcam (5520)
Von Willbrand Factor (2F2-A9) raised in mouse	BD Biosciences (555849)
Secondary Fluorescein isothiocyanate, anti-mouse	Sigma (F8521)
NorthernLights™ 557-conjugated, anti-mouse	R&D Systems (NL007)
Phycoerythrin,, anti-mouse	BD pharmingen (550589)
Phycoerythrin, anti-rabbit	Santa Cruz (3745)
Phycoerythrin, anti-rat	BD pharmingen (550767)

6.1.3 Primary cells

- **Bone marrow human mesenchymal stem cells** (Lonza, CH, PT-2501)

The manufacturer tested their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages and showed positive expression for CD105, CD166, CD29, and CD44 and negative expression for CD14, CD34 and CD45 by flow cytometry²⁷¹.

- **FibroGRO™ Xeno-Free human foreskin fibroblasts** (Millipore, USA, SCC058)

FibroGRO Xeno-Free Human Foreskin Fibroblasts derived from normal human foreskin under xeno-free conditions²⁷².

- **Human cardiac ventricular fibroblasts** (Lonza, CH, CC-2904)

Cardiac ventricular fibroblasts (cvFB) were isolated from normal, adult heart tissue. The manufacturer performed routine characterization which included positive immunofluorescent staining for collagen I and negative staining for von Willebrand factor VIII related antigen by flow cytometry²⁷³.

- **Clonetics™ Umbilical Vein Endothelial Cell Systems** (Lonza, CH, CC-2517)

Human umbilical vein endothelial cells were certified by the manufacturer positive on Willebrand factor VIII related antigen, CD105 and CD31 endothelial markers and negative for alpha smooth muscle actin marker at passage four²⁷⁴.

- **Porcine cardiac fibroblasts**

Porcine cardiac fibroblasts were previously isolated and cultured in our laboratory.

6.1.4 Culture reagents for cell maintenance

Media for all cells cultured on tissue culture plate (static conditions) were supplemented with either 1% P/S and 0.4% fungizone or 1-2% ABAM. All cultures were maintained in 37°C and humidified atmosphere of 5% CO₂. The amount of antibiotics was doubled when working with the bioreactor (dynamic conditions).

Material	Manufacturer (cat. no.)
Antibiotic-Antimycotic (100X) ABAM (containing 10'000 units of penicillin, 10'000 µg of streptomycin and 25 µg of amphotericin B/ml)	Gibco, USA (15240-062)
Fetal bovine serum (FBS)	GE healthcare, UK (A15-151)
FGM-3 BulletKit™	Lonza, CH (CC-4526)
Fibroblast growth factor human recombinant	Millipore, USA (GF003)
FibroGRO™ Complete Media Kit	Millipore, USA (SCMF001)
Fungizone	Invitrogen, USA (15290026)
L-glutamine at 200 mM	Invitrogen, USA (25030-081)
MEM-Alpha	Biological Industries, IL (11-042)
Penicillin & Streptomycin,	Invitrogen, USA (15140-122)
TryPLE™ Select 10X	Invitrogen, USA (A12177-01)
Trypsin (0.5%, 10X, with 5.4mM EDTA)	Invitrogen, USA (15400-054)

6.1.5 Buffers and solutions

- Elution protein buffer
20 mM TrisHCl pH 7.7
150 mM NaCl
0.1% SDS, 2mM EDTA
- Formalin
0.5% formalin in PBS diluted from 37% stock solution
- Gelatin

- 0.2% Gelatin in PBS, sterilized
- Phosphate buffer saline (PBS) sterile
 - 8.1mM Na₂HPO₄
 - 1.47mM KH₂PO₄
 - 0.137M NaCl
 - 2.68mM KCl
 - pH= 7.4
- PFA
 - 2% PFA in PBS (diluted from purchased ampule of 16% PFA in DDW).
- Wash buffer before harvest bmMSC from pcECMs
 - EDTA at 0.625 mM in PBS

6.1.6 Transfection of cardiac fibroblasts with eGFP

- pTK113– eGFP plasmid²⁷⁵ (generously provided by Prof. Ben-Zion Levi, Faculty of Food and Biotechnology, Technion – Israel Institute of Technology)
- XL1-Blue competent cells (Stratagene, Cat. 200249)

The XL1-Blue strain allows blue-white color screening for recombinant plasmids.
- Lipofectamine® 2000 Transfection Reagent (Invitrogen, Cat. 11668-019)
- NanoDrop (N100, Thermo Scientific)

It measures microvolume by UV-Vis spectrophotometry and fluorospectrometry, fundamental for DNA, RNA and protein analysis.
- Opti-MEM® I Reduced Serum Medium (Invitrogen, Cat. 31985-070)

It has reduction in fetal bovine serum but with no change on the cell growth or cell morphology according to the manufacturer²⁷⁶.
- PureLink® HiPure Plasmid Filter MaxiPrep Kit
- ViraPower™ modified Lentiviral Packaging Mix (Invitrogen, Cat. K4975-00)

It contains all three packaging plasmids (pLP1, pLP2, and pLP/VSVG) to supply the functions, structural and replication proteins required to produce a recombinant lentivirus.

- Polybrene (hexadimethrine bromide, Sigma H9268)
- Zeocin™ (Invitrogen, Cat. 11006-33-0)

According to the manufacturer²⁷⁷, this antibiotic is a copper-chelated glycopeptide and causes cell death by intercalating into DNA and cleaving it.

6.1.7 Gel electrophoresis

6.1.7.1 stock solution

- 0.5 M Tris (HCl) buffer SDS pH 6.8 (kept at RT)

Tris (Trizma base minimum T1504-500g)	6.05 gr
SDS – sodium dodecyl sulfate	0.4 gr
Use HCl solution to fix pH at 6.8	
- 1.5 M Tris (HCl) Buffer SDS pH 8.8 (kept at RT)

Tris (Trizma base minimum T1504-500g)	45.5 gr
SDS – sodium dodecyl sulfate	10 ml
Use HCl solution to fix pH at 8.8	
- 10% SDS (keep at RT)

SDS (Trizma base minimum T1503-500g)	10 gr
Distilled H ₂ O	100 ml
- Sample Buffer (x4) (keep at -20°C)

0.5M Tris (HCl) buffer SDS pH 6.8	1 ml
SDS (10% 2/v)	1.6 ml
Glycerol	0.8 ml
2-mercaptoethanol	0.4 ml
0.5% Bromo phenol blue in ethanol	0.2 ml
Distiller H ₂ O	4 ml
- Running buffer (x5) (keep at RT)

Tris (Trizma base minimum T1503-500g)	15.5 gr
Glycine	72 gr
SDS	5 gr
Use HCl solution to fix pH at 8.8	
- Running buffer (1x) (keep at RT)

Running buffer (5x)	200 ml
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Distilled H₂O

800 ml

6.1.7.2 Gel electrophoresis apparatus

The gel electrophoresis apparatus was from Bio-Rad (Mini-PROTEAN Tetra Cell, 1.5 mm) and contained the common equipment components²⁷⁸.

6.1.7.3 Solutions for Silver staining

- Fixing solution
50% EtOH, 10% Glacial acetic acid
- Rinse solution
50% EtOH
- Sensitizer
0.02% Sodium Thiosulphate
- Stain
0.1% Silver nitrate (Sigma Ultrapure)
- Developer (for 500 ml)
2% Sodium carbonate (10 gr) , 200 ul (od 37% v/v) formaldehyde, 20 ml sensitizer
- Stop solution
1% acetic acid

6.1.8 Solutions for histology

6.1.8.1 Masson Trichrome staining

- Phosphate buffer saline (PBS) non sterile
8.1mM Na₂HPO₄
1.47mM KH₂PO₄
0.137M NaCl
2.68mM KCl
pH= 7.4
- Ascending alcohol concentrations for dehydration
50%, 70%, 80%, 90% and 100% EtOH in distilled water

6.1.8.2 Hematoxylin and eosin (H&E) staining

- Acid alcohol
0.5% hydrogen chloride in 70% ethanol
- Ammonium
0.05% ammonia in double distilled water
- Ascending alcohol concentrations for dehydration
80%, 95% and 100% EtOH in distilled water

6.2 Specific instruments and equipment

6.2.1 Agilent 2100 Bioanalyzer system

The Agilent 2100 bioanalyzer system (Agilent Technologies, USA) can provide sizing, quantitation and quality control of DNA, RNA (notably RNA integrity number), proteins and cells on a single platform.

6.2.2 BeadBug™ microtube homogenizer

The BeadBug™ (D1031E, Benchmark Scientific, USA) is a benchtop homogenizer that can simultaneously homogenize up to 3 samples. Rapid tissue disruption through constant impact is caused through high velocity impact from the hardened micro-beads chosen according to the type of tissue.

6.2.3 Cryotome

The cryostat (CM1950, Leica, DE) allows quick, reliable and safe cutting of frozen sections. Temperature and slide thickness can be adjusted.

6.2.4 Fluorescence-activated cell sorting (FACS) Calibur

Flow cytometer from BD Bioscience (USA) uses excitation at 488 nm and 635 nm with 4 filters: FL1 (530 nm, green), FL2 (585 nm, orange), FL3 (670 nm, red), FL4 (661

nm, far-red), SSC (488 nm, cell scattering), FSC (488 nm, cell dimension).

6.2.5 Fluorescence-activated cell sorting (FACS) GUAVA™

Similarly, the flow cytometer from Millipore (USA) uses two lasers, one red (635 nm) and one blue (488 nm) to achieve simultaneous 4-color detection (FITC, PE, PE-Cy5.5 and APC).

6.2.6 Fourier Transform Infrared Spectrometer (FTIR)

FTIR spectrometer (Frontier™) from Perkin Elmer (USA) with advanced Far-IR capability and electronic force gauge to ensure optimum reproducibility. It also includes a universal ATR sampling accessory.

6.2.7 Liquid scintillation analyzer

The liquid scintillation analyzer (Tri-carb 2800TR, Perkin-Elmer, USA) is a computer-controlled benchtop liquid scintillation analyzer for measuring small amounts of beta, gamma and alpha radioactivity.

6.2.8 Nanophotometer®

The NanoPhotometer® is a spectrophotometer manufactured by Implan (DE) and is optimized for quantification and purity assessment of nucleic acid and protein samples.

6.2.9 Microscopes

- Binocular microscope - Olympus SZX16. Filters (ex/em): green (460-480/495-540nm), red (535-555/570-625nm), UV (370-390/400-460nm). Objectives: SDFPLAPO 0.8x (NA=0.3, WD=81mm) and SDFPLAPO 1.6xPF (NA=0.3,

WD=30mm)

- INCell Analyzer 2000 – General Healthcare (GE). Automated cellular and subcellular imaging system for fixed and live cells. Objectives: x2 (NA=0.1, WD=8.5mm), x10 (NA=0.45, WD=4mm), x20 (NA=0.45, WD=7.5mm), x60 (NA=0.95, WD=0.15mm). Excitation and emission filters for DAPI, CFP, FITC, YFP, Texas Red, Cy3, Des Red, CY5
- Inverted fluorescent microscope (in Singapore) – Nikon Eclipse Ti. Filters (ex/em): green (488/507nm), red (558/583nm), UV (340/480nm). Objectives: x10 (NA=0.25, WD=0.7 mm), x20 (NA=0.5, WD=2.1mm).
- Inverted fluorescent microscope (in Israel) - Nikon eclipse TE 2000-S. Filters (ex/em): green (488/507nm), red (558/583nm), UV (340/480nm). Objectives: x4 (NA=0.1 , WD=0.3 mm), x10 (NA=0.3 , WD=0.16 mm), x20 (NA=0.45 , WD=0.74 mm), x40 (NA=0.6 , WD=0.27-0.37 mm).
- Inverted confocal microscope (LSM700, Carl Zeiss, Germany). The system includes 4 visible solid state lasers: UV laser - 405 nm (5mW), Blue laser - 488 nm (10mW), Green laser - 555 nm (10mW) and Red laser - 639 nm (5mW). Objectives: x10 (NA=0.3), x20 (NA=0.8), x40 (NA=0.6), x40 (oil, NA=1.3) and x63 (oil, NA=1.4).

6.2.10 pH meter

pH meter (Seven Easy™) from Mettler Toledo (CH) with Inlab micro pro sensor (5 mm diameter).

6.2.11 CFX96™ Real-time polymerase chain reaction detection system

Real-time polymerase chain reaction (RT-PCR) machine (CFX96, C1000 thermal cycler) manufactured by Bio-Rad (USA) combines a thermal cycler with an optical

reaction module for singleplex and multiplex detection of fluorophores and features thermal gradient functionality.

6.2.12 Varioskan™ Flash multimode plate reader

The Varioskan Flash reader from Thermo Scientific (USA) includes fluorescence intensity, time-resolved fluorescence (TRF), photometric and optional luminometric detections with unlimited wavelength selection for spectral analysis and measurement at any single wavelength.

6.2.13 Softwares

- Bio-Rad CFX manager (version 1.5) – Bio-Rad, for experiment setup and data analysis for the CFX96™ real-time PCR detection system.
- CellSens – Olympus, for acquisition and analysis on binocular microscope (Olympus SZX16)
- Flowjo (version 7.6.3), for analysis of flow cytometry data.
- Guavasoft (version 2.2.2) – Milippore, for flow cytometry data acquisition and analysis on FACS Guava™.
- ImageJ (version 1.440), a Java-based image processing program.
- InCell Analyzer 2000 (version 1.9.1) – GE Healthcare, for auto image acquisition
- InCell Investigator and Developer (version 3.7.1) – GE Healthcare, for advanced imaging segmentation and analysis.
- NIS Elements (version WR 3.2) – Nikon, for image acquisition and image processing on Inverted fluorescent microscope (Nikon eclipse TE 2000-S).
- ZEN light edition 2012 (version 1.1.1.0) – Carl Zeiss, for acquisition and analyses for confocal microscopy (LSM700).

6.3 Bioreactor materials and equipment

6.3.1 Customized equipment

- Customized seeding chamber for dynamic pcECM (as described in 7.3.2)
- Customized bioreactor (as described in 7.3.3).

6.3.2 Bioreactor equipment

Bioreactor equipment	Manufacturer (cat. no.)
2-L medium reservoir	Radnoti, USA (140143-2)
24G Catheters, 8CM long (non-sterile raw material)	Biometrix, IL (#3CT240)
50-CM manometer line M/F sterile	Biometrix, IL (AW-10500)
Autoclavable plastic luer tubing connectors	Cole-Parmer Instrument, USA
Female and male adapters at 1/4", 1/8" and 1/16" dimensions	
Combi stopper M/F (red)	Biometrix, IL (CW-02080)
Controllable peristaltic pump	Masterflex, DE (7523-70)
Glucose meter Freestyle™	Abbott laboratories, USA
Heparin lock (for sampling ports)	Biometrix, IL (CW-02050)
Lactate meter (Lactate scout)	EKF diagnostics, DE
Oxygenators	Radnoti, USA (C1468-130144)
Tubing in silicone (L/S 14, 16, 25)	Masterflex, DE
Tubing in tygon R-3603 (1/32"x3/32")	Saint-Gobain, FR (00057)
Spinner flask	Corning, USA (4500-3L)
Surgical thread	Assut Sutures, CH (650)

7 Methods

7.1 Studying and characterizing the porcine extracellular matrix in terms of its cell support ability under static conditions

7.1.1 Initial cell attachment and density studies on pcECMs

Human bone marrow mesenchymal stem cells (bmMSCs) were cultured in 10 cm-diameter cell culture plates with 25 ml/plate of medium (containing 500 ml of α -MEM with 50 ml FBS, 5 ml of antibiotic-antimycotic solution, 5 ml of L-glutamine at 200 mM and bFGF at $5 \cdot 10^{-3}$ μ g/ml). The medium was changed every 2-3 days. In the meantime, porcine extracellular matrix (pcECM) scaffolds were cut using surgical scissors and a punch hole of 8 mm in diameter. The pcECMs were disinfected with three washes of 70% EtOH in distilled water and three washes in PBS containing 10 ml of antibiotic-antimycotic solution under sterile conditions. They were subsequently placed into a low binding 96-well plate with the epicardium (whitish fatty-like tissue) positioned at the bottom part of the well while the endocardium facing up. Finally, 100 μ l of medium were added in each well to moisturize the pcECMs while the cells were being harvested.

For attachment study (see Fig. 4), cells were harvested by trypsinization and were diluted in medium to reach 0.3×10^6 cells per 80 μ l (seeding volume per well per pcECM, corresponding to $0.6 \times 10^6/\text{cm}^2$). The 100- μ l medium was removed from each pcECM prior seeding to slightly dry (1.5-2hr) the pcECM rendering it more cell-medium absorbent. Cells were seeded into each pcECM by pipettation using a normal 200- μ l pipette at different positions on the surface of the pcECM. The seeding process was

performed in 96-well plate placed at 37°C (5% CO₂) to let the cells attach for different incubation times (5, 45, 90 and 180 min, n=4 for each group). This was followed by quick PBS dipping to remove non-attached cells and immersion in a low-binding 12-well plate containing 2 ml of culture media per well (per ECM). For viability measurement, media was replaced with 2 ml media containing 10% AlamarBlue™. After 5 hours incubation with AlamarBlue™, 100 µl of each well in four replicates were transferred to a black 96-well plate for fluorescence reading on the Varioskan flash reader (excitation 540 nm, emission 585 nm).

Fig. 4 Schematics of experimental protocol for cell attachment and cell density studies in static conditions

Cells were seeded into pcECMs by injection with a standard 200-µl pipette at different positions on the surface of the pcECM. Various seeding densities at 0.1×10^6 , 0.5×10^6 , 2×10^6 and 4×10^6 cells per pcECM or different period of incubation time (5 min, 45 min, 90 min and 180 min) were compared in term of cell attachment.

For the density study (see Fig. 4), cells were harvested by trypsinization and were diluted in medium to reach the various seeding densities (at 0.1×10^6 , 0.5×10^6 , 2×10^6 and 4×10^6 cells, n=4 for each group) in 80 µl seeding volume per well per pcECM. The pcECMs were similarly prepared as in the attachment study. Cells were similarly seeded

into each pcECM by a 200- μ l pipette. The 96-well plate was placed at 37°C, 5% CO₂ for 90 min to let the cells attach. Each pcECM was subsequently dipped into PBS, washing non-attached cells, and was subsequently transferred into a low-binding 12-well plate well. The cell viability was similarly performed as described above (immediately after cell attachment).

Cell viability measurements obtained by AlamarBlue™ were translated into cell quantities or cell densities by using a calibration curve and dividing by the surface of one well from a 96-well plate. The calibration curve was determined by measuring the fluorescence of AlamarBlue™ added to 6-well plates (2 ml volume with 10% AlamarBlue™) with known cell concentrations in duplicates (usually at 0, 0.1875x10⁵, 0.375x10⁵, 0.75x10⁵, 1.5x10⁵, 3x10⁵ cells per well). These 6 points were used to create a linear curve to obtain an equation ($y = a \cdot x$) corresponding cell quantity (x-value) to the fluorescence level (y-value). Each cell type required its own calibration curve as the fluorescence values may slightly vary for different cell types.

7.1.2 Cell support improvement by bio-chemical modifications

7.1.2.1 Bio-chemical modifications of pcECMs

BmMSCs and pcECMs were similarly prepared as described in 7.1.1. After washing in 70% EtOH, pcECMs (n=5 per treatment) were immersed in 100 μ g/ml of laminin (LAM) in PBS, 10 μ g/ml of fibronectin (FN) in PBS, 0.4% gelatin (GEL) in PBS, 100% FBS, 0.65%wt of polycaprolactone (PCL) in acetone, and 0.1 cm²/ml or 1cm²/ml of nitrocellulose (NC) in MeOH (low and high concentration respectively) for 24h. PcECMs treated with PCL or NC were subsequently immersed in PBS for 1 hour to remove acetone or MeOH solvent respectively. In addition, some pcECMs were immersed in 1M NaOH or 1M HCl (sterile-filtered solutions) for 2 hours. Those were subsequently

washed in PBS until the pH from the last wash reached 7.4. We have chosen to use the above concentrations, which are within the ranges already reported in the literature or product protocol for each bioactive material (LAM^{279 280}, FN²⁸¹, GEL²⁸², NC²⁸³, PCL²⁸⁴, NaOH²⁸⁵, HCl²⁸⁵). All treated pcECMs were subsequently transferred into a low-binding 96-well plate and were seeded with 300,000 cells in 80 µl medium per pcECM. BmMSCs were let to adhere on pcECMs for 90 min at 37°C 5% CO₂. PcECMs were subsequently dipped into PBS to remove the non-attached cells and transferred into a low-binding 12-well plate. AlamarBlue™ was performed for each pcECM with 2ml volume medium (10% AlamarBlue™) right after the attachment incubation and every 2-3 days. Cell quantities were deducted from known cell-concentrations based calibration curve in 6 well plates using similar sampling ratios and incubation times. After every AlamarBlue™ reading, the medium was replaced with 2 ml fresh medium in each well.

7.1.2.2 FTIR characterization

PcECMs were treated with NC similarly as in 7.1.2.1. A single treated pcECM was cleaned either 1, 3 or 6 times in 1 ml PBS over 2 hours in total. Each pcECM was carefully disposed in the center of the FTIR platform and secured with a constant pressure of 85 Pa. The background (only air) and samples were measured with similar parameters (reading range from 400 to 4,000 cm⁻¹, 8 scan number per reading, resolution 4 cm⁻¹). After each reading, each FTIR curve was subtracted by the background and subsequently adjusted using baseline and smoothing corrections from the software. Cell attachment (with high seeding cell density at 3.0x10⁶/cm², one time point at day00) and cell viability (with seeding cell density at 0.6x10⁶/cm², during 10 days) of NC-treated pcECMs washed 1 and 6 times were further characterized by AlamarBlue™.

7.1.3 Cell support improvement by cell co-culturing

7.1.3.1 Porcine cardiac fibroblasts harvesting and culturing

Briefly, 5-6 months old porcine hearts were purchased from the Animal Research Institute at Kibbutz Lahav and were inserted into sterile 50 ml conical tubes and quickly rinsed (5min) with diluted (1% in PBS) Cetrin solution (1.5%w/v chlorhexidine gluconate and 15% Cetrimide) for disinfection. All subsequent procedures were performed in sterile conditions. Disinfected hearts were transferred into sterile 50-ml plastic tubes containing 20 ml of sterile PBS with 2% Pen-Strep® and 0.8% Fungizone® (PBS-2) for three times (20 min each) to wash excess Cetrin solution. The atria and right ventricles were then discarded and samples of the left ventricle (10x10mm) were minced in a sterile 10-cm plate containing 2 ml of PBS-2 into 1x1mm slices. Slices were sterile transferred (as dry as possible) to a different sterile 10 cm plate and positioned at a distance of approximately 1cm from one another. After 10 min subjected to sterile air, complete culture media (DMEM high containing 20% FCS (Fetal Calf Serum), 2%Pen-Strep® and 0.8% Fungizone®) was slowly added from the side. Plates were carefully transferred to a standard CO₂ incubator (5% CO₂ and 37°C) and cultured for 5 days prior to media replacement. From this point on, media was replaced twice a week. Adherent cells, which migrated from the explants into the plate (starting to appear at around 17 days post harvesting), were termed porcine cardiac fibroblasts (pcFBs). These cells were produced from the same explants for 4 consecutive cycles. Passaging was performed with standard trypsin digestion (0.05% cell culture certified trypsin. Freezing media: 50% complete culture media + 40% FCS + 10% DMSO).

7.1.3.2 Comparison of fibroblasts on pcECM for optimal model

Human Cardiac ventricular fibroblasts (cvFB), human foreskin fibroblasts (fFB) and porcine cardiac fibroblasts (pFB) were seeded on pcECMs similarly as in 7.1.1 (n=5 per group, 300,000 cells/pcECM, 90 min attachment, replenishment of 2 ml medium/well

every 2 days) and cultured on pcECM for over 20 days. Cell viability was measure by AlamarBlue™ every 2-3 days (10% in 2 ml volume for 5 hrs).

7.1.3.3 Cardiac ventricular fibroblast characterization by flow cytometry on tissue culture plate

CvFBs were cultured in similar medium to bmMSCs (aMEM + bFGF) on tissue culture plate. Immunofluorescence staining was performed following the flow cytometry protocol (7.4.1). CD73, CD90, CD44 (Biolegend 103001), CD105 and CD31 required no permeabilization, whereas DDR2, vWF and α -SMA required a permeabilization step with Triton-X-100. Fluorescent staining was performed with either PE anti-mouse (BD Biosciences 550589, dilution 1:100) or PE anti-rat (BD Biosciences 550767, dilution 1:100).

7.1.3.4 Stem cell and fibroblast co-culture systems on pcECMs

Human cvFBs or fFBs and bmMSCs were cultured on pcECMs at different ratios (0-100, 25-75, 50-50, 75-25 and 100-0%) similarly as in 7.1.1 (300,000 cells/pcECM, 90 min attachment, 2 ml medium/well replenished every other day) and cultured on pcECM for over 20 days. Cell viability was measure by Alamar Blue™ every 2-3 days (10% in 2 ml volume for 5 hrs). Similar experiment was performed with HdffFB and bmMSCs. At the end of experiment, samples were finally subjected to OCT freezing and Masson Trichrome staining (see 7.4.2 and 7.4.4).

7.1.3.5 Transfection of cardiac ventricular fibroblasts with eGFP

Plasmid amplification:

PTK-eGFP plasmids containing ampicillin resistance and aminoglycoside phosphotransferase (PGK-NeoR) for selection were generously given by Prof. Ben-Zion Levi (Faculty of Food and Biotechnology, Technion – Israel Institute of Technology, Haifa, Israel). This construct consisted of two transcriptional units that were incorporated into a lentiviral vector backbone (pTK113, a self inactivating (SIN) HIV-1 vector). The first unit included the HygR–eGFP fusion protein under the transcriptional control of the human MLC-2v promoter. The second transcriptional unit contained the PGK promoter driving the expression of aminoglycoside phosphotransferase (PGK-NeoR), which was meant to allow selection.

XL-1 blue competent cell suspension was thawed on ice for 40 minutes. 0.5 µl of 2.5 µg/µl plasmid concentration was added to 100µl (approximately 10^9 cells/µl) cell suspension and allowed to incubate on ice for another 40 min. The mixture was heat-shocked at 42°C for 90 s followed by 5 min incubation on ice. Cell suspension was cultured in LB (Luria-Bertani) broth for 1 hour in a shaking incubator at 37°C. Subsequently, a 100-µl sample was evenly spread (using the Drigalski method/spatel) on a standard agar plate containing ampicillin (100mg/l in LB broth). The bacterial colonies were cultured overnight in the incubator at 37°C and single colony was collected using a sterile pipette tip and cultured in 100 ml LB broth in a sterile conical flask in shaking incubator for 12-18 hours. The amplified plasmid DNA was harvested using PureLink® HiPure Plasmid Filter Maxiprep Kit. Briefly, cells were harvested by centrifugation at 4,000 g for 10 min and cell pellet was resuspended in RNase A and lysed with lysis buffer. The lysate was washed through the DNA Binding Column, which combines both steps of clearing the bacterial lysate and binding the DNA directly to the anion-exchange

resin. After several washing to remove RNA, proteins, carbohydrates and other impurities, only plasmid DNA remained bound to the resin, which was eluted and concentrated by washing with isopropanol and 70% ethanol with centrifugation at $>12,000$ g for 30 min at 4°C after each wash. DNA pellet was air dried for 10 min, resuspended in TE buffer, and stored at -20°C until further use. The DNA concentration and purity were checked using NanoDrop (N100, Thermo Scientific) with values reasonably close to 1.8 and 2.0 for OD 260/280 and 260/230 respectively).

Lentivirus production:

The lentiviral stock (containing pLenti expression construct with the gene of interest, i.e. eGFP) was produced by co-transfecting the packaging plasmid mix and pLenti expression construct into the 293FT cell line using the ViraPower™ kit (Invitrogen).s Briefly, 293T cells were plated in T75-flasks at 85-90%. The next day, the medium was removed and replaced with 5 ml Opti-MEM (with serum). For each transfection flask, DNA-Lipofectamine™ 2000 cocktail was prepared as followed: i) 9 μg of the ViraPower™ Packaging Mix and 3 μg of pLenti expression plasmid DNA (gene of interest) were diluted in 1.5 ml Opti-MEM in a sterile 5-ml tube ii) 36 μl Lipofectamine™ 2000 was added in 1.5 ml of Opti-MEM in a separate 5-ml tube iii) both tubes were incubated for 5 min at room temperature and were mixed gently iv) both 5-ml tube contents were combined and incubated for 20 min at RT to allow the DNA-Lipofectamine™ 2000 complexes to form and DNA-Lipofectamine™ 2000 complexes were added dropwise to one T75-flask. After 6 hrs, the medium containing the DNA-Lipofectamine™ 2000 complexes was removed and replaced with 10 ml complete medium for cardiac fibroblasts. For all transfection steps, no antibiotic was used as it decreases considerably the efficiency of lipofectamine. The virus-containing media supernatants were harvested 72 hours post-transfection (Day 04) and centrifuged once at 3,000 rpm for 15 minutes at

4°C to remove debris. Supernatants were finally aliquoted in 1.5-ml microcentrifuge tubes and stored at -80°C until further use.

Transduction:

P2 human cvFB were cultured on T75-flasks. At 50% cell confluency, the medium was changed to 7 ml medium (with serum but no antibiotics) and 1 ml of frozen PTK virus-containing supernatant for each flask (ratio determined from previous optimization experiment). Polybrene was added to reach a final concentration of 10 µg/ml to increase cell transduction efficiency with lentivirus. After an overnight incubation, the viral medium was changed and replaced by fresh complete medium. The cell were split when they reach confluency and re-transduced with PTK viruses after splitting (similarly as the first transduction).

Selection:

Three days after transduction, cvFBs were treated with Zeocin at 50 µg/ml concentration in cell medium for another 3 days to select the successfully transduced cvFBs.

7.1.3.6 Quantification of Connexin43 in stem cell/fibroblast co-culturing system on tissue culture plate and pcECMs

CvFB-eGFPs and bmMSCs were seeded at different concentrations (0-100, 25-75, 50-50, 75-25 and 100-0%) with 150,000 cells per well on a 24-well optical-bottom plate. 24 hours later, cells were fixed with 0.5% formalin for 10 min at room temperature, washed 3 times with PBS, and subsequently blocked with 0.5% BSA for 1 hour. All wells were then incubated with 200 µl volume of rabbit anti-human Cx43 (Sigma C6219, dilution 1:1000) overnight at 4°C. After 3 washes in PBS, wells were incubated with anti-rabbit PE conjugated secondary antibody (Santa Cruz 3745, dilution 1:200) for 1 hour in the dark and washed again twice in PBS. The cells were finally stained with Hoescht

33258 (dilution 1:2000) for 15 min and rehydrated in PBS.

The bmMSC/cvFB co-cultures were imaged with the INCELL analyzer, using a Nikon 60x/0.95 objective (20 fields per well randomly). Nuclei staining (Hoescht) was measured with excitation wavelength of 350 nm, emission wavelength of 455 nm and exposure time of 120 ms. CvFB-eGFP (eGFP): excitation wavelength 490 nm, emission wavelength 525 nm and 150ms exposure time. Cx43 organelles (PE): excitation wavelength 534 nm, emission wavelength 605 nm, exposure time 400 ms. Image quantification analyses were performed using the INCELL investigator and Microsoft Excel software. Cell counts and Cx43 organelle counts per field were obtained from RAW data sheet obtained from INCELL investigator and transferred to an excel file. Those parameters were averaged according to cell number. Finally, one field per well was chosen for image post-processing by imageJ.

On pcECMs, cvFBs and bmMSCs were seeded at different concentrations (0-100, 50-50 and 100-0%) with 300,000 cells/pcECM (attachment time 90 min, 10 days cultivation time). PcECMs were fixed in 4%PFA and stained overnight with Connexin43-FITC (Invitrogen 138388) at 1:200. Organelle quantification was performed using ImageJ software. Each image was first converted into greyscale before proceeding (*Image → Type → 16-bit*) where “*scale when converting*” option should be selected (*Edit → Options → Conversion*). To select the desirable structures (in our case cell nuclei or Cx43 organelle), the threshold (*Image → Adjust → Threshold*) was adjusted so that only those structures were highlighted in red. A new image was subsequently created with two pixel intensities only (white = 0 and black = 255). All images (n=3 per group) were analyzed for particle counting (*Analyze → Analyze particles*, with 0 or 50 to infinity for size pixel and 0-1 for circularity, show *outlines*). Total number of counts and dimension for each area were

collected and divided by total cell number to obtain an average organelle count per cell.

7.2 Characterizing the effects of porcine extracellular matrix on reseeded cells and possible differentiation induction under static culture conditions

7.2.1 Phenotypic characterization of stem cells seeded on pcECMs

BmMSC and pcECMs were similarly prepared as described in 7.1.1. PcECMs were seeded with an initial number of 300,000 bmMSCs, culture media was replenished every other day for 4 weeks to achieve maximal cell quantity on the pcECM for FACS analysis. To harvest bmMSCs from pcECMs, each pcECM was washed twice with EDTA at 0.625 mM in PBS and was transferred into 0.5 ml of Tryple 10X in a 1.5-ml eppendorf tube. After 8 min incubation, pcECMs were removed and 0.5 ml of complete culture media (containing 10-20% FBS) was added in each eppendorf before centrifugation at 300 g for 5 min. All cell pellets were collected in one single tube, washed twice with cold (4°C) PBS and followed immunofluorescence staining protocol with formaldehyde against CD29 (dilution 1:50), CD44 (Biolegend 103001, dilution 1:50), CD73 (dilution 1:50), CD90 (dilution 1:50), CD105 (dilution 1:50) and CD31 (Biolegend 303102, dilution 1:50) with either PE anti-mouse (BD Biosciences 550589, dilution 1:100) or PE anti-rat (BD Biosciences 550767, dilution 1:100) for flow cytometry analysis (7.4.1).

7.2.2 Quantitative RT-PCR of stem cells seeded on pcECMs for endothelial-related genes

BmMSCs were seeded and cultured on pcECMs in static conditions similarly as in 7.1.1 (300,000 cells/pcECM, 90 min attachment, 2 ml medium/well replenished every other day). After 25 days of culturing, seeded pcECMs (n=4) were snap frozen by placing them in empty 2-ml tubes immersed in iso-propanol at -80°C or in dry ice for at least 2

hours. BmMSCs were also cultured on simple tissue culture plates to serve as control.

In the meantime, 3 Beadbug™ pre-filled tubes (3mm, zirconium), sterile culture plates, sterile tweezers and sharp scalpels were cooled down at 4°C for an hour. Each Beadbug™ tube was subsequently filled with 700 µl Qiazol lysis reagent under a chemical hood. The frozen pcECM samples were then quickly shredded as small as possible on cold sterile tissue culture plate using sterile scalpels and tweezers, and dispensed into the 3 Beadbug™ tubes in equal amount. All 3 tubes were placed in the Beadbug™ homogenizer (Benchmark Scientific) and shaken for 3 min at 3,000 rpm. The homogenates were subsequently incubated at RT for 5 min. The miRNeasy mini kit (Qiagen) was used to isolate and purify RNA from the bmMSC-seeded pcECMs. Briefly, 140 µl chloroform was added in each tube which was subsequently shaken vigorously for 15 sec and incubated for 2-3 min at RT. After centrifuging the tubes for 15 min at 12,000 x g at 4°C, the upper aqueous phase was transferred to new sterile collection tube (avoiding transferring any interphase) and 525 µl 100% EtOH was mixed by pipetting thoroughly to each tube. The three samples were subsequently pipetted into one single RNeasy® mini column in a 2-ml collection tube which was centrifuged at 8,000 x g for 15 sec to discard the flow-through (this step was repeated several times to concentrate 3 samples into one single RNeasy® mini column). The RNeasy® mini column was subsequently centrifuged at 8,000 x g with 700 µl RWT buffer at for 15 sec, 500 µl RPE buffer for 15 sec and again 500 µl RPE buffer but for 2 min (after each centrifuge, the flow-through was discarded). The RNeasy® mini column was transferred into a new collection tube and 50 µl RNase-free water was pipetted directly into the column. The RNA was eluted in the 2-ml collection tube after centrifuging for 1 min at 8,000 x g, divided into 10-µl samples and stored for further usage at -80°C. RNA quality was assessed by measuring the optical density (OD) at 260/280 (nucleic acids / protein ratio),

the OD at 260/230 (nucleic acids / guanine), the nucleic acids concentration and the RNA integrity number (RIN) using the nanophotometer® (Implen) and the bioanalyzer (Agilent 2100). The RNA from cell pellet was isolated similarly to seeded pcECMs but without using the Beadbug™ homogenizer. In brief, bmMSCs were harvested and the cell pellet was washed once in PBS. The cell pellet was subsequently resuspended in 700 µl Qiazol lysis reagent by pipetting thoroughly. The next steps were similarly performed as for the seeded pcECMs (starting from the 5-min incubation of the Qiazol homogenates). For all experiments, RIN values of >7 and 260/280 OD ratios of 1.8-2.0 were used to ensure RNA quality.

Prior using the RT² PCR profile array kit for endothelial-related gene profile (Qiagen), the frozen purified RNA sample to be tested, reagents from RT² First-strand kit and RT² SYBR Green qPCR mastermix (Qiagen) were put on ice for 20 min to thaw. In the meantime, DNA-free workspace was prepared by spraying surface with EtOH and exposing pipettors to UV. The protocol for RT² PCR profile array (n=2 per sample) included 4 steps: 1) preparation of cDNA from purified RNA using RT² first strand kit (containing buffer GE, RNase-free water, 5x buffer BC3, Control P2, RE3 reverse transcriptase mix) 2) mixing cDNA to RT² SYBR Green Mastermix (containing 2x RT² SYBR® Green Mastermix) 3) aliquoting the mix into RT² profiler PCR arrays and 4) temperature cycling in real time cycler. The following reagent amounts were prepared for one single RT² PCR profile array. Briefly, the genomic DNA elimination mix was first prepared by adding 0.5 µg purified RNA, 2 µl buffer GE and RNase-free water to reach a total volume of 10 µl. It was subsequently incubated at 42°C for 5 min using the CFX96 RT-PCR system (Bio-Rad) and placed immediately on ice for at least 1 min. In the meantime, the reverse-transcription mix was prepared by mixing 4 µl of 5x buffer BC3, 1 µl control P2, 2 µl RE3 reverse transcriptase mix and 3 µl RNase-free water. The reverse-

transcription mix was subsequently mixed by gently pipetting to the genomic DNA elimination mix. The 20- μ l mix was then incubated at 42°C for exactly 15 min, followed by 5 min at 95°C to stop the reaction. 91 μ l RNase-free water was added and mixed by gentle pipetting. The final mix (cDNA synthesis reaction) was put on ice while preparing the RT² SYBR[®] Green Mastermix reaction. In 5-ml sterile tube, 1248 μ l RNase-free water and 1350 μ l of 2x RT² SYBR[®] Green Mastermix were mixed together and 102 μ l cDNA synthesis reaction was subsequently added to the mix giving the final PCR component mix (for a total volume of 2700 μ l). The RT² PCR profile array was carefully opened and 25 μ l of PCR component mix were dispensed in each well using either a single pipettor or a multipipettor and a RT² PCR array loading reservoir with filtered tips. A N95 mask was worn during this process to avoid contamination. The array was subsequently tightly sealed with the optical 8-caps strips provided by the manufacturer and was centrifuged at 1,000 x g at RT for 1 min to remove bubbles. Persistent bubbles were removed with 25 G needles. The RT² PCR array was finally placed inside the CFX96™ RT-PCR system with the following cycling conditions: 1 cycle at 10 min at 95°C, 40 cycles including one step for 15 sec at 95 °C, one step for 1 min at 60°C and collection of fluorescence data.

The entire analysis methodology is based from the analysis section from the handbook as provided by the manufacturer, Qiagen (NL). For all sets of data, the threshold (from the parameter settings of the CFX96 software) was set similarly and as low as possible (avoiding background noise) when looking at the C(t) curves in log view. After setting the threshold, the positive PCR controls (PPC) present on the RT PCR array should reach approximately 18 C(t) values. The baseline was manually selected by choosing the x-axis range area where there is no C(t) curve, most frequently from 2 to 12. The C(t) values were exported and copied in a excel template (provided by Qiagen) which

was uploaded on their web-based software. Quality control, fold regulation and plot charts were obtained and easily exported for further analysis processing. Data quality control for each RT PCR array run included 2 criteria. By comparing to Positive PCR control (PPC), the Reverse Transcription control (RTC) is passed if $C(t)_{\text{RTC}} - C(t)_{\text{PPC}} \leq 5$. If ≥ 5 , it suggests that the reverse transcription step was slightly altered. In these experiments, we obtained values between 5 to 6.5 which was still acceptable according to the company help support team. In addition, Genomic DNA control (GDC) is supposed to be higher than 35 to confirm no DNA contamination in RNA sample (as obtained in most data sets, except a few data sets with ≥ 32 which was still acceptable). Briefly, fold regulation (which represents fold-change results in a biologically meaningful way) was obtained by the web-based software using the following equations. First of all, automatic selection from Housekeeping Gene (HKG) panel is used to perform normalization of all data sets. ΔC_T was thus calculated for each pathway-focused gene in each plate using the C_T values for the gene of interest (GOI) and the housekeeping genes used for normalization (HKG) using the equation (1). Statistical significance was obtained by comparing ΔC_T values from samples to control replicates. When biological and/or technical replicates are performed, the average ΔC_T value of each gene (each well) was calculated across those replicate arrays for each treatment group. $\Delta \Delta C_T$ for each gene across 2 RT2 Profiler PCR Arrays (or groups of samples) was further calculated using equation (2) where group 1 is the control sample or group of control samples and group 2 is the experimental sample or group of experimental samples. The fold-change for each gene was calculated from group 1 to group 2 using equation (3). Finally, if the fold-change is greater than 1, the result may be reported as a fold upregulation. If the fold-change is less than 1, the negative inverse of the result may be reported as a fold downregulation. However, a cut-off value was set at ± 2 , with upregulated genes with positive fold regulation values and for downregulated genes with negative values. In

addition, statistical p-values were calculated using t-test from ΔC_T values comparing group 1 and group 2. To efficiently associate the genes showing down/up RNA expression, GNCpro, a free online software developed and maintained by SABiosciences (from Qiagen, NL), was used to collate those genes and deduce potential biological pathways and processes by viewing the interactions among multiple genes simultaneously.

$$\Delta C_T = C_T^{\text{GOI}} - C_T^{\text{AVG HKG}} \quad \text{equation (1)}$$

$$\Delta \Delta C_T = \Delta C_T (\text{group 2}) - \Delta C_T (\text{group 1}) \quad \text{equation (2)}$$

$$\text{Fold change} = 2^{(-\Delta \Delta C_T)} \quad \text{equation (3)}$$

$$\text{if Fold change} > 1 \quad \rightarrow \text{Fold regulation} = \text{Fold change} \quad \text{equation (4)}$$

$$\text{if } 0 < \text{Fold change} < 1 \quad \rightarrow \text{Fold regulation} = - (\text{Fold change})^{-1} \quad \text{equation (5)}$$

7.2.3 Quantitative RT-PCR of stem cells seeded on pcECMs for cardiac related genes

BmMSC were seeded and cultured on pcECMs in static conditions similarly as in 7.1.1 (300,000 cells/pcECM, 90 min attachment, 2 ml medium/well replenished every other day). After 25 days culturing, seeded pcECMs (n=4) were snap frozen by placing them in empty 2-ml tubes immersed in iso-propanol at -80°C or in dry ice for at least 2 hours. Additionally, bmMSCs were cultured on simple tissue culture plates to serve as control.

RNA isolation from seeded pcECMs was similarly performed with the Beadbug™ homogenizer (Benchmark Scientific) and miRNeasy mini kit (Qiagen) as in 7.2.2. RNA quantification with QBiomarker iPSC PCR Array for cardiomyocytes differentiation (Qiagen) was performed using RNA isolated from bmMSC cell pellet (n=2) and bmMSCs

seeded on (n=2) after 25 days. Because the array contains 4 replicates of each cardiac-related genes (and housekeeping genes), all 4 RNA extracted samples were run using one single array. Briefly, the RT² First-strand kit was used similarly as in 7.2.2. To prepare each four RT² SYBR[®] Green Mastermixes, 310.5 µl RNase-free water and 337.5 µl of 2x RT² SYBR[®] Green Mastermix were mixed together and 27 µl of each cDNA synthesis reaction was subsequently added to the mix giving the final PCR component mix (for a total volume of 675 µl) in individual 1.5-ml tube. 25 µl of each cDNA reaction mix (total of 4) was finally dispensed in all 4 gene replicates. The qPCR on CFX94 machine and analysis were performed similarly as in 7.2.2.

7.2.4 Collagen metabolic assessment by H³-proline measurement from cardiac ventricular fibroblasts on pcECMs

CvFBs (p8) were seeded and cultured on pcECMs (day 0) in static conditions similarly as in 7.1.1 (300,000 cells/pcECM, 90 min attachment, 2 ml medium/well replenished every other day). At day 1, 4, 10, 15 and 18, the following steps were performed. All pcECMs were taken for cell viability measurement by Alamar Blue[™] (10% in 2 ml volume for 5 hrs) and re-suspended in fresh media except for 2 pcECMs without cells (control) and 3 pcECMs seeded with cvFB (sample, n=3). Those pcECMS were transferred into a sterile 24-well plate and were incubated in 1 ml medium volume with 10 µl H³-proline/ml for 24 hrs at 37°C. Each pcECM was subsequently washed 3x in 15 ml PBS with mild shaking for 30 min to remove un-metabolized radioactive proline and minced with surgical scissors. PcECMs were solubilized in 500 ul 0.5 M acetic acid with 10mg/ml pepsin bovine in a microcentrifuge tube with a magnetic stirrer at 4°C for 3-4 days before neutralizing each collagen sample solution with 50 µl 5M NaOH.

SDS-PAGE gel preparation was performed as followed. The gel cassette stand was assembled for 1 gel with a spacer plate, a short plate and a casting frame. In one 50-ml

tube, 6% running gel was prepared by mixing 5.8 ml H₂O, 2.475 Tris pH 8.8, 1.5 40% acrylamide, 200 μ l glycerol and subsequently 10 μ l Temed and 50 μ l APS. As the reaction happens fast, the mixture was quickly poured into the casting frame. Water was added gently on top of it to settle the gel. After the running gel solidified (40-60 min), the water was gently removed and 4% stacking gel was prepared by mixing 2.25 ml H₂O, 0.875 ml Tris 6.8 pH, 0.3475 40% acrylamide and subsequently 4 μ l Temed and 15 μ l APS. The mixture was poured on top of the solidified running gel and a 10-well comb was quickly placed in the casting gel. After the stacking gel has solidified (30 min), the entire gel cassette sandwich (the polymerized stacking and running gels in between the spacer plate and short plate) was fixed to the electrode assembly, which was transferred slowly into the tank filled with running buffer 1x (the gel was immersed). The comb was slowly removed before loading the samples.

Fifty μ l of each sample was mixed with 17 μ l sample buffer (sample buffer 4x to 100% glycerol ratio of 5:1) and were pre-heated at 90°C for 10 min. Each well was loaded with 50 μ l sample and the gel ran at 100 V for 1.30 hrs. The gel was then carefully moved onto a gridded glass surface and according to the visible BioRad ladder, each column up to 100 kDa was cut and transferred into a 2-ml scintillation glass vial. The gel pieces were cut with a surgical scissors and immersed in 1 ml elution protein buffer (see Materials) for 24 hrs on mild shaking at 40° C (including 1-2 hrs at 60°C). Subsequently 15 ml QuickSafe A was added to each 2-ml vial and Beta-radiation measurement was read using a scintillation counter. In one single experiment, the gel was not cut for scintillation counting but prepared for silver staining to verify collagen dissolution process and the position of the collagen bands. All steps were performed under a chemical hood with mild shaking. Briefly, the gel was removed from the glass cover and placed in several gel volumes of milli-Q water for 30 min. It was subsequently fixed in

approximately 200 ml of fixing solution (50% EtOH, 10% glacial acetic acid) for 10 min. The gel was rinsed for 5 min in the rinsing solution (50% EtOH) for 2 min and subsequently rinsed in the sensitizing solution (0.02% sodium thiosulphate) for 2 min. The gel was washed with milli-Q water for another 2 min. It was then stained in cold staining solution (0.1% silver nitrate) for 30 min and subsequently washed twice in water for 1 min. The gel was immersed in developer solution (2% sodium carbonate (10 gr), 200 μ l (od 37% v/v) formaldehyde, 20 ml sensitizer) for 1 min (no more than 10 min) until bands appeared. To stop the developer reaction, the stop solution (1% acetic acid) was added for at least 5 min. The gel was finally washed 3 times in milli-Q water before scanning. The entire experiment is represented in schematic in Fig. 5.

Fig. 5 Schematics of experimental protocol of cvFB collagen metabolic assessment from cvFBs seeded on pcECMs

CvFBs were seeded and cultured on pcECMs. Seeded pcECMs were incubated for 24 hours with H3-proline, which was incorporated in the newly synthesized collagen. After several washes, pcECMs were solubilized and samples run in SDS-PAGE gel. Because collagen bands are known to appear in between 100 and 150 MW kD, the areas corresponding to those lengths were cut and prepared for scintillation counting.

Radiation safety routine was followed in accordance with the regulations approved by the Radiation Safety Unit at the Technion Israel Institute of Technology. All contaminated equipment and working areas were well labeled. Radioactive solid waste was accumulated separately from normal waste and transfer to confined radioactive area

for further disposal. Liquid waste was disposed in a plastic container filled with absorbent. Since tritium is a low beta-radiation emitter, there was no need of plexiglass shield. Tritium is not dangerous externally (no skin penetration) but could be hazardous by inhalation or absorption through skin. Thus, common safety wear included laboratory coat, goggle and double gloves.

7.2.5 Quantitative RT-PCR of cardiac ventricular fibroblasts seeded on pcECMs for ECM modeling-related genes

CvFBs were seeded and cultured on pcECMs in static conditions similarly as in 7.1.1 (300,000 cells/pcECM, 90 min attachment, 2 ml medium/well replenished every other day). After 10 and 20 days culturing, seeded pcECMs (n=4) were snap frozen by placing them in empty 2-ml tubes immersed in iso-propanol at -80°C or in dry ice for at least 2 hours. CvFBs were also cultured on simple tissue culture plates to serve as control.

RNA isolation from seeded pcECMs was similarly performed with the Beadbug™ homogenizer (Benchmark Scientific) and miRNeasy mini kit (Qiagen) as in 7.2.2. RNA quantification with RT² profiler PCR arrays of genes related to extracellular matrix and adhesion molecules (Qiagen) were performed using RNA isolated from cvFB cell pellet (n=2) and cvFBs seeded on pcECMs (n=2) after 20 days.

7.3 Achieving dense recellularization under dynamic conditions

7.3.1 Decellularized porcine extracellular matrices

Decellularized porcine extracellular matrices (pcECMs) were obtained from our research group in Technion-Israel Institute of Technology (Drug Delivery & Mammalian Cell Technology, Faculty of Biotechnology & Food Engineering, Haifa - Israel). Our group has previously reported a decellularization procedure based on trypsin/triton perfusion of porcine heart left ventricles, which is described elsewhere for thicker tissue

slabs (10-15 mm)²⁸⁶. The results have showed after decellularization that the remaining scaffolds were thick (10-15mm), non-immunogenic and cell-supportive pcECM scaffolds, preserving both native tissue ultra-structural properties and inherent vascular network (see Fig. 6).

Fig. 6 Decellularized pcECM and its inherent vasculature

PcECM slab after decellularization and corresponding inherent vasculature by mold casting after ECM removal.

7.3.2 Seeding chamber for static pre-seeding

A customized rectangular seeding chamber for dynamic pcECM slab was developed to be able to seed cells on the scaffold endocardial surface (see Fig. 7). The chamber and window chamber are made of polyethylene and polycarbonate respectively to allow sterilization by autoclaving. It is composed of 3 rectangular wells (5x2 cm), which usually fit the surface of our pcECM slabs.

Fig. 7 Schematics of customized rectangular seeding chamber

A seeding chamber pcECM slab was customized to allow seeding on the myocardial surface of pcECM slab. The chamber and window chamber are made of polyethylene and polycarbonate respectively to allow sterilization by autoclaving. It is composed of 3 rectangular wells (5x2 cm), which usually fit the surface of our pcECM slabs.

7.3.3 Bioreactor system and equipment

The bioreactor is mainly composed of a round platform with two chambers (see Fig. 8). In each chamber, a pcECM can be mounted and remains fixed using a blocker at each of its extremities. The pcECM slab vascular network main inlet and outlet are sewed to two catheters, which are connected to the bioreactor catheter connection inlet and outlet respectively via male-female luer-connections. The medium flows from the tubing system to enter the inlet connection to the inlet catheter and infiltrate the pECM via the vasculature. It will overflow from the chamber and is rapidly drained through a drainage hole as the bioreactor platform is slightly inclined (see Fig. 8B). A hard glass cover closes the bioreactor and protects it from any contaminated air. Finally, an air conduit allows bringing the desired oxygen/CO₂ environment inside the bioreactor.

The bioreactor also offers the possibility to induce mechanical and electrical stimuli to the pECMs. A silicone tubing is placed in each chamber under the pECM. As these tubings can inflate and deflate with pressure, it provides a mechanical stimulus that mimics the physical beating of the heart. Another conduit provides an entrance in the bioreactor for electrodes in order to electrically stimulate the pECMs.

Fig. 8 Schematics of customized bioreactor (colors are fictive)

(A) The bioreactor contains two chambers where a pECM is placed. The medium infiltrates the pECMs, fills up the chambers and is drained by the drainage hole using the inclination of the bioreactor platform (B). The bioreactor also allows mechanical and electrical stimuli through an inflating tube and an electrode entrance (C-D).

The complete bioreactor set-up is composed of the bioreactor itself and the oxygenator (placed in the incubator), the medium reservoir and the peristaltic pump (placed under a sterile hood). In brief, by the peristaltic pump transported the medium in the reservoir to the oxygenator filled with the suitable oxygen/CO₂ level from the incubator. As the tubings are made of silicone, it allows gas exchange to the medium. The medium reaches the bioreactor through a tygon tubing (to prevent any gas exchange) and enters the pECM through the inlet catheter, which is connected to the main vasculature. The medium fills the chamber and overflows to be finally drained by the drainage hole and comes back to the reservoir via the peristaltic pump (see Fig. 9).

Fig. 9 Schematics of bioreactor system (colors are fictive)

The medium is pumped through the peristaltic pump and sent to the oxygenator for gas transfer of oxygen/CO₂ concentration suitable for cell activity. It reaches the bioreactor and infiltrates the pECMs and comes back to the medium reservoir.

7.3.4 Bioreactor assembly

All tubings and luer connectors were assembled to connect the entire perfusion system in the following order: peristaltic pump, oxygenator, bioreactor, medium reservoir and back to the peristaltic pump. All tubings were in silicone except the tubing going from the oxygenator to the bioreactor, which was in tygon to avoid any gaz exchange. Particular attention was paid to the first tubing entering and exiting the peristaltic pump (and going towards the oxygenator) as it would determine the flow rate going in the bioreactor. In addition, the tubing draining back the medium to the reservoir was either similar or bigger dimension than the first tubing to ensure that no medium would accumulate in the bioreactor. To switch from different tubing dimension, female and male luer connectors were used to make the bridge and additional plastic clamps were applied to secure the tubing-luer connection.

For practical reasons, a standard CO₂ incubator was located next a standard biological hood to allow easy transfer of the bioreactor between both sterile environments without disconnecting any tubings. The bioreactor was located in the incubator when culturing cells and in the biological hood whenever manipulation related to the pECM or

the bioreactor was required. To supply suitable gaz conditions to the medium, the oxygenator was connected (in addition to the medium system) to an air pump and both were located in the incubator. At all time, the medium reservoir was placed in the biological hood whereas the peristaltic pump was located outside.

Any new material or equipment entering a sterile environment (like the incubator or the biological hood) was either autoclaved or ethanol sprayed. In addition, any manipulation related to the perfusion system (adding or changing any tubing or luer connector) was also ethanol sprayed.

7.3.5 Standard operating procedure

7.3.5.1 Bioreactor sterilization

Prior any dynamic experiment, the oxygenator, the bioreactor and the glass cover were autoclaved and assembled to the perfusion system. The entire bioreactor system (including the tubings, the bioreactor and the oxygenator) was perfused several times with 70% EtOH for a few days, subsequently with PBS containing 10% antibiotics (ABAM) for another few days using the peristaltic pump.

7.3.5.2 Culturing routine

Culturing cells on pcECM slabs in dynamic conditions requires several procedures to be performed routinely and parameters to be controlled to maintain suitable cell homeostasis and viability.

Medium replacement

Similarly as in static culturing conditions, cells in dynamic conditions require medium to be changed every 2-3 days. The bioreactor with all its connected tubings was slowly

transferred from the incubator to a sterile biological hood. Three-way luers were located at both the inlet and outlet of the bioreactor (see Fig. 10). The medium was dispensed by simply switching the 3-way luer located right before the bioreactor entrance (inlet), which deviated the normal direction of the medium to a disposal container. Freshly prepared complete medium was in the meantime absorbed from another 3-way luer right after the bioreactor exit (outlet). Some old medium remains in the ECM chamber so the pcECM stays moisturized during the old-to-new medium transfer. When the old medium was completely replaced by the new medium in the tubing system, the remaining medium volume in the bioreactor bath was discarded by simply inverting the bioreactor and vacuuming through the drainage hole. The bioreactor was finally transferred back in the incubator when the new medium has fill up completely the ECM chamber. Similar procedure was performed when changing the medium to Alamar Blue™ medium for cell viability measurement.

Fig. 10 Schematics of medium replacement in bioreactor system

Medium was dispensed into a container by simply switching the 3-way luer located before the bioreactor entrance. Freshly prepared complete medium was in the meantime absorbed from another 3-way luer after the bioreactor exit.

Monitoring parameters

The oxygenator provides suitable O₂-CO₂ ratio to the medium, which goes through the tubings in contact with the incubator air. The medium color is a good indicator to monitor whether the medium is being well supplied by O₂. CO₂ level in the medium is related to the pH and thus the medium color (yellow = too high CO₂ level, dark pink = no CO₂). If the color is being abnormal, the whole CO₂ supply system must be checked: tubings from pump to oxygenator and oxygenator to released area, CO₂ incubator controller and finally main CO₂ tank supply. However, extra precautions should be taken when assessing the color of media as color change could also mark microbial growth or simply anaerobic metabolism yielding high lactic acid into the media.

Medium pH measurement

The medium pH was monitored every 2-3 days (before changing medium). 1-ml medium was taken with a syringe and needle from a heparin lock (sampling port). The sample was quickly transferred into a 0.5-ml microcentrifuge tube and pH was measured right away with a thin pH probe. It was important not to let the sample in contact with air as it will change the pH value.

Lactate/glucose measurement

The level of lactate and glucose in the medium was monitored every 2-3 days (before changing medium). Medium sample was taken similarly as for pH measurement and readings were taken from a few drops using the glucose LifestyleTM (Abbott Laboratories) and the Lactate-ScoutTM (EKF diagnostics) meters.

7.3.5.3 AlamarBlueTM measurement

To measure cell viability, 5% AlamarBlueTM in medium was perfused for several

hours where fluorescent measurements were taken at three different time points. The entire medium volume was drained and replaced with 130 ml AlamarBlue™ medium. This number was found to be the minimal volume, which allowed filling the entire perfusion system (tubings and the bioreactor chamber) while excluding the medium reservoir from the perfusion loop. In addition, the conventional perfusion loop (where the medium overflows from the ECM chamber and then drained from the drainage hole located on the bioreactor round platform) was not used and was replaced by, what we called, the “small” perfusion loop for all cell viability measurement (see Fig. 11). The medium was carried out of the ECM chamber by a small tubing directly connected to the ECM chamber, which minimized the amount of medium used (as compared when using medium overflowing from the ECM chamber). At each measurement time point, a medium sample was collected using a heparin lock and 25G needle. Those samples were subsequently subtracted to an AlamarBlue™ control, which was kept statically in a static and sterile eppendorf since the beginning of the AlamarBlue™ perfusion. From the three fluorescent measurements, a slope was obtained and was plotted as a function of cell concentration to deduce the actual cell quantity within the dynamically cultivated construct.

Fig. 11 Schematics of “small” loop perfusion

In the “small” loop perfusion, the medium was carried out of the ECM chamber by a small tubing directly connected to the ECM chamber, which minimized the amount of medium used (as compared when using medium overflowing from the ECM chamber).

7.3.6 Single time-point injection seeding method

BmMSCs were cultured and harvested as described in 7.1.1. In the meantime, a thick pcECM slab sutured with catheters was mounted onto the bioreactor. It was subsequently disinfected with 3 washes of 70% EtOH in distilled water (over 2-3 days) and 3 washes in 250 ml PBS containing 5 ml of antibiotic-antimycotic solution (over 1-2 days). This was followed by perfusion of 250 ml of complete culture medium until bmMSC seeding.

On the day of seeding, the pcECM slab was transferred from the bioreactor to a sterile surface. Ten million bmMSCs (2 million cells/ml) were injected inside the pcECM bulk using a 25G needle. The total 5 ml volume was distributed at different locations from both sides of the pcECM. After seeding, the pcECM slab was re-allocated immediately to the chamber filled with 50 ml of medium. The entire bioreactor system remained at 37°C for 1.5, 8 or 24 hrs with no medium perfusion to let the cells attach. Perfusion at 5ml/min ensued following the attachment period. After 24 hrs perfusion, cell viability was measured by Alamar Blue™ (5% in 130 ml volume at 3 different time points). The pcECM slab was finally immersed in 2% PFA for 1 hr and prepared for H&E staining (see 7.4.2 and 7.4.4). Cross sections stained by H&E were further processed for cell counting (see 7.3.6).

To assess viability over a long period, medium was changed every 2-3 days and cell viability was measured by Alamar Blue™ every 3-4 days (5% in 130 ml volume at 3 different time points). Cell metabolism was assessed every 2-3 days using digital meters and media pH was maintained at physiological levels (7.3-7.4) by adapting the incubator CO₂ % level. The pcECM slab was finally immersed in 2% PFA for 1 hr and prepared for

H&E staining (see 7.4.2 and 7.4.4).

7.3.7 Multi time-point injection seeding method

BmMSCs cultures and sterilization of pcECM and bioreactor system were prepared similarly as in 7.3.3. The thick pcECM slab was multi-seeded with 10 millions bmMSCs (2 million cell/ml) every 7 days (day0, day7 and day 14) by needle injection with 1.5 hr attachment time similarly as in 7.3.3. Alamar Blue™ was performed before and after each seeding (5% in 130 ml volume at 3 different time points). The pcECM slab was finally immersed in 2% PFA for 1 hr and prepared for H&E staining (see 7.4.2 and 7.4.4).

7.3.8 Static pre-seeding before dynamic cultivation method

BmMSCs were cultured and harvested as described in 7.1.1. In the meantime, a pcECM slab was disinfected and treated with nitrocellulose as described in 7.3.3 and 7.1.2.1 respectively. Five million bmMSCs in 5 ml (1 million cells/ml) were seeded by pipettation on the endocardial surface of the dynamic pcECM slab in our customized rectangular seeding chamber (sterile foam was used to fill the vacant space left in the chamber). The cells were let 1.5 hr at 37°C to attach, similarly as in static-condition experiment. Subsequently the seeded dynamic scaffold was maintained in static conditions for 30 days (60 ml medium of 20% FBS α -MEM replenished every 2-3 days, which allowed the cells to statically stabilize prior transferring the slab to the bioreactor system (see Fig. 12). Using sterile pipettes, the pcECM slab was always maintained immersed and pcECM endocardial surface facing up when cultured under static conditions. Following the 30-day period (static growth) sterile surgical threads, scissors, and tweezers were prepared in a biological hood. Disinfected catheters were carefully re-sutured with a sterile suturing thread to the main vasculature conduits of the statically seeded pcECM slab. During the suturing, the pcECM was immersed in 37°-heated

medium on a sterile 10-cm petri dish so it remained moist for cell survival. After the suturing, 10-ml medium is slowly injected into the inlet catheter to confirm the catheter was well sutured with no blockage. Finally, the pcECM was transferred to the bioreactor.

Dynamic cultivation followed for additional 14 days (160 ml of double α -MEM, replenished every 2-3 days). During this, time cell viability and cell metabolism were measured with Alamar Blue™ similarly as in 6.3.2. At the end of the experiment, the pcECM slab was immersed in 2% PFA for 1 hr and prepared for Masson Trichrome staining (7.4.2 and 7.4.4) and immunofluorescence staining against CD44 (BD Bioscience 555476, dilution 1:50) with FITC anti-mouse (Sigma F8521, dilution 1:50) and against CD31 (R&D Systems BBA7, dilution 1:25) with NorthernLights™ 557-conjugated Anti-mouse (R&D Systems NL007, 1:100) (see 7.4.3). In addition, z-stack (~20 μ m thick) and tile-scanned images were taken at 20x magnification by confocal microscopy of DAPI- or Hoescht-stained pcECM cross sections. Single images were also taken at 63x magnification (n=4 images, regions per distance interval (0-100, 100-500 and 500-1000 μ m) repeated on at least 3 sections taken from our recellularized construct.

Fig. 12 Schematics of experimental protocol of static pre-seeding followed by dynamic cultivation method

Catheters were removed from pcECM slab so it can be seeded with bmMSCs on its endocardial surface in sterile a rectangular chamber. Static cultivation ensued for 30 days to let bmMSC stabilized before transferring the seeded pcECM slab into the bioreactor for another 14 days.

7.3.9 Cell counting by ImageJ

To count automatically colored-specific structures using ImageJ software, each image was first converted into greyscale before proceeding (*Image → Type → 16-bit*) where “*scale when converting*” option should be selected (*Edit → Options → Conversion*). To select the desirable structures (in our case cell nuclei), the threshold (*Image → Adjust → Threshold*) was adjusted so that only those structures were highlighted in red. A new image was subsequently created with two pixel intensities only (white = 0 and black = 255). From these binary images (n=3), 3 to 4 different rectangle areas were selected for particle counting (*Analyze → Analyze particles*, with 0 or 50 to infinity for size pixel and 0-1 for circularity, show *outlines*). For visual purpose, some of them were enlarged in size (with increased contrast). Total number of counts and dimension for each area were collected to obtain an average cell density.

7.4 Histology and fluorescence staining

7.4.1 Immunofluorescence staining for flow cytometry

Paraformaldehyde fixation

Unless stated otherwise, cells were fixed in 4% paraformaldehyde at RT for 10 min and subsequently washed once in PBS and then once in wash buffer (0.5% BSA in PBS). Permeabilization step was performed if needed just after fixation with incubation in 0.1% Triton X-100 for 4 min at 4°C. The cells were conjugated with primary antibodies markers in 100 µl volume wash buffer at room temperature for 30 min. Subsequently, the cell pellets were washed twice in wash buffer and conjugated with the corresponding secondary antibodies in 100 µl volume wash buffer for 30 min at room temperature in the dark. The cells were then washed twice with wash buffer and resuspended in 500 µl 0.5% BSA. The samples were finally run on the FACS Calibur.

7.4.2 Fixation and OCT freezing

After 1 hr immersion in 2% PFA, dynamically cultivated pcECM slabs were subsequently immersed in 1% PFA for another hour. PFA solution was slowly injected through the catheters using a syringe to make certain the pcECM slab was entirely fixed. The scaffolds were then perfused and immersed with OCT for 2 hrs and transferred into plastic mold filled with OCT. The molds were frozen over dry ice and were kept at -80°C until use. Cross-sections, 10-40 μm , were sliced from the blocks using a cryostat and mounted onto positively charged glass, which were kept at -20°C until staining was performed. For static pcECMs, they were simply immersed in 4 % PFA and then washed with PBS before being frozen in OCT, similarly as for pcECM slabs.

7.4.3 Slide fixation and immunofluorescence staining

Staining

OCT-embedded sections were allowed to reach RT equilibrium for 1hr prior to fixation. Slides, facing upward, were carefully immersed in -20°C cold methanol at 4°C for 30 min. To enhance fixation, slides were subsequently allowed to air-dry inside a chemical hood for 5 min before rinsing 3x in PBS. Each specimen was carefully circled using a PAP pen. If permeabilization needed, samples were incubated with 1% Triton X-100 for no more than 10 min at RT. Blocking unspecific protein binding sites was performed with 5% FBS for 1 hr at RT. Specimens were incubated with primary antibodies diluted in 3% FBS (+0.1% Triton X-100 in PBS if using intracellular antibody) at 4°C overnight. After 3x washes in PBS, the samples were subsequently incubated with secondary antibodies diluted in 3% FBS in PBS for 1 hr at RT (protected with aluminium foil). Samples were then washed 3x in PBS (DAPI or Hoescht staining in PBS for 10 min can be performed at that stage) and finally mounted with fluoromount for bleaching prevention. The slides

were kept at 4°C in the dark.

For whole pcECM staining (with no OCT), fresh reseeded pcECMs were directly fixed in 4% PFA at RT for 20 min. The samples were washed 3 times in PBS and blocked with 5% FBS for an hour at RT. The pcECMs were subsequently immersed with the first antibody overnight at 4°C and washed 3 times in PBS. They were finally immersed with the secondary antibody for 1 hour in dark at RT and subsequently washed 3 times in PBS again. If required, nuclei staining was performed by immersing the samples for 30 min in Hoescht 1:1000.

Imaging

Samples were fluorescently imaged either by normal fluorescent microscopy (Nikon) or using a confocal microscope (20x or 63x, LSM700, Carl Zeiss) where tile-stacks and Z-stacks (20-40 μ m) were taken and merged to form one single image. In addition, fluorescent images with larger view were taken using the binocular microscope (Olympus).

7.4.4 Hematoxylin & Eosin and Masson trichrome stainings

Hematoxylin & eosin staining (H&E):

All glass slide containers were readily prepared with the solutions and located under a chemical hood. For fixation, the glass slides were immersed in pre-cooled (-20°C) MeOH for 15 min at 4°C or on ice. They were subsequently washed in DDW 3-5 times to remove OCT compound. For staining, the glass slides were then transferred in hematoxylin for 5-10 min and washed in running tap water until water became clear. They were quickly dipped in 0.5% HCl in 70% EtOH, followed by 1 wash under tap water, 0.5% ammonia in DDW for 30 sec, 1 wash again under tap water, immersed in

80% EtOH for 2 min and finally in eosin for 2 min. Subsequently, the dehydration step included to immerse the glass slides in 95% EtOH for 1 min, then twice 100% EtOH for 1min, xylene for 1 min and then another 2 min, and finally mount the glass slides in Enthylene.

Masson Trichrome staining:

Staining procedure was followed as stated in the technical data sheet of ready-to-use Masson trichrome staining with aniline blue (cat number 04-010802, Bio-Optica). Briefly, the fixation step included immersion of glass slides in pre-cooled (-20°C) MeOH at 4°C for 15 min, then one wash in DDW to remove OCT compound. A hydrophobic pen was used to circle each tissue sample and thus to minimize the volume used from the staining solutions. The staining followed closely the staining procedure from the product. Briefly, samples were incubated with a few drops of reagent A and B (Weigert's iron hematoxylin solution) for 10 min. After removing the drops (by drainage, no washing), they were subsequently incubated with reagent C (Picric acid alcoholic solution) for 4 min. The samples were washed quickly (3-4 sec) in DDW. They were further incubated with reagent D (Ponceau acid fuchsin) for 4 min. After one wash in DDW, the samples were immersed in reagent E (phosphomolybdic acid solution) for 10 min, drained with no washing, then immersed in reagent F (Masson aniline blue) for 5 min. The dehydration step included washing all glass samples in DDW and dehydrating rapidly through ascending EtOH (less than 1 min each and 1 min in 100% EtOH). Finally, the samples were cleared in xylene and mounted with a few drops of enthylene.

7.5 Faculty services

Advanced Molecular Pathology laboratory (AMLPL)

H&E and Masson trichrome stainings obtained in Singapore were performed by the

histopathology facility provided by the Advanced Molecular Pathology laboratory (AMLP) from the Institute of Molecular and Cell Biology (IMCB) at Agency for Science, Technology and Research (A*Star).

Life Sciences and Engineering Infrastructure Unit (LS&E infrastructure unit)

The images from the binocular microscope (SZX16, Olympus), the confocal microscope (LSM700, Leica) and the INCell analyzer (2000, General Electric) were taken under the assistance and supervision of accomplished scientists from the Life Sciences and Engineering Infrastructure Unit (LS&E infrastructure unit) at the Israel Institute of Technology (Technion) in Haifa – Israel.

Centre for Translational Medicine

RNA integrity number (RIN) values from RNA isolated samples were measured through the services provided by the Centre for Translational Medicine from the Cancer Science Institute of Singapore at the National University of Singapore (NUS).

7.6 Statistical analysis

Data is expressed as mean values $\pm$ standard deviation. Two sided t-tests were used for comparisons using Excel (Microsoft Office) $p < 0.05$ was considered significant for all comparisons.

8 Results and discussion

8.1 Studying and characterizing the porcine extracellular matrix in terms of its cell support ability under static conditions

8.1.1 Initial cell attachment and density studies on pcECMs

Before attempting to recellularize thick porcine cardiac extracellular matrix (pcECM) slabs under dynamic conditions, where many parameters are at play, it is important to assess the optimal parameters for cell attachment and growth on an easier experimental model under static conditions. Cell attachment and density studies were investigated by seeding bone marrow mesenchymal stem cells (bmMSCs) on pcECMs with varying attachment durations (5, 45, 90 or 180 min, Fig. 13A) and initial cell seeding densities (0.2×10^6 , 1×10^6 , 4×10^6 or 8×10^6 cells/cm², Fig. 13B). With constant initial cell seeding density (0.6×10^6 cells/cm²), cell viability was highest at 90 min attachment time, statistically significant ($p < 0.05$) from the values obtained at 5 and 45 min, but non-significant compared to 180-min value ($p > 0.05$). This seeding time duration was therefore chosen as the optimal attachment time and used throughout the rest of this entire study. When varying initial cell seeding densities, results demonstrated overall correlation whereby a higher attachment density was observed for a higher initial seeding cell density, reaching a plateau with maximal value at approximately 30×10^4 cells/cm² above initial seeding density of 4×10^6 cells/cm². Above 90 min incubation, cells most likely failed to attach because of nutrient shortage, which may be attributed to the minimal culture media volume within the culture dish used (96 well plate using $\sim 80 \mu\text{l}$ media per pcECM piece).

Fig. 13 Cell attachment and cell density studies in static conditions

BmMSCs were seeded on *pcECMs* with (A) varied attachment durations (5, 45, 90 or 180 min) and (B) various initial cell seeding densities (0.2×10^6 , 1×10^6 , 4×10^6 or 8×10^6 cells/cm²). Cell number was determined by cell viability measurement with *AlamarBlue* and standard curve, and finally divided by matrix surface area (in cm²). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

Varying the initial cell seeding density over a fixed attachment time has demonstrated to follow a saturation behavior that describes natural phenomena like ligand binding and enzyme kinetics. Kepner²⁸⁷ has defined a general saturation curve in terms of its independent (x) and dependent (y) variables with two empirical constants, the initial slope of the data plot and the limiting value at saturation (Equation 1). Using Microsoft Excel SolverTM, the optimal constant C ($878,652$ cells/cm²) and $y_{\max}$ ($306,762$ cells/cm²) were found to fit the model through systematic trial and error with solver defined condition of obtaining maximal R^2 value ($R^2=0.9549$). For this experiment, the plateau obtained from the mathematical model is easily related to the limiting space for cell attachment on *pcECM* surface. However, the model does not take into account the nutrient shortage, which increases with time and cell seeding number. Both parameters are limiting factors that would decrease cell viability. $Y_{\max}$ gives a theoretical approximation of *pcECM* maximum cell attachment in those particular conditions ($80 \mu\text{l}$

seeding volume, 90 min for cell attachment in 96-well plate). Furthermore, by considering cell penetration to be equal to 0.01 cm (which corresponds to limited oxygen diffusion depth in static conditions, see also Fig. 14), pcECM cell capacity was estimated (3.07×10^7 cells/cm³) and found comparable to the physiological cell density of cardiomyocytes in the human heart (2×10^7 cells/cm³)²⁸⁸. This simple methodology to quantify and characterize a scaffolding biomaterial for its biological cell support ability has not been described elsewhere to the best of our knowledge. Some studies have investigated varying initial seeding cell densities focusing mainly on difference in cell behavior²⁸⁹ and but none has reported any model describing cell capacity over the later parameter^{290 291}.

$$f(x) = \frac{x}{C + x} y_{\max} \quad \text{Equation (1)}$$

where

$f(x)$ = Attached cell density as a function of the initial seeding density [cells/cm²]

C = half-life constant [cells/cm²]

$Y_{\max}$ = maximal attachment cell density [cells/cm²]

Fig. 14 Schematic of theoretical maximal cell capacity on pcECM in static conditions

(A) Cross section of bmMSC seeded on pcECMs after 4 weeks stained by H&E. (B) Considering cell penetration to be equal to 0.01 cm (which corresponds to oxygen diffusion depth limit in static conditions), pcECM cell capacity was estimated and found comparable to physiological cell density of a human heart ($2-3 \times 10^7$ cells/cm³).

8.1.2 Cell support improvement by bio-chemical modifications

To improve cell adhesion and migration on pcECM, the scaffolds were treated with different proteins/polymers/chemicals, which have already demonstrated such capabilities. Before bmMSC seeding, pcECMs were immersed in solutions of fibronectin (FN), laminin (LAM), fetal bovine serum (FBS), gelatin (GEL), sodium hydroxide (NaOH), hydrochloric acid (HCl), polycaprolactone (PCL) and nitrocellulose (NC),

8.1.2.1 Bio-chemical modifications of pcECMs

PcECM controls (no treatment) reached 19.69% ($\pm$ 3.46%) of attached cells, whereas LAM, FN and GEL treatments exhibited slightly improved cell attachment at 23.16% ($\pm$ 3.01%), 24.34% ($\pm$ 4.53%), 24.41% ($\pm$ 4.66%) respectively, but those results were not significant ($p > 0.05$, Fig. 15A). Only FBS treatment exhibited a lower initial cell attachment value ($15.98\% \pm 2.06\%$, $p > 0.05$). It is well known that higher concentration of FBS increases cell proliferation in common cell culture²⁹² but FBS-treated pcECMs exhibited no enhancement in cell attachment nor in cell proliferation over 10 days. FBS treatment might have electrostatically masked some of the pcECM binding functional groups during the 24-hour treatment period, which could have induced lower cell attachment. Tedder *et al.*²⁹³ have reported using high concentration of FBS to neutralize glutaraldehyde groups from their scaffolds to reduce toxicity. Regarding LAM, FN and GEL treatments, they demonstrated improved cell adhesion but exhibited very similar cell growth than the control group (Fig. 15D). After 10 days, total cell numbers remained lower than initial cell seeded numbers for each group of treatment. Initially, the first intuitive choices to improve cell attachment and growth were to coat pcECMs with native ECM proteins, like LAM and FN (even though our pcECM has previously showed to preserve at some extent those proteins after decellularization²⁹⁴). Both molecules are non-

collagenous glycoproteins that contribute to the ECM structure and influence the behavior of associated cells, such as adhesion, migration and differentiation²⁹⁵. LAMs and FNs are usually cell-secreted and bind to integrins that are cell surface receptors that link directly the ECM to the intracellular cytoskeleton²⁹⁶. Because both proteins play a major role in regulating cells in different extracellular matrices, numerous studies have already reported the capabilities of such protein coating strategies. For instance, Sales *et al.*²⁹⁷ have demonstrated improved cellularity of endothelial progenitor cells seeded on FB pre-coated elastomeric scaffolds, as well as a differential effect on cell phenotype according to the type of coatings (FN vs LAM). Aiming for adhesive support, Tate *et al.*²⁹⁸ have also used both proteins in injectable collagen-based scaffolds for neural transplants but demonstrated enhanced cell survival only after acute cell loss upon injection. Finally, Garcia-Nieto *et al.*²⁹⁹ have reported no difference in cell viability of dendritic cells between FN-, LAM- and non-coated plates but only superior endocytic capacity. Taken together, these studies demonstrate that surface treatments are specific for the cell type and the scaffold used, and no particular treatment is regarded as generally useful. Therefore, in this study, we evaluated each material coating as a sole treatment in terms of its improvement in cell attachment and growth. BmMSC adhesion and viability on pcECM with either LAM or FN protein coating did not show any significant improvement over a period of 10 days. Conforming to previously published findings, FN and LAM coatings do not always support cell adhesion and growth and may solely act on cell differentiation and phenotype. Seeing as cell adhesion and growth improvements were the main focus of this experiment, no further study was performed to uncover any phenotypic difference of bmMSC seeded on coated pcECMs. This may be the scope of future studies as some interesting findings were reported in other published papers with that respect^{300 301 302}.

Fig. 15 Cell attachment and cell density studies on treated pcECMs in static conditions

Before cell seeding, pcECMs were treated by immersion in fibronectin (FN), laminin (LAM), fetal bovine serum (FBS), gelatin (GEL), sodium hydroxide (NaOH), hydrochloride acid (HCl), polycaprolactone (PCL) and nitrocellulose (NC). Cell number was determined by AlamarBlue and by a standard curve. (A) and (D) Cell attachment and cell viability, respectively, of LAM-, FN-, GEL- and FB-treated pcECMs over 10 days. (B) and (E) Cell attachment and cell viability, respectively, of NaOH- and HCl-treated pcECMs over 14 days of culture under static culture conditions. (C) and (F) Cell attachment and cell viability, respectively, of PCL-, NC-low-, NC-high-treated pcECMs over 16 days of culture. All performed under static culture conditions.

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

In addition to LAM and FN coatings, GEL coating was also attempted on pcECM to improve cell adhesion and growth. Despite reported enhancement of cell adherence and cell growth in literature, GEL coating has showed no significant improvement in those criteria on pcECM. It was probably insufficient to provide a suitable platform to enhance those parameters in a 3D system, such as pcECM scaffolds. GEL is a natural material and has always retained considerable attention in tissue engineering^{303 304 305} considering its gel-forming ability³⁰⁶ and bioactive properties³⁰⁷ that should enhance cell performance in biological systems. Obtained from partial hydrolysis of collagen from different animal sources (skins, bones, tendons), gelatin benefits from a varied range of physical and chemical properties³⁰⁸, which makes it employable in different forms like electrospun³⁰⁹, hydrogel³¹⁰ or sponge scaffolds³¹¹. On the other hand, GEL coating is mostly applied on surfaces for biocompatibility or cell adherence features (electrodes³¹², particles³¹³ or arterial grafts³¹⁴) and routinely used to attach specific cell types on plastic culture dishes. Based on those facts, gelatin coating on pcECMs was expected to increase cell attachment. Nevertheless in this study, bmMSC adherence and viability were found similar to non-coated pcECMs even though improved cell attachment on other surfaces materials by GEL coating has been well documented. There could be a few reasons why GEL treated pcECMs failed to exhibit improved cell growth. When looking at the two major parameters affecting cell adhesion and proliferation, specific cell binding ligands (as opposed to scaffold compliance, i.e. mechanical/physical properties) include structural protein based (i.e. Arg-Gly-Asp- containing (RGD) moieties such as in collagen/GEL/LAM/FN) and glycosaminoglycans/glycosidic moieties³¹⁵. As our pcECM exhibited preserved ultrastructural properties including defined collagenous structures²⁴⁰, the addition of other structural proteins like GEL, LAM and FN may have no consequence in increasing cell attachment and that may be why no improvement with

such treatments was observed. In addition, GEL-coating capabilities, obtained in either manufactured coated tissue culture plates or planar surfaces from laboratory-made scaffolds, may not be sufficient to support cell growth in 3-dimension (3D) systems like the studied pcECM. It has been well reported that flat surfaces induce an artificial polarity that influences the entire cell behavior³¹⁶ and generate important differences on morphology, adhesion, migration and gene expression on 2D vs 3D systems^{317 318}. Even though this experiment was conducted in static culturing conditions, pcECM constructs do offer a dense porous collagen fiber system²⁸⁶ where cells, once attached, are able to migrate in all 3 dimensions.

In addition to LAM, FN and GEL coatings, pcECM were also treated in NaOH and HCl solutions. Mild alkali or acidic treatments may expose different side groups within the scaffold that may alter the electrostatic and chemical interactions with it. PcECM controls (no treatment) reached $20.05\% \pm 4.04$ of attached cells (Fig. 15B), whereas NaOH and HCl treatments exhibited improved cell attachment at $25.80\% \pm 3.38$ (non significant, $p>0.5$) and $27.34\% \pm 4.64$ (significant, $p<0.05$) respectively. By comparing cell viability over 14 days, chemically treated pcECMs exhibited no extensive enhancement (Fig. 15B). When treating collagen with alkali or acid solutions, hydrolysis occurs and breaks down the collagen-helix organization^{319 320} by cleaving collagen fibrils and disrupting collagen crosslinks^{321 322}, which results in a denatured form of collagen and eventually gelatin. Several studies have reported enhanced cellular functions such as cell differentiation^{323 324}, cell affinity³²⁵ and cell remodeling³²⁶ when seeding cells on denatured collagen in comparison to native collagen. This improved cellular activity was explained by greater accessibility of RGD motifs in denatured collagen^{323 327}. Even though most of these studies were investigated under the main theme of cell recruitment in wound healing and that they used preferably heat treatment to denature collagen, these

results may suggest that NaOH- and HCl-treated pcECMs were at some extent denatured and may have beneficated enhanced cell attachment for similar reasons (i.e. enhanced RGD-motif accessibility in partially denatured collagen).

Synthetic polymer coatings with PCL and NC have also been selected to potentially support cell growth and adhesion on pcECM for the following reasons. PCL has been extensively used in various biomaterial applications like in drug delivery^{328 329} and tissue engineering^{330 331}. As for NC, it is mostly used in Western blot – a well-known molecular analytical technique to detect protein. Proteins are usually transferred from a gel to a NC membrane for further specific staining. Knowing the potential of NC to bind proteins (and thus, probably cell membranes), several references were found reporting the utilization of such binding capabilities in other applications^{332 333} including cell adherence for applied biomedical purposes³³⁴. PcECM coating with PCL, NC_{low} and NC_{high} demonstrated higher cell attachment values at $22.70\% \pm 4.53$, $24.98\% \pm 1.87$ and $21.51\% \pm 4.16\%$ respectively as compared to the control pcECMs ($19.6 \pm 4.29\%$) (Fig. 15C). Unlike native ECM protein treatments, cell growth was enhanced on treated pcECMs with synthetic polymers. By comparing cell viability from each group at day 16 (Fig. 15F), bmMSC density was shown significantly higher ($p < 0.05$) for NC-low- (1.22 fold higher), NC-high- (1.59 fold higher) and PCL- (1.24 fold higher) treated pcECMs as compared to control group. It is interesting to note that NC has similar backbone chemical structure to glycosaminoglycans (Fig. 16). Since treatments with RGD-containing moieties (i.e. LAM, FN and GEL) did not exhibit any improvement but moieties similar to glycosaminoglycans (i.e. NC) did, it could suggest that the initial decellularization process to obtain pcECMs may have washout the native glycosaminoglycans (and not as much degraded the RGD-containing moieties), which could explain the different results observed from the bio-chemical modifications. Loss of glycosaminoglycans during

decellularization has indeed been well reported in literature^{335 336}. However, the chemical backbone similarity is unlikely the main reason to explain this NC coating effect as glycosaminoglycans / glycosidic moieties still contain many groups (uronic acid, acetyl amine and sulfate groups) absent from NC.

Fig. 16 Chemical structures of glycosaminoglycans, nitrocellulose and polycaprolactone

Nitrocellulose (NC) chemical structure contains a polysaccharide backbone, similarly to common glycosaminoglycans (chondroitin sulfate and hyaluronan), whereas polycaprolactone (PCL) is a polyester widely used in biomedical applications for its biodegradability in physiological conditions.

In addition, such synthetic polymer coatings probably improved pcECM compliance (mechanical support) to allow cell migration in the dense fibrous pcECM network. It is possible that these synthetic polymer coatings may have offered a better physical support on top of the initial pcECM fibrous system (in comparison to protein coatings). O'Brien *et al.*³³⁷ have indeed reported a decreasing cell attachment rate with increasing pore size (from 95 to 150 μm) on collagen-glycosaminoglycan scaffolds made from a lyophilization technique. It has been suggested that too large pores reduce the ECM surface area where cells can interact with and thus compromise cell adhesion, proliferation and migration³³⁸. In addition, both PCL and NC were unlikely to give the

most suitable coating for the following reasons. First of all, PCL is a hydrophobic polymer as it contains mainly ester bonds and lacks of -OH or -CH₃ groups and thus, it is generally agreed that PCL is inadequate for optimal cell adhesion and cell growth due to its hydrophobicity³³⁹. Even though the superiority of hydrophilic surfaces for cell adherence over hydrophobic surfaces is not clear yet in molecular level, scientists have widely reported in literature low cell affinity for hydrophobic polymers³⁴⁰, which led to different methods to increase hydrophilicity including plasma treatments³⁴¹, chemical treatments³⁴² or bioconjugation³⁴³. In this study, cell growth might have improved by reaching a favorable hydrophilic/hydrophobic balance between pcECM and PCL coating. A study by Groth *et al.*³⁴⁴ has indeed identified the optimal contact angle to be between 55-75° on a fibroblast model, showing that optimal cell adhesion may fall at a certain level of hydrophilicity (or hydrophobicity). As for NC, its protein adsorption capability was expected to influence positively cell affinity on pcECM. Cell growth on NC-coated pcECMs has indeed exhibited the most significant improvement ($p < 0.05$) as compared to all protein or polymer coatings attempted in this study. How NC binds to protein is still not fully understood but is probably based on different forces, including hydrophobic interactions, electrostatic interactions and hydrogen bonding³⁴⁵. The most accepted model suggests that proteins are initially attracted to NC membrane by electrostatic interaction, followed combination of hydrogen bonding and hydrophobic interaction for long-term binding^{346 347}. Because cell membrane is composed of proteins, cell attachment may have been supported considering protein affinity to NC. Alternatively, NC coating could have absorbed proteins from the serum present in the medium that subsequently formed a surface favorable for cell attachment and proliferation³⁴⁸. If NC harbors such advantageous cell attraction feature, its use is still very limited in biomedical applications. It should be noted however, that in terms of biodegradability, NC is unfortunately not able to degrade in physiological conditions without complex chemical-bacterial

digestion³⁴⁹. Despite this flaw, NC use has still been reported for a few medical implants in neural generation³⁵⁰ or vascular devices³⁵¹.

Without any acute modification of the acellular pcECM, several protein-based and polymer-based coatings were attempted to improve pcECM surface in term of cell adhesion and cell growth. LAM and FN, proteins found in the native ECM, and GEL, chemically processed collagen and well established in cell cultivation for supporting cell adhesion, have reported no cell-supportive capability when used as a non-covalent coating on pcECM. NaOH and HCl treatments have exhibited significant improvement in cell attachment and but have demonstrated little proliferation capabilities. On the contrary, synthetic polymers, i.e. PCL and NC, have demonstrated significant support in cell growth. Because NC coating have exhibited the most significant improvement in cell proliferation, NC coating was further applied in studies under dynamic conditions. Overall, the fact that none of the coating (including native ECM protein coatings) has remarkably improved cell support on pcECM may suggest that the acellular scaffold is sufficient platform as it is for cell survival. On the other hand, it is a possibility that bmMSCs have reached a quiescent state. Studies have already reported bmMSCs becoming quiescent on different substrates with potential initiation to terminal differentiation with appropriate cues^{352 353}. Future work should focus on cell cycle analyses to investigate this matter.

8.1.2.2 FTIR characterization

Fourier Transformed Infrared Spectroscopy (FTIR) was used to further characterize the NC-treated pcECMs in order to verify post-treatment NC presence within the pcECM scaffolds. Absorbance spectrums were compared between NC-treated pcECMs, pure NC film, pcECM controls (non treated) and methanol (MeOH, used as NC dissolving solvent)

-treated pcECMs (no PBS wash).

Pure film of NC exhibited a peak at approximately 1280 cm⁻¹ (symmetric NO₂ peak, Fig. 17A) due to the vibrations of nitrate groups³⁵⁴. NC-treated pcECMs exhibited identical stretching at 1280 cm⁻¹, which was absent from non-treated pcECMs. However, MeOH residuals were also visible on the spectrum of NC-treated pcECMs (at 1018 cm⁻¹), which corresponds to residual solvent of MeOH from the NC treatment step as verified against MeOH control spectrum. Because of its toxicity, 6 washes were performed after treatment instead of a single wash. Removal of MeOH peak was achieved with 6 washes after treatment while the nitrate groups were still visible. The resulting pcECMs have reached an undetectable level of MeOH by FTIR but still harbored the specific peak for NC.

FTIR is an efficient and fast method to characterize materials / surfaces according to their respective light absorption spectrum. NC-treated pcECM spectrum revealed a specific peak corresponding to nitrate groups, which were absent in non-treated controls. This suggests the effect on molecular basis behind the successful cell support ability of this treatment is at least mediated in part by NO₂ groups. As NC-high treatment demonstrated previously higher attachment value than control group but was found not significant, we speculated that the initial seeding density used (0.6×10^6 cells/cm²) was too low to be able to observe significantly the cell attachment capability of NC treatment. Therefore, bmMSCs at 3×10^6 cells/cm² (higher density as normally used) were seeded on NC-treated pcECMs (after 1 and 6 washes, Fig. 17B) in an alternate experiment similarly as in 8.1.2.2. This time, NC-treated pcECMs (6 wash) obtained significantly higher cell attachment percentage ($11.38 \pm 0.25\%$ cells attached, $p < 0.05$) than control group ($8.43 \pm 0.44\%$ cells attached). In addition, to show that additional washes did not compromise

the NC coating capabilities, bmMSC viability and growth were compared between NC-treated pcECMs washed once or 6 times after treatment (with 0.6×10^6 cells/cm² initial seeding density, Fig. 17C). Compared to control pcECMs (non treated), NC-treated pcECMs have demonstrated enhanced cell growth for both groups (1 or 6 washes) implying that 6 extra washes did not alter the advantages of NC coating on pcECM in those respects.

Fig. 17 FTIR spectrum of NC-treated pcECMs

(A) Absorbance spectrums were compared between non-treated pcECM (CONT), pure NC film, methanol (MeOH)-treated pcECMs (no PBS wash) and NC-treated pcECMs. Because NC-treated pcECM spectrum presented a peak corresponding most probably to MeOH residues, NC-treated pcECMs were washed in PBS 1 (NC 1wash) and 6 times (NC 6 wash) and characterized by FTIR. (B) Attachment study on NC_{high}-treated pcECMs with higher cell density was performed to significantly prove the enhanced capability of such treatment in term of cell affinity (C) Cell viability studies of non-treated pcECMs (CONT), NC-treated pcECMs washed 1 and 6 times were compared to ensure that NC coating improvement still remains even after 6 washes. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

8.1.3 Cell support improvement by cell co-culturing

As far as the decellularization processes used to produce the pcECM are concerned, the question is not whether damage was caused to the ECM but to what extent that

damage is³⁵⁵. It was hypothesized that re-populating pcECM scaffold with FBs could reproduce an environment more similar to the native ECM as FBs would secrete new ECM proteins. Hence, functionalizing pcECM surface with FB-secreted proteins was expected to promote cell proliferation and migration. Other scientists have suggested this theory and demonstrated improvement of those characteristics by using FB co-culture but in disease-related models^{356 357}. More relevant to tissue engineering, FB-derived ECM constructs were shown to support cell proliferation by culturing FB either on tissue culture dish^{358 359} or synthetic scaffold³⁶⁰ and then removing them, leaving only the remaining ECM-secreted proteins behind. In this study, bmMSCs and FBs, human cardiac ventricular fibroblasts (cvFBs) or human foreskin fibroblasts (fFBs), were seeded on pcECMs in co-culture systems to assess interactions between both cell models in term of cell viability.

8.1.3.1 Comparison of fibroblasts on pcECM for optimal model

Cardiac ventricular fibroblasts (cvFB) consist more than 70% of total cell number in the human heart and play a critical role in the maintenance of cardiac ECM homeostasis³⁶¹. Under physiological conditions, they contribute not only to structural and mechanical properties of the heart, but also provide biochemical and electric support between myocytes³⁶¹. Thus, cvFB may be considered as an essential asset for generating a functional cardiac construct. Aside from studying bmMSC as a cell model on pcECM and possibly future source for myocyte cells, cvFBs were hypothesized to provide i) cell support in a co-culture system with bmMCSs, ii) ECM remodeling effect on pcECM scaffolds and iii) electrical support by cell-cell interactions. CvFB (from Lonza, CH) is a primary cell type which was newly introduced to our laboratory and originated from a delicate tissue with possible limited proliferation capacity on the pcECM, We decided to compare our results to already available fibroblast cell types in our laboratory (of human

and porcine origin) as control. Some investigations have already reported the use of human dermal and foreskin FBs instead of using cardiac FBs in cardiac tissue engineering (CTE)^{362 363}.

Therefore, human cvFBs, fFBs and porcine cardiac fibroblasts (cFBs) were first seeded on pcECMs and cultured in static conditions as single culture (Fig. 18C-D). Cell viability was measured by AlamarBlue™ assay over a period of 20 days. Cell attachment study demonstrated that fFBs had significantly ($p < 0.05$) lower binding affinity to pcECMs as compared to cvFBs and cFBs. Human fFBs have indeed exhibited an almost three fold lower initial cell attachment rate at day 0 compared to the other two cell types (4.94×10^6 cells/cm² $\pm$ 0.44 for fFB against 14.12×10^6 cells/cm² $\pm$ 1.94 and 13.01×10^6 cells/cm² $\pm$ 3.21 for cvFBs and cFBs respectively, Fig. 18A). No significant difference ($p > 0.05$) was found between human and porcine cardiac FBs for cell attachment. We can suggest that fibroblasts originally coming from cardiac tissue probably express specific surface markers, more suitable for attachment as compared to the surface markers from foreskin fibroblasts. The fact that the pcECM itself is obtained from cardiac tissue may promote better attachment for cardiac-related cells as the proteins preserved on the pcECM surface may correspond to the receptors express by cardiac fibroblasts.

Cell viability curves exhibited similar tendency for all three types of FBs over 20 days (Fig. 18B). Total cell number seemed to converge to a cell baseline set at approximately 8×10^4 cells/cm² per pcECM after 20 days of culture. Those results reported a similar trend to the bmMSC growth profile (see Fig. 15D) with a comparable cell number. Even fFBs, known to have a particularly aggressive proliferation behavior, did not show any superior growth than cvFBs. Low proliferation of FB on pcECM might not necessarily be a disadvantage. Excessive FB proliferation should be avoided, as it is

frequently associated with fibrosis, which plays a key component in heart disease. Cardiac fibrosis refers to an abnormal thickening of the heart wall (caused by disproportionated deposition of collagen by FBs³⁶⁴) and is being investigated for potential therapeutic targets³⁶⁵. Finally, cell viability of porcine cFBs, which were initially populating the cellularized pcECM scaffold, demonstrated similar behavior to human FBs, suggesting no conflict in using a xenogeneic (human) cell source. In addition, the overall steady state value achieved of cell density may be a result of the pcECM reaching a saturation value of maximal cell holding capacity limited by static culture conditions provided for these experiments. This suggests that though initial attachment could be higher for cells that are more prone to attach to the pcECM (such as cvFB), culture conditions should also be taken into consideration, pointing to the need for the dynamic cultivation system reported in aim 3.

As the end result exhibited no substantial difference between the growth profiles of human and porcine (allogeneic to the pcECM) cells, only human cells were further evaluated in co-culture systems. This also suggests that human cells could be applicable and well supported as a xeneogeneic cell source on the porcine pcECM.

Fig. 18 Cell attachment and growth of several FBs sources seeded on pcECMs.

Human foreskin fibroblasts (fFBs), human cardiac ventricular fibroblasts (cvFBs) and porcine cardiac fibroblasts (cFBs) were seeded on pcECMs and cultured in static conditions as single culture. Cell viability was measured by AlamarBlue™ assay over a period of 20 days. (A) Initial cell attachment study of single FB type cultures on pcECMs (B) Cell viability study of single FB type cultures on pcECMs over 20 days. (C)-(E) Brightfield images of porcine cFBs, human cvFBs and human fFBs in tissue culture plate, respectively. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

8.1.3.2 Stem cell and fibroblast co-culture systems on pcECMs

Because no significant difference was found between cvFB and fFB single culture, both FB types were co-cultured with bmMSC on pcECMs in static conditions. To assess interactions between those two cell types in term of cell proliferation, FBs and bmMSCs were cultured at different ratio (0:100, 25:75, 50:50, 75:25 and 100:0 bmMSC/FB ratios) with a fixed initial cell seeding number. Cell viability was measured by AlamarBlue™ assay over a period of 30 days. Because cell standard curves were similar (all slopes

equaled 0.003, intercept set at zero) for both cvFB and bmMSC, measured fluorescent intensity (FI) was corresponded to cell number as if only one and single cell type was cultured.

Cell viability of human fFB/bmMSC (Fig. 19A) and human cvFB/bmMSC (Fig. 19B) co-cultures demonstrated no significant difference in any cell system ratio. All cell viability curves reported similar trend, showing little cell growth during the first 7 days followed by a limited proliferation phase. Only bmMSC/cvFB 0:100 data (cvFB single culture) exhibited constant cell viability, which was significantly lower than the other co-culture ratios ($p < 0.5$). Cross sections of bmMSC/cvFB constructs characterized by Masson trichrome staining confirmed the lower cell viability in 0%bmMSC ratio compared to its counterparts when looking at the cell layer thickness (Fig. 20).

Co-culturing human FBs with bmMSCs have demonstrated no effect on the overall proliferation of such co-culture systems. FB interactions with bmMSC had either no influence on total cell growth signaling or were insufficient to trigger any improvement detectable in this study. This lack of apparent influence of the various FB ratios on the total pcECM-cell densities in bmMSC co-cultures may be the result of several independent causes which could be broadly regarded as either cell-cell, cell-pcECM or cell-external factor related. Assuming controlled culture parameters in all experiments (including culture media type and quantity) the latter cause may be neglected. Therefore if true, this surprising observation, may also suggest a clever feedback mechanism in which homeostasis of cell supporting capacities is maintained on the pcECM throughout the experimental durations. This homeostatic phenomena may be mitigated through either cell:cell or cell:pcECM first order interactions with possible triple (pcECM:cell:cell) more complex interactions. The exact interactions at play, whether this homeostasis is a

result of equal cell division and death rates, or the results of pcECM-induced canonical cell-quiescence remains unclear and merits further elucidation.

Fig. 19 Cell viability studies obtained from bmMSC/fFB and bmMSC/cvFB co-cultures

To assess interactions between those two cell types in term of cell proliferation, both FB types were co-cultured with bmMSC on pcECMs in static conditions at different ratio (0:100, 25:75, 50:50, 75:25 and 1000 bmMSC/FB ratios). Cell viability was measured by AlamarBlue™ assay over a period of 30 days. (A) bmMSC/fFB coculture viability (in separated graphs (a1) and (a2) for clearer view). (B) bmMSC/cvFB coculture viability (in separated graphs (b1) and (b2) for clearer view).

Fig. 20 Crossed-sections of bmMSC/cvFB co-cultures constructs by Masson Trichrome staining

Increasing ratio of bmMSC demonstrated thicker cell layer. It suggested higher amount of cell number, which confirmed results obtained by cell viability measurement.

Taken together, cvFB exhibited similar proliferative capacity and bmMSC supportability similar to other human and porcine FB cells. Human fFBs have demonstrated no particular influence on bmMSCs compared to cvFBs and thus, it was decided to pursue bmMSC/FB characterization with cvFB only. Although bmMSC/cvFB co-cultures have shown no particular improvement in term of cell proliferation, they might stimulate others cellular signaling pathways (i.e. cell differentiation, proteins expression, cellular junctions etc). The fact that the pcECM itself is obtained from cardiac tissue may promote better cellular response for cardiac-related cells as the proteins preserved on the pcECM surface may interact with the receptors expressed by cardiac fibroblasts. Hence, further studies were performed to assess those alternative cellular functions.

8.1.3.3 Cardiac ventricular fibroblast characterization by flow cytometry on tissue culture plate

Ultimately, we hypothesized human cvFBs to be the most appropriate cell type to work with due to their intrinsic functional cardiac related characteristics already reported³⁶¹. This is why this type of cells only was thoroughly characterized by fluorescence-activated cell sorting (FACS) from tissue culture plate for various positive (DDR2, α -SMA), negative markers (CD31, vWF) and common bmMSC positive markers (CD44, CD90, CD73 and CD105) (Fig. 21). Those results were compared to bmMSCs (Fig. 22) to possibly determine specific markers for these two cell types for future work in co-culture systems and possibly tri co-culture systems with human umbilical vein endothelial cells (HUVECs, Annex 11.1).

Fig. 21 FACS characterization of cvFBs

Cardiac ventricular fibroblasts (cvFBs) were thoroughly characterized by FACS from tissue culture plate for various positive (DDR2, α -SMA), negative (CD31, vWF) markers, and bmMSC positive markers (CD105, CD90, CD73 and CD44).

Fig. 22 FACS characterization of bmMSCs

Bone marrow mesenchymal stem cells (bmMSCs) were thoroughly characterized by FACS from tissue culture plate for various positive (DDR2, α -SMA), negative (CD31, vWF) markers, and their common positive markers (CD105, CD90, CD73 and CD44).

CvFBs exhibited positive expression of DDR2 and α -SMA (>90%) in concordance with already published studies^{366 367}. DDR2 (Discoidin Domain Receptor 2) belongs to a family of collagen specific receptor tyrosine kinases³⁶⁸, which is involved in the conversion of extracellular stimuli into cellular responses (i.e. growth, migration, morphology and differentiation). In addition, DDR2 is considered a specific marker for cardiac fibroblasts absent from cardiomyocytes, cardiac endothelial and smooth muscle cells^{366 369}. But this marker expression was also observed in bmMSCs (>95%) in agreement with previous studies^{370 371}. Similarly, α -SMA (alpha smooth muscle actin) involved in cell motility, structure and integrity³⁷² was expressed in both cell types (>90%). CvFBs were further characterized by FACS against bmMSC positive markers (CD44, CD73, CD90 and CD105, Fig. 21) and similarly compared to bmMSCs (Fig. 22). CD44 and CD105 were expressed in both cell types ($\geq 90\%$). However, different batch of cvFBs (supplied by the manufacturer) demonstrated some variability (see CD90 insert,

Fig. 21) where CD90-positive cvFBs reached 70% or close to 4%. This dissimilarity is not surprising, as fibroblast has already been reported to represent a dynamic population of cells, exhibiting functional heterogeneity within and among tissues using a separation criterion using CD90(+) and CD90(-)^{373 374 375}. The manufacturer (Lonza, CH) only certified the expression of α -SMA and absence of expression of vWF in cvFB population leaving CD90 with no expression control from batch to batch. In addition, CD73 exhibited lower expression (12%) in cvFB population whereas bmMSCs obtained more than 90% positive.

In this study, it has been attempted to determine distinguishing surface markers by flow cytometry that could be specific for cvFBs or bmMSCs for future immunofluorescent stainings of co-cultures. While studies of fibroblast from dermal origin were reported in comparison to mesenchymal stem cells, it is to the best of our knowledge, the first time cardiac ventricular fibroblasts were characterized and compared to bmMSCs. Unfortunately, no specific marker was found for cardiac FBs compared to bmMSCs. Because cardiac FBs can arise from MSCs³⁶¹, more effort should focus in understanding the differentiation process between both cell types by investigating additional markers (cardiac related) than the ones presented in this study. Differentiating dermal FBs from bmMSCs in term of phenotype expression has been also widely investigated in the literature^{376 377 378} and is to date not fully understood. The heterogeneity of FB population complicates the discovery of universal specific markers and it has been suggested by Halfon *et al.*³⁷⁸ that focus on gene and protein level could be used to identify robust markers that have differences of expression in MSCs and FBs.

8.1.3.4 Transfection of cardiac ventricular fibroblasts with eGFP

CvFBs were transduced with eGFP by lentiviral transfection as described in the

method section 7.1.3.5. CvFB images were taken on brightfield and fluorescence microscope at 1 and 3 days after transduction (Fig. 23). Transduction with eGFP reached 69.6% ($\pm 7.2\%$), calculated from three sets of bright-field and green fluorescent images taken by fluorescence microscope of cvFBs cultured on tissue culture plates. Thus, no Zeocin selection after transduction was required.

Fig. 23 Images of transduced cvFBs on tissue culture plate

Taken on bright-field and fluorescence microscope at 1 and 3 days after transduction. Transduction with eGFP reached satisfying fluorescence efficiency.

8.1.3.5 Quantification of Connexin43 in stem cell/fibroblast co-culturing system on tissue culture plate and pcECMs

CvFBs are the most abundant cell type in the cardiac muscle, and thus, every myocyte cell is located nearby at least one or several cardiac FBs. Although cvFB main function is to synthesize ECM proteins like collagen and fibronectin to maintain mechanical support for CMs, there is stronger evidence that they also participate in the electrical activity of the cardiac muscle. It was indeed reported that FBs could be electrically coupled to CMs in cell culture³⁷⁹ and native cardiac tissue³⁸⁰ by gap junctional channels, called connexin proteins, which allow metabolic intercellular communication. Gaudesius *et al.*³⁸¹ have reported that cardiac FBs were capable of synchronizing electrical activity of CM strands interrupted by cardiac FBs over a defined distance. Those results supported the hypothesis that non-excitable cells should increase the heterogeneity of the excitation wavefront. In addition, bmMSCs were also reported to

form gap junctions. Murely *et al.*³⁸² have demonstrated that bmMSCs transplanted into infarcted cardiac areas alleviated arrhythmias because of the upregulation of Cx43 through paracrine signaling. Valiunas *et al.*³⁸³ have also reported formation of gap junctions in bmMSC co-culture with HeLa cells transfected with Cx43 and canine ventricular myocytes. Based on those reported studies, cvFBs were hypothesized to support formation of gap junctions when co-cultured with bmMSCs, which was not reported to date.

To test this hypothesis, cvFB-eGFP and bmMSC were seeded on tissue culture plate at different concentrations (0:100, 25:75, 50:50, 75:25 and 100:0 ratios). At 24 hours post-seeding, cells were stained against connexin43 (Cx43) and immunofluorescence images (Fig. 24C) were taken using the INCELL analyzer. Image quantification analyses were performed by INCELL investigator software and Microsoft Excel to obtain organelle count average (per field, per cell, OCA) and organelle area average (per field, per cell, OAA) of Cx43. Both OCAs and OAAs were calculated according to each individual bmMSC/cvFB co-culture. Results have demonstrated that OCA and OAA of bmMSC single culture (0.62 ± 0.18 and 0.27 ± 0.08 respectively) were significantly lower than their counterparts from 0% to 75% bmMSC (for OCA: from 1.48 ± 0.54 to 1.88 ± 0.43 , for OAA: from 0.70 ± 0.21 to 1.01 ± 0.272). However, cvFB single culture and co-cultures with bmMSC reported comparable OCAs and OAAs with no significant difference (Fig. 24A). Similar trend was obtained on pcECMs where OCA reached 18.06 ± 4.61 , 20.23 ± 3.86 and 12.10 ± 2.19 for 0:100, 50:50 and 100:0 ratios of bmMSC-cvFB co-cultures respectively (Fig. 25). Cell cultures containing cvFB demonstrated higher OCAs compared to bmMSC single cultures (even though only statically significant for 50:50 ratio), confirming the results obtained in tissue culture plate.

Fig. 24 Comparison of Cx43 organelle counts in bmMSC/cvFB-eGFP co-cultures on tissue culture plate using INCELL analyzer and investigator software

CvFB-eGFPs and bmMSCs were seeded on tissue culture plate at different concentrations (0:100, 25:75, 50:50, 75:25 and 100:0 ratios), stained against Cx43 and imaged for immunofluorescence (IF) by the INCELL analyzer. (A) Cx43 organelle count average (OCA) and Cx43 organelle area average (OAA) according to bmMSC/cvFB-eGFP ratios quantified by INCELL investigator. (B) Porcine cardiac heart tissue stained by IF against Cx43 (red). (C) IF images of bmMSC/cvFB-eGFP co-cultures were taken by INCELL analyzer where nuclei were stained in blue (Hoescht) and Cx43 in red (PE). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

Fig. 25 Comparison of Cx43 organelle counts in bmMSC/cvFB co-cultures on pcECMs

CvFBs and bmMSCs were seeded on pcECMs at different concentrations (0:100, 50:50 and 100:0 ratios), stained against Cx43 and imaged for immunofluorescence (IF) using confocal microscopy. Cx43 organelle count average (OCA) quantified automatically by ImageJ demonstrated similar trend as obtained in tissue culture. IF images of bmMSC/cvFB co-cultures were taken using confocal microscope where nuclei were stained in blue (Hoescht) and Cx43 in green (FITC). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

The results obtained conformed to the previously published data that cvFB plays a key role in electrical cell communication. They demonstrated that co-culturing cvFB with bmMSC induced gap junction formation in cell culture conditions. In addition, the results suggested that cvFB presence only was sufficient to promote a certain level of Cx43 junction expression in bmMSC/cvFB co-cultures as compared to bmMSC single culture. Indeed, increase of cvFB ratio had no influence on Cx43 junction number. This latter observation implies that cvFB-induced Cx43 formation might involve exclusively paracrine signaling mechanisms. If Cx43 expression was caused by juxtacrine signaling (contact-dependent signaling), co-cultures with higher cvFB number would have obtained

higher number of Cx43 gap junctions. Little has been published on the effects caused by cardiac FB-secreted molecules through paracrine signaling. Pedrotty *et al.*³⁸⁴ have reported slow conduction and prolong action potential from cardiac FB paracrine factors (but unchanged Cx43 expression) but together with CMs (and not bmMSCs).

8.2 Characterizing the effects of porcine extracellular matrix on phenotypic expression of reseeded cells and possible differentiation induction under static culture conditions

8.2.1 Phenotypic characterization of stem cells seeded on pcECMs

The phenotypic characterization study revealed that bmMSCs preserved their positive phenotypic profile as MSC common markers³⁸⁵ (CD29, CD44, CD90, CD73 and CD105, >90%, Fig. 26) were all expressed even after a period of 4 weeks cultivation on pcECM scaffolds. However, CD31 (or PECAM-1), a well-accepted negative marker for bmMSC, which was not expressed in non-pcECM exposed cells, has been expressed more predominantly on pcECM-seeded bmMSCs after 2 and 4 weeks at 10% and 55% respectively (Fig. 27A). This result was verified by confocal immunofluorescence staining after 4 weeks only (Fig. 27B).

Fig. 26 Flow cytometry analyses of bmMSCs from reseeded constructs against positive surface markers
Cultured bmMSCs were harvested from reseeded constructs following 4 weeks cultivation on pcECM for flow cytometry analyses against positive surface markers: CD29, CD44, CD90, CD73 and CD105.

Fig. 27 Flow cytometry analyses of bmMSCs from reseeded constructs and confocal immunofluorescence staining against negative surface marker CD31

(A) Cultured bmMSCs were harvested from reseeded constructs following 2 and 4 weeks cultivation on pcECM for flow cytometry analyses against negative surface marker CD31. CD31, which was not expressed in non-pcECM exposed cells, has been expressed more predominantly on pcECM-seeded bmMSCs after 2 and 4 weeks. (B) Expression of CD31 was verified by confocal immunofluorescence staining after 4 weeks on seeding (DL-557 for CD31 and dapi for nuclei staining).

Commonly expressed on endothelial cells, neutrophils and monocytes, this adhesion molecule is concentrated at intercellular junctions to establish cell-cell contact³⁸⁶. The progressive expression of CD31 suggested that bmMSCs might undergo endothelial transdifferentiation. This phenomenon was already reported in previous studies where scientists have transdifferentiated mesenchymal or progenitor stem cells into endothelial-like cells in tissue culture plate using a transdifferentiation medium including notably vascular endothelial growth factor (VEGF)^{387 388}, which is known to induce angiogenesis. Others studies have also described stem cell differentiation into endothelial phenotype *in vivo*^{389 390}. To date, this study to the best of our knowledge may be the first one to report endothelial transdifferentiation of bmMSCs seeded on acellular ECM *in vitro* without any endothelial-specific factor treatment. The differentiation was probably triggered by pcECM surface environment. There is indeed supportive evidence that ECM proteins play an important role in endothelial homeostasis. Sottile³⁹¹ reviewed a significant list of studies, which have reported various effects obtained from individual ECM proteins on angiogenesis *in vitro* and *in vivo*. Furthermore, some investigations have focused more precisely on the ECM influence towards endothelial differentiation by using the commercial Matrigel™ from BD Bioscience (which contains mainly laminin, fibronectin and collagen) to mimic the native ECM and by using no endothelial factor treatment^{392 393}. They have demonstrated tubule formation with derived endothelial cells, which affirms the influence of ECM on cell fate towards endothelial phenotype differentiation. In addition, VEGF, which is normally found in soluble form, has also been reported to bind to the extracellular matrix (ECM)^{394 395}. The pcECM scaffold might contain VEGF-bound molecules that remained after decellularization, which could be responsible for the transdifferentiation phenomena observed. Hoganson *et al.* have indeed demonstrated the retention of VEGF in porcine decellularized mesothelium³⁹⁶ and dermis³⁹⁷ tissues by ELISA after protein extraction. As they suggested in their studies, the decellularized

material, when remodeled *in vitro* or *in vivo*, could release those VEGF molecules and promote either endothelial differentiation if using stem cells or angiogenic ingrowth of vessels into the material. Wang *et al.*³⁹⁸ have come to the same conclusion with their decellularized musculo-facial matrix. They have implanted subcutaneously their constructs in rats and demonstrated neovascularization as verified by immunohistochemical staining of CD31.

The possibility that bmMSC can transdifferentiate into endothelial cells via pcECM protein background solely would be a major advantage in terms of endothelialization and angiogenesis. PcECM scaffold has retained its inherent vasculature, but the smallest blood vessels (called capillaries) are obviously absent as they are normally formed by one lining of endothelial cells only (and were destroyed in the decellularization process as a result). To achieve dense recellularization, strategies to achieve endothelialization and to trigger capillary angiogenesis have to be developed to obtain viable thick tissue engineered constructs. Even though this study does not focus on this topic, endothelial transdifferentiation of bmMSCs could give some prospect of 'self-induced angiogenesis'. This study therefore suggests that bmMSCs could possibly give rise to both endothelial and cardiomyocyte-like cells neglecting the need to use external other cell sources for co-culturing. Nevertheless, the nature of this effect (in terms of spatial and temporal distribution), the maturity level and functional benefits of the resulting transdifferentiated cells should be further explored.

8.2.2 Quantitative RT-PCR of stem cells seeded on pcECMs for endothelial-related genes

To further characterize the potential transdifferentiation of bmMSCs in endothelial-like cells, RNA expression levels of genes related to endothelial biology were compared between bmMSCs cultivated for 25 days on pcECMs to control sample (bmMSCs

cultured on tissue culture plate). Quality of all RNA extracted samples was validated by obtaining 260/280 OD values between 1.8 and 2.0, and RNA integrity number (RIN) for all tested samples as required from the manufacturer (Qiagen) for high-throughput RNA expression arrays (Fig. 28). 260/230 OD was not considered as essential and reached approximately 1.0 value or higher.

Fig. 28 Example of electropherograms obtained from well-preserved and degraded RNA samples

The RIN software from Agilent allows classifying total RNA based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this study, all samples have obtained RIN values higher than 7. As RNA degradation increases, there is a decrease in the 18S and 28S ribosomal band ratio, which results in a low RIN number.

Out of 84 genes (see annex 11.3.1), 23 genes have been upregulated considering a fold regulation value (FR) ≥ 2 , including 9 genes (IL1B, IL6, KDR, MMP1, MMP9, PECAM1, THBD and VCAM1) with a FR ≥ 10 (Fig. 29). On the other hand, 17 genes were downregulated considering a FR ≤ -2 , including only 1 gene (KIT) with a FR value ≤ -10 (Fig. 29). Unfortunately, the latter genes with $|\text{FR}| \geq 10$ were not proven significant

(except for MMP9) but others down-/up- regulated genes ($10 \geq |FR| \geq 2$) were proven statistically significant (Fig. 29). This may be attributed to the relatively small biological repetition size ($n \geq 4$, $r=2$) and so future work may focus on reducing the current standard deviations to get a clearer statistical and biological picture of the forces at play. Still, when analyzing the different fold regulation results reported herein, particular attention was given to those genes with consensus literary importance (such as common gene clusters related to a cellular process or defining a specific cell type) and/or genes which in our case displayed considerably high FR values ($|FR| > 10$) or statistical significance ($p < 0.05$) with lower $2 < |FR| < 10$ values for up- (VEGFA, MMP9, COL18) and down- (ENG, FGF2) regulated genes. The following discussion will refer to those two groups, respectively.

Fig. 29 Fold regulation values of RNA expression levels from genes related to endothelial biology of pcECM-seeded bmMSCs

Fold regulation values (FR) of genes related to endothelial biology were obtained from pcECM-seeded bmMSCs compared to control (bmMSCs cultured on tissue culture plate). Out of 84 genes (see annex 11.1.1), 24 genes have been upregulated considering a fold regulation ≥ 2 (right panel). On the other hand, 17 genes were downregulated considering a fold regulation ≤ -2 (left panel). Particular attention were given to genes with $|FR| \leq 10$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

First, when looking at genes commonly used as specific markers for endothelial characterization^{388 399 400} (FLT1, KDR, NOS3, PECAM1, VCAM1, VEGFA and vWF, Fig. 30A), most of them have exhibited considerably higher than threshold ($|FR| \geq 2$) FR values even if non-statistically significant, ($p > 0.05$). Thus, KDR, PECAM1 and VCAM1 have all exhibited FR higher than 10 (17.3, 14.4 and 47.4 respectively), followed by VEGFA ($p < 0.05$) and vWF with FR higher than 2 (6.0 and 2.6 respectively). Much minor changes were observed with FLT-1 and NOS3 which did not exceed fold cutoff ($p > 0.05$, $FR = 1.7$ and -1.0 respectively). Of above mentioned markers list, two markers are routinely used to specifically identify endothelial cells (PECAM1 also known as CD31, and vWF) and distinguish them from other cell types⁴⁰¹. Both were found to be upregulated in this study by the seeding and cultivation of bmMSCs on pcECMs. Even though PECAM1 up-regulation was not significant ($p > 0.05$), this observation was in agreement with the results obtained previously at the protein level (8.2.1) by flow cytometry and immunofluorescence staining, further supporting our claim for pcECM induced MSC to endothelial transdifferentiation. Though PECAM1 FR values were high, another specific endothelial marker, vWF had a milder increase in expression ($FR = 2.6$). This milder increase may be attributed to transdifferentiation towards a particular endothelial subtype, as it has been previously observed in the murine model that vWF distribution and expression is tissue-dependent and that endothelial cells of micro-vessels and arteries have in general much lower vWF expression than their corresponding large vessels and venous endothelial cells⁴⁰². In addition, PECAM1 is a specific endothelial lineage adhesion molecule, which is part of the immunoglobulin gene superfamily⁴⁰³. It may thus be expected that this marker of endothelial differentiation will be most apparent in our case as bmMSCs interact with the pcECM through such molecules.

Fig. 30 Fold regulation values of RNA expression levels from genes related to specific endothelial characterization and angiogenesis

(A) Fold regulation (FR) values of genes characterizing specifically endothelial cells. (B) Schematic view of interactions (as reported in literature) between highly upregulated angiogenic genes found in this study from right-sided graph. (C) FR values of angiogenic genes were compared in a single graph, including those genes exhibiting no upregulation nor downregulation. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant.

Another member of the same immunoglobulin superfamily is VCAM1 (CD106) which expression level was also found herein to be substantially (though not yet significant) up-regulated ($p > 0.05$, $FR = 47.4$). Whereas PECAM1 is specific to endothelial

cells, VCAM1 can also be found in subpopulation of bmMSCs⁴⁰⁴. Thus, increased RNA expression of VCAM1 could be related not only to endothelial transdifferentiation but might also arise from enhanced cell migration processes as already reported for bmMSCs⁴⁰⁵. Thus, it could be that in the heterogeneous MSC population, some cells migrate, some transdifferentiate and in both cases these processes are mediated through the VCAM1 molecule, potentially explaining the high FR value measured on the pcECM compared to the plate control. Another extensively reported molecule, VEGFA, has been shown to induce endothelial transdifferentiation^{388 406}. Furthermore, the VEGF signaling pathway has indeed demonstrated crucial impact on endothelial cell division and migration during angiogenesis^{407 408}. It consists of five VEGF members (including VEGFA), which have been implicated into promoting activation of the VEGF tyrosine kinase receptors (VEGFR) like FLT1 (or VEGFR1) and KDR (FLK1 or VEGFR2)⁴⁰⁹. In this RNA expression study, VEGFA (FR=6.0, $p<0.05$) and its corresponding receptor KDR (FR=17.3, $p>0.05$) exhibited substantial up-regulation, suggesting the pathway has been activated. Interestingly, KDR FR value was 10-fold that of FLT1 FR value (FR=1.7, data not shown), in concordance with already published data⁴⁰⁸ stating that FLT1 is usually expressed at low levels and that KDR is the major mediator of VEGF-driven responses in endothelial cells. Accordingly, it seems that amongst the most common markers previously reported to be related to endothelial biology (Fig. 30A), most of them were upregulated in the bmMSCs populations seeded on pcECMs and cultured for 25 days. These results suggest activation towards endothelial-like transdifferentiation.

Consequently, to further investigate additional pathways that could have influenced this potential differentiation, FR values of these as well as additional genes, tested herein and related to angiogenesis (as defined by Qiagen literature list⁴¹⁰) were compared in a single graph (Fig. 30C). Analysis of this graph revealed that, in addition to the most common endothelial markers already mentioned above (Fig. 30, red font),

several other genes (Fig. 30C, blue font) were apparently involved as well in the process of this pcECM induced differentiation. In particular, IL1B, IL6, MMP1, MMP9, KIT and THBD exhibited absolute FR values which were higher than the FR=10 threshold (12.2, 15.6, 64.0, 145.6, -13.0 and 42.0 respectively) and COL18, ENG and FGF2 (FR= 3.4, -5.7 and -3.3, respectively) which absolute FR values were higher than 2 in a significant manner ($p<0.05$). Using the GNCPro software (Qiagen, DL) we were able to identify common pathways of previously reported interactions between most of those genes, as graphically exported in Fig. 30C. In that figure, arrows point to literary reported up- (green) or down (red) regulation relationship exerted by the gene at the arrow origin on the gene at the arrow end. Interestingly, it was noted that VEGFA has obtained the highest number of interactions with the other up-/down- regulated genes, underlying the fact that VEGFA plays a key role in angiogenesis⁴¹¹, and probably in our system as well. After going through the references to those interactions, we concluded that IL1B (interleukin1-beta) and IL6 (interleukin-6) are both pro-inflammatory cytokines that were demonstrated to induce VEGF expression (and tumor vasculature growth^{412 413}). In our case, these pro-inflammatory cytokines expressed by the bmMSCs to the construct culture environment, seem to act in an auto-/para-crine manner on neighbouring reseeded bmMSCs, pushing them towards endothelial transdifferentiation which may be mediated, in part, through the VEGFA to KDR pathway mentioned above and described in Fig. 30B. Indeed, previous studies have shown that those cytokines were suggested to up-regulate VEGF expression also via the P38 mitogen-activated protein kinase pathway^{414 415}. In addition, MMPs (matrix metallo-proteinases, such as MMP9 and MMP1) are involved in the breakdown of ECM in normal physiological processes such as tissue remodelling⁴¹⁶ and has also exhibited capabilities into promoting angiogenesis by increasing VEGF expression^{417 418 419}. Bergers *et al.*⁴¹⁹ have further suggested that MMP9 could be the angiogenic switch during carcinogenesis in pancreatic islet model by

demonstrating the unique triggering effect of MMP9 rendering normal islets angiogenic. This concept is particularly interesting when looking at MMP9 FR value in this study, as it was the highest significant value observed. Similar observation was also reported several years ago by our group relating to cardiac ventricular fibroblasts, which were seeded on the pcECM scaffold²⁴⁰.

Interestingly, some of the trends observed in this assay were contrary to the literary reported ones. For instance, FGF2, which has been previously reported to be upregulated by the VEGFA expression⁴²⁰, was significantly though mildly reduced in our case (FR=-3.3, $p<0.05$). FGF2 has broad angiogenic and mitogenic activities^{421 422}, and it is known to have at least 5 different mRNA isoforms⁴²³, some of which act through intracrine and others through para-/auto-crine manners. Therefore, this finding may represent a yet unclear regulation which can be the subject of further studies. Another interesting significant yet mildly up-regulated gene, COL18A is a known ECM protein which may be secreted by the pcECM remodelling MSCs. Surprisingly, it has also been reported that when proteolitically cleaved, Collagen type 18 produces a C-terminal fragment known as endostatin. This fragment is a potent anti-angiogenic protein⁴²⁴ and may thus form a secondary and regulatory pathway for the VEGFA induced differentiation. Last, three other genes, THBD, KIT and ENG (Fig. 30C) appear to have no directly reported connections to the known pathway mentioned above. Of these, only THBD was upregulated. THBD (Thrombomodulin) is another endothelial specific marker which binds thrombin and though not commonly used as endothelial marker, this further supports the notion of the observed transdifferentiation. The mechanism by which THBD expression is induced obviously differs from the pathway presented herein. Future studies may be required to elucidate this phenomenon. As for the other two genes, KIT and ENG, those were significantly downregulated. Much to our delight, in both cases, positive

conclusions can be drawn from their down-regulation. KIT was previously reported as an oncogene in cancer related angiogenesis⁴²⁵. Thus, in our assay, it appears no transformation occurs as result of these culture conditions. Finally and much to our surprise, ENG (CD105, Endoglin) has been shown to be significantly downregulated ($p < 0.05$, FR=-5.7). As CD105 is one of the most important markers for MSC lineage integrity⁴²⁶, its down-regulation suggests that at least some of the reseeded bmMSCs may start to lose their lineage integrity. Even significant ($p < 0.05$), the endoglin mRNA concentration in both population samples (pcECM and plate controls) was still relatively high (C(t) values of ~20-23), thus this reduction may not be with a population wide biological influence at that time point. This is further supported by our FACS results showing that harvested cells after 4 weeks of culture did still possess the CD105 marker on their membranes (section 8.2.1).

In conclusion, even though not all statistically significant yet, these results strongly suggested that bmMSCs seeded on pcECMs have undergone RNA expression alteration pointing towards endothelial transdifferentiation. Several mechanisms may be complementarily taking place in the observed pcECM induced bmMSC to endothelial transdifferentiation. Though under normal culture conditions, only subpopulations of bmMSC usually express VEGF receptors⁴²⁷. Hypoxia has already been reported to increase the VEGFA and VEGFR1 expression in relation to MSCs⁴²⁸. As evidenced in sections 8.1.1 and 8.3.3 (static and dynamic sections, before and after, respectively), oxygen gradients play a central role in MSC penetration depth, survival and proliferation on the pcECM and hence hypoxic conditions may at least partially exist within the reseeded construct. Nevertheless, in our hand, the VEGFR2 (and not VEGFR1) levels were elevated concomitantly to the increase in autocrine VEGFA expression pointing to possible additional mechanisms, which are not necessarily hypoxia related. Thus, several

other alternative mechanisms may lead to the observed transdifferentiation which is mediated, in part, through the VEGFA – KDR pathway in either a paracrine or an autocrine manner. First, it has been previously documented that decellularized ECMs of different origins may contain pockets of growth factors such as bFGF and VEGF (as reported in section 8.2.1) that could be released upon cell seeding and induce a paracrine effect on those pcECM remodelling cells. Though the existence of such molecules in the scope of this dissertation was not directly studied on our pcECM, indirect evidence from parallel work in our lab *in vivo* may point to this mechanisms validity. In those studies a considerably high angiogenic effect was transiently observed within implanted acellular pcECM scaffolds in both acute and chronic rat myocardial infarction models. These observations may be explained through a burst release effect of such growth factors released from the remodelled pcECM (data not shown). Second, it has been previously noted that Platelet derived growth factor (PDGF) and its receptors (PDGFR, which expression in both the control and pcECM samples remained constant and relatively high) may be a strong mediator of MSC to endothelial differentiation induced by VEGFA⁴²⁷. This was associated with increased levels of CD31, VEGFR2, vWF expression (and also VE-cadherin which in this study did not change substantially) and may point to a possible differentiation mechanism occurring in our case. Furthermore, prior studies⁴²⁹ have indicated that functional association of PDGF receptors to ECM may increase signalling in an integrin specific manner (like $\alpha_5\beta_1$)⁴²⁹. It should also be noted that because an inflammatory area is often hypoxic and thus pro-angiogenic, inflammation is often associated with aberrant angiogenesis in number of pathological conditions (i.e. cancer, diabetic retinopathy, ischemic cardiovascular diseases and chronic inflammatory diseases)⁴³⁰. As for tissue remodelling, cell invasion for the formation of new blood vessels rely mainly on ECM degradation⁴³¹. Therefore the results obtained from our mRNA expression study suggested that similar molecular pathways (including

inflammatory cytokines and tissue remodelling proteases) are being activated or upregulated in bmMSCs seeded on pcECMs leading to angiogenic effects. Indeed Aplin *et al.*⁴³² have proposed that VEGF could stimulate the production of pro-angiogenic inflammatory cytokines and might represent an early element of the molecular cascade responsible for the angiogenic response.

Future studies may focus on elucidating the role of PDGF signalling and related integrin localization within the pcECM, the possible growth factors' release from the pcECM and the pro-inflammatory mediated response to the apparent pcECM – bmMSC interactions. Another interesting research question arising from these results is whether this phenomenon may also be observed in other more synthetic or natural 3D scaffolds, at this or shorter time points which at the moment remains unclear. The answer to these questions may shed better light on the specific pcECM contribution to this process and clarify the possible mechanism behind it.

8.2.3 Quantitative RT-PCR of stem cells seeded on pcECMs for cardiac related genes

Because mRNA expression from bmMSCs cultivated on pcECMs have exhibited trend towards endothelial transdifferentiation, further studies were conducted for RNA expression levels of genes related also to cardiogenicity. Quality of all RNA extracted samples was validated similarly as in 8.2.2. Out of 20 cardiac specific genes (see annex 11.3.2), only desmin (DES) and myosin light chain (MYL7) exhibited a fold regulation value which was either $FR \geq 10$ (DES $FR=15.4$) or of statistical significance (MYL7, $FR=1.9$, $p<0.05$, Fig. 31).

Fig. 31 Fold regulation values of RNA expression levels from genes related to cardiomyocytes

(A) Fold regulation (FR) values of genes related to cardiomyocytes were compared in a single graph. Only MYL7 value was found significant and DES value ≥ 10 . *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant.

DES is a muscle-specific protein in cardiac muscles mainly located at the periphery of Z-disk of striated muscles⁴³³ and contributes to structural integrity and function of the myocardium. Interestingly, Weitzer et al.⁴³⁴ demonstrated that skeletal myogenesis was totally inhibited in DES null mutant embryo bodies. Another group⁴³⁵ has further suggested that DES could play a role in early cardiomyogenesis by demonstrating that increased DES expression led to the upregulation of early cardiac genes (like *nkx2.5*), which resulted in the development of synchronously beating cardiomyocyte clusters. In this RNA expression study, DES was upregulated but even though not significant, this result suggests activation of DES RNA expression, which could potentially represent a crucial and initial step leading towards cardiac-like differentiation. Assuming DES is part of the initial signals for cardiac gene differentiation, future work may focus targeting the DES activation pathway. MYL7 (myosin light chain 7) is a myosin subunit and was demonstrated to be significantly upregulated ($p < 0.05$). From this result, it is difficult to

suggest any conclusion as MYL is more of a cardiac differentiation end product and none of the early cardiac genes tested have demonstrated any significant change in RNA expression. Overall, these results suggest that pcECM environment does not influence significantly bmMSC fate towards cardiac differentiation but may provide a basic initial platform (as pcECM is originally cardiac ECM), more suitable than synthetic scaffolds. Cardiac differentiation process may require extra external stimulations (i.e. factors or physical stimulations). Furthermore, as the results presented herein refer to a steady state status (i.e. 4 weeks post seeding and static cultures), further studies should be performed to elucidate the kinetics of these phenomena as well as the effect of dynamic culture conditions on the expression of those markers.

8.2.4 Collagen metabolic assessment by H³-proline measurement from cardiac ventricular fibroblasts on pcECMs

Collagen secretion by cvFB seeded on pcECMs was assessed by radioactive measurements of newly synthesized collagen molecules, which have incorporated H³-proline (tritium-bound proline). H³-proline CPM (count per minute) measurements from cvFB-secreted collagen have demonstrated progressive increase within the first days and subsequently decreasing to below detection limits at the end of the experiment (Fig. 32A). At day 4, radioactive measurement reached a peak value of 2,478±496 CPM, which was doubled compared to day 1 (1,088±66). But measurements returned to low values at day 10, to reach undetectable radioactivity at day 18.

Fig. 32 Collagen metabolic assessment of cvFB seeded on pcECMs

Radioactive measurements of tritium-proline incorporation in newly synthesized collagen were performed over 18 days. **(A)** H³-proline CPM over time by cvFB cultured on pcECMs with cell viability. **(B)** Acid-dissolved pcECM, run by SDS-PAGE and stained by silver staining method, demonstrating clear collagen I bands between 100 and 150 kDa.

In light of the results obtained, cvFB behavior on pcECM might be comparable to FBs following cardiac repair, a process in which extensive collagen secretion is usually observed. The heart is ultimately healed by forming a collagen-based scar⁴³⁶. After this post-inflammatory phase, FBs usually return to a quiescent state where all ECM synthesis activity is inhibited⁴³⁷. In parallel, cell viability was measured throughout this study by AlamarBlue™, assuring that any change of collagen level was not correlated to cell densities/quantities. Other studies have also observed decrease in ECM synthesis over time when culturing fibroblasts^{438 439}. To increase or maintain collagen synthesis from fibroblasts, chemical or mechanical stimulation may be needed (previously reported by other studies^{440 441}).

8.2.5 Quantitative RT-PCR of cardiac ventricular fibroblasts seeded on pcECMs for ECM modeling-related genes

To further characterize the remodeling activity of cvFBs on ECM, RNA expression levels of genes related to ECM and adhesion molecules were compared between cvFBs cultivated for 20 days on pcECMs to control sample (cvFBs cultured on tissue culture plate). Similarly as in 8.2.2, quality of all RNA extracted samples was validated by obtaining 260/280 OD values between 1.8 and 2.0, and RNA integrity number (RIN) higher than 7 as required for high-throughput RNA expression arrays (Fig. 28). 260/230 OD was not considered as essential and reached approximately 1.0 value or higher.

Out of 84 genes (see annex 11.3.3), 43 genes have been upregulated considering a fold regulation value (FR) ≥ 2 (Fig. 33), including 16 genes with significant ($p < 0.05$) upregulation values (COL6A1, COL6A2, COL7A1, HAS1, ITGA5, ITGA8, ITGB2, LAMA1, LAMA2, LAMB3, MMP10, MMP11, MMP13, MMP9, TNC and VCAM1). On the other hand, only 5 genes were downregulated considering a $FR \leq -2$ (Fig. 33) including 4 genes (CDH1, CNTN1, CTGF and ITGA4) with significant ($p < 0.05$) downregulation values.

Fig. 33 Fold regulation values of RNA expression levels from genes related ECM and adhesion molecules of pcECM-seeded bmMSCs

Fold regulation values (FR) of genes related to ECM and adhesion molecules were obtained from pcECM-seeded bmMSCs compared to control (bmMSCs cultured on tissue culture plate). Out of 84 genes (see annex 11.1.2), 47 genes have been upregulated considering a fold regulation ≥ 2 (right panel). On the other hand, 6 genes were downregulated considering a fold regulation ≤ -2 (left panel). Particular attention were given to genes with $|FR| \leq 10$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant

In view of the number of upregulated vs downregulated genes, there is a clear trend towards higher cvFB activity on pcECM substrate as compared to cell culture plate, as demonstrated by the upregulated RNA expression profile obtained. Even though collagen metabolic assessment by radioactivity measurements (section 8.2.4) demonstrated a decrease in collagen protein expression after day 9, the reported RNA expression profile related to ECM remodeling suggests still high cellular activity after 20 days of culturing on pcECMs. With the results available, it is difficult to explain such discrepancy, as the RNA expression were obtained at 20 days only (RNA expression at day 10 might have resulted in even higher values). Similarly as in 8.2.2, particular attention was given to those genes with consensus literary importance (such as common gene clusters related to a cellular process) and/or genes which displayed considerably high yet not necessarily significant FR values ($|FR| > 10$, ITGA7, MMP3 and TIMP1) or statistically significant FR values ($p < 0.05$) as mentioned above. The following discussion will refer to those two groups ($|FR| > 10$ and $p < 0.05$) respectively.

CvFB primary function is to maintain cardiac ECM homeostasis by synthesizing structural components like collagen, fibronectin, laminin, elastin as well as glycoaminoglycans which impact cardiac cells and thus heart function³⁶¹. Additionally, they produce ECM-regulatory proteins, matrix metalloproteinases (MMPs) for ECM protein degradation and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs)⁴⁴². A well-controlled balance between both MMPs and TIMPs is critical to maintain ECM homeostasis⁴⁴³. The MMP family is divided into three groups⁴⁴⁴. The collagenase subgroup (MMP13) predominantly degrades interstitial collagen type I, II, and III⁴⁴⁵, whereas gelatinases (i.e. MMP9) degrade gelatins and basement membrane collagen such as collagen IV^{446 447}. The last subgroup, called stromelysins (MMP3 and MMP10) digest a broad range of substrates such as collagen IV, proteoglycans, laminin, gelatins, and

fibronectin⁴⁴⁴. MMP-11, also a stromelysin, does not cleave extracellular matrix components unlike the other MMPs^{448 449} and seem to play an important role in human cancer⁴⁵⁰. In this RNA expression study, significant upregulation values were obtained from genes related to ECM proteases (Fig. 34A, considerably high FR or significant FR highlighted in red), including MMP10 (FR=439, $p<0.05$), MMP11 (FR=3, $p<0.05$), MMP13 (FR=38, $p<0.05$), MMP3 (FR=3038, $p>0.05$), MMP9 (FR=556, $p<0.05$) and TIMP1 (FR=15, $p>0.05$). A special feature of MMP3 is that this protease has also been reported to activate other MMPs^{451 452}, which is particularly interesting as it has demonstrated in this study the highest fold regulation value (even though not significant yet). In addition, the proteolytic activity of MMPs is being controlled by the TIMPs. Amongst them, TIMP1 RNA expression was significantly upregulated. This metalloproteinase inhibitor was reported at low level expression in the healthy heart but higher in diseased hearts⁴⁵³. Altogether, these results suggested high cellular activity related to ECM remodeling through degradation, which could be beneficial in term of cell infiltration. A critical function for a tissue-engineered construct lies in its ability to foster cell invasion. These cells should initiate the remodelling process by partially degrading the ECM with secreted proteases and by secreting molecular signals enabling cell migration and proliferation⁴⁵⁴. Some groups have indeed demonstrated bioactive hydrogels with protease-sensitive peptides to enable facilitated degradation (and thus cell infiltration) of their hydrogel platform^{455 456 457}. Even though those groups were using kinetics to determine the optimal peptide formulation to obtain suitable enzymatic response, our pcECM substrate has demonstrated capabilities to enable enhanced remodelling effects from the cell type seeded (in our case cardiac ventricular fibroblasts).

Fig. 34 Fold regulation values of RNA expression levels from genes related to specific subgroups in ECM and adhesion molecules

Fold regulation (FR) values of genes characterizing specifically ECM proteases (**A**), FR values of cell/matrix adhesion related genes (**B**), FR values of ECM constituent related genes (**C**) were compared in a single graph. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant.

In this RNA expression study, genes related to cell-matrix adhesion molecules have also demonstrated mostly upregulation with FR values for ITGA5 (FR=2, $p < 0.05$), ITGA7 (FR=11, $p > 0.05$), ITGA8 (FR=19, $p < 0.05$), ITGB2 (FR=17, $p < 0.05$) and only one significantly downregulated FR value for ITGA4 (FR=-12, $p < 0.05$) (Fig. 34B, considerably high FR or significant FR highlighted in blue). ITGs, called integrins, are a large family of transmembrane glycoprotein receptors, which connect the intracellular and extracellular environments. They mediate cell-matrix by binding ECM glycoproteins (i.e. collagens, fibronectins, laminins) and cellular receptors (i.e. VCAM-1 or ICAM)⁴⁵⁸, and also mediate cell-cell interactions by modulating signal transduction pathways that control biological and cellular functions including cell adhesion⁴⁵⁹. Integrins are formed by two non-covalently bound A and B subunits, which pair to form heterodimers. Up to date, 18 A and 8 B subunits have been identified in mammals, but not all combinations of A/B heterodimers can form⁴⁶⁰. ITGB2 when associated to ITGAL and ITGAM (tested but not up/downregulated) binds to cellular receptors such as the ICAM family receptors and

fibrinogens⁴⁶¹ suggesting cell-cell interactions. Interestingly, this B integrin has demonstrated to be expressed during cardiogenesis in embryonic mouse and chicken heart⁴⁶². In addition, ITGA5 and ITGA8 can generally join with ITGB1 (FR=3, $p>0.05$) to form a fibronectin receptor^{463 464}. Significant upregulation of both ITGA5 and ITGA8 RNA expression (even though not extensively high) is not surprising as our pcECM has previously demonstrated preserved fibronectin proteins after decellularization²⁴⁰. Both ITGA5 and ITGA8 expression were demonstrated in myocytes and cardiac fibroblasts^{460 465} (in concordance with the results obtained in this study), allowing the cells to anchor the ECM matrix through fibronectin connections. Interestingly, ITGA7 was reported as a receptor for the basement membrane protein laminin and may be involved in differentiation and migration processes during myogenesis^{466 467}. It is expressed in skeletal and cardiac muscles only⁴⁶⁷, and not in cardiac fibroblasts as they normally contain no laminin in their basal membrane⁴⁶⁰. The fact that in this study, cvFBs on pcECMs did exhibit RNA expression of ITGA7 may result in response to the upregulation of laminin-related genes (LAMA1 and LAMA2, FR=17 and FR=3 respectively, $p<0.05$). Finally, ITGA4 is a receptor that mediates either cell-ECM or cell-cell adhesions by interacting with fibronectin and VCAM1 respectively. It is unclear why ITGA4 exhibited a significant downregulation value in this study as VCAM1 on the other hand showed significant upregulation (FR=27, $p<0.05$) and probably requires further study. Nevertheless, the fact that ITGA4 is significantly downregulated concomitantly with upregulation of VCAM1 may be indicative of inhibition of particular cell-fibronectin (of pcECM) interactions at the measured time point. Whether this is a time dependant result remains unclear at the moment and requires further studies. Overall, integrins were demonstrated to be upregulated suggesting enhanced interactions between cvFBs and pcECM surface. To clarify the mechanisms which could play in cell attachment, further studies may focus on determining the exact combinations of integrins forming in this

particular case (A and B units).

RNA expressions of genes related to ECM constituents have demonstrated only upregulated FR values of which statistical significance was observed only for COL6A1 (FR=3), COL6A2 (FR=5), COL7A1 (FR=19), HAS1 (FR=27), LAMA1 (FR=17), LAMA2 (FR=3), LAMB3 (FR=23) and TNC (FR=20) (Fig. 34C, significant $p < 0.05$ FR values are highlighted in green). Amongst the collagen-related genes, COL1A1, COL4A2 and COL5A1, which encode the main collagen structures⁴⁶⁸, have not exhibited FR values exceeding the cut-off value (data not shown). Surprisingly, secondary collagen structures, such as microfibrillar and anchoring fibrils encoded by COL6 and COL7 respectively⁴⁶⁸, were upregulated in this RNA expression study, suggesting cvFBs strengthen the initial collagen-based ECM with smaller collagen structures to allow cell adhesion and migration. Furthermore, HAS1 (hyaluronan synthase 1) was significantly upregulated (FR=27). This high molecular weight unbranched polysaccharide is actively produced during tissue repair for ingrowth of blood vessels⁴⁶⁹. This result is even more encouraging as we hypothesized previously (section 8.1.2.1) that our pcECM may lack some of the relevant glycosaminoglycans (such as hyaluronan) which could explain the improvement in cell adhesion and proliferation following nitrocellulose treatment (due to the relevant increase in similar glycosidic moieties present). CvFBs may have responded accordingly to express ECM missing features, which according to this assay include the hyaluronan as well as LAMA1, LAMA2 and LAMB3 (all significantly upregulated). The latter proteins are part of the laminin adhesion mechanism to the basal membrane (ECM structures located in the immediate vicinity of most cells)⁴⁷⁰. Their upregulation could enhance cell migration and adhesion but also formation of gap junctions as reported by Imbeault *et al.*⁴⁷¹. Finally, TNC (tenascin C) which was also significantly upregulated (FR=20) plays an important role in tissue remodelling (like cardiac repair), such as MMP upregulation

and activation, angiogenesis or fibrosis⁴⁷². It can bind to integrins which transmit multiple signals for cellular fate (i.e. cellular adhesion or de-adhesion, proliferation, death, etc). Overall, many genes related to ECM constituents were upregulated when seeding cvFBs on pcECMs. This suggests cvFBs beneficial interactions with our pcECMs, as ongoing deposition of important ECM constituents even after 20 days was observed through RNA expression. Further studies needs to be performed on control 3D collagen scaffolds to elucidate whether the effect observed herein is directly related to the pcECM unique biophysical features or to its main collagenous structural composition.

In addition, CDH1 (E-cadherin) was significantly downregulated (FR=-21). This calcium dependent cell-cell adhesion glycoprotein mediates not only mechanical adhesion between cells but also coordinate cell movements as well as structural and functional cellular characteristics⁴⁷³. Many studies have reported results in cancer research which associated increased cell mobility (resulting in invasiveness) to the reduction or loss of CDH1 expression^{474 475}. In our case, CDH1 downregulation may be beneficial as it could allow cells to invade and migrate easier on the pcECM scaffold. Surprisingly, when upregulated, CNTN1 (contactin-1) which in our hands was also significantly downregulated ($p<0.05$, FR=-20), was previously reported to downregulate⁴⁷⁶ CDH1 (FR=-20), underlying some alternative pathways influencing both proteins. Furthermore, CTGF (connective tissue growth factor) has exhibited significant downregulation (FR=-3, $p<0.05$). This secreted matricellular protein modulates many signalling pathways leading to cell adhesion/migration, angiogenesis, myofibroblast activation, ECM deposition and remodelling⁴⁷⁷.

Taken together, those results suggest enhanced functional cvFB activity on pcECMs as demonstrated by the increased FR values for ECM- and adhesion molecules related

genes, particularly those genes related to proteases (MMP), cell-matrix adhesion molecules (ITG) and ECM constituents (COL and LAM) (Fig. 34A-C). There have been several suggested mechanisms on how cvFB are being activated (leading to cardiac remodelling), mostly described in diseased conditions⁴⁴². For instance, changes in ECM production may occur following enhanced secretion of growth factors, such as angiotensin (not tested)⁴⁷⁸, endothelin (not tested)⁴⁷⁹ or TGFB1 (transforming growth factor- β_1 , FR = 2.0, $p > 0.5$)⁴⁸⁰. However, angiotensin and endothelin are normally secreted in the kidney⁴⁸¹ and by vascular endothelial cells⁴⁸² respectively, and thus, those growth factors are not relevant in this study. TGFB1 was found upregulated (although not significant yet) and might have been secreted by cvFB to stimulate collagen production as already suggested by other studies^{483 484 485}. Future work may focus on comparing our pcECM and synthetic scaffold in term of cvFB activity and determining specific proteins enabling enhanced pcECM remodelling as compared to tissue culture plate. Finally, even though overactive fibroblasts have been highly described in cardiac diseases, upregulated activity of cvFB in this study suggested enhanced cell-matrix interactions and may be favourable to promote cell integration in such tissue constructs.

8.3 Achieving dense recellularization under dynamic conditions

8.3.1 Single time-point injection seeding method

Several seeding strategies have been reported to optimize cell distribution and migration within a thick scaffold. Amongst them, some have conceived sophisticated techniques to induce cell infiltration. For instance, Shimizu *et al.*⁴⁸⁶ have magnetically labeled fibroblasts and seeded 3D collagen sponges located on a magnet. Cell seeding efficiency was improved compared to traditional surface seeding. In addition, Li *et al.*⁴⁸⁷ have used surface acoustic waves (SAWs) to drive cell suspension into scaffolds by forming a longitudinal pressure, which resulted into a liquid recirculation towards the

scaffold. Despite these few alternative approaches, most studies however have used conventional seeding techniques like surface seeding, direct injection with a needle, centrifugation and dynamic shaking. For instance, Thevenot *et al.*⁴⁸⁸ have compared all four seeding methods and obtained satisfying cell distribution and cell penetration with centrifugation and dynamic shaking methods, whereas the other two methods have given poor results. But those latter seeding methods (i.e. centrifugation and dynamic shaking) were attempted on pcECM by other students in our lab and exhibited no substantial improvements over conventional injection or pipettation (data not shown). It was deducted that the pcECM slabs were presumably too thick (10 mm) to suit centrifugation-seeding technique, as the fibrous network would have probably collapsed on itself during centrifugation, forming a thick and dense tissue in which cell diffusion was very limited. In addition, in some preliminary experiments (data not shown), bmMSC seeded on pcECM surface have demonstrated poor survival in dynamic shaking conditions (where small pcECM scaffolds were floating with medium in an orbital shaker), which eliminated any consideration in using such method for pcECM slab seeding.

In our hands, cell seeding using direct cell injection (Fig. 35A) with a needle revealed itself an easy and fast methodology. While injecting the medium volume in pcECM bulk, the pcECM slab was observed to swell with minimum medium loss. However the main disadvantages of this method relate to major variations dependent on manual injections in multiple locations. Though our strategy was to reseed in high volume to assure all pcECM matrix is properly inflated, many parameters which are not controlled can affect the cell final distribution including the depth of needle penetration, the number and location of the random injection sites, the speed of injection and the volume injected in each site. In addition, pcECM slabs have some biological variances. Thus, diffusion characteristics could also influence the final cell attachment efficiency

obtained using needle injection methodology. Nevertheless as this method was already successfully reported for rat whole heart ECM²³⁵, it was also evaluated herein serving as a baseline for further possible improvements, which were evaluated in subsequent stages.

Fig. 35 Cell attachment study on variable incubation periods in pcECM slabs by single time point needle injection seeding method

PcECMs slabs were seeded with 10 millions bmMSCs by needle injection. Cell viability was measured by AlamarBlue™ immediately after 1.5, 8 or 24 hrs incubation time (static culture to allow for cell attachment) followed by 24 hr perfusion. (A) The seeding injection method is illustrated before and after seeding. A pcECM was positioned with the myocardium side facing up and was punctured at different locations with a needle containing suspended cells. (B) Cell viability study performed 1 day after attachment incubation period (1.5, 8 and 24 hrs) (C) Cross section obtained by Hematoxylin & Eosin staining of pcECM seeded with 8 hrs attachment time. This cross section demonstrated a “cell pocket” which was also observed in others pcECM samples.

As the seeding methodology was changed from the pipettation used under static culture conditions in previous aims, the optimal attachment period was also evaluated herein. Furthermore, an optimization is required to elucidate whether the optimal attachment parameters deducted under static conditions (90 min seeding, density of 6×10^5 cells/cm²) are still sufficient to enable proper cell survival using dynamic perfusion. This optimization relates to a tradeoff between premature perfusion which flow shear stress may inhibit proper attachment to delayed perfusion which may also affect cell viability due to accumulation of waste products and the deprivation of oxygen and nutrient supply⁴⁸⁹. In these experiments, pcECMs slabs were seeded with 10 million bmMSCs by needle injection and were allowed to attach for various pre-incubation times (1.5, 8 and 24 hrs, with no perfusion). This was followed by 24 hrs of dynamic perfusion, at the end of which, cell viability was measured by AlamarBlue™. We assumed that wherever cells could survive and proliferate through medium consumption (i.e. under both static and dynamic culture conditions), the medium, as well as the AlamarBlue reagent which is a fairly small molecule, could also be suitably metabolized. Nevertheless, negative controls of matrices soaked with AlamarBlue media without cells were performed (data not shown) which results were not significantly different from the blank, suggesting that every measured effect is the result of the presence of cells. In that sense, the results obtained are stringent as they report the minimal measurable effect of cell metabolism.

On another set of experiments, multiple injection seeding events through time (section 8.3.2) and pipettation seeding of static steady-state attachment followed by dynamic perfusion (section 8.3.3) were used to increase the seeded densities and are further discussed below. Results (Fig. 35B) have demonstrated a significantly higher ($p < 0.05$) cell attachment at 8 hours with $4.4 \times 10^5 \pm 0.3 \times 10^5$ cells attached compared to

$1.8 \times 10^5 \pm 0.1 \times 10^5$ and $1.8 \times 10^5 \pm 0.9 \times 10^5$ for 1.5 and 24 hrs respectively. Overall, the attachment percentage in dynamic conditions using needle injection seeding method was approximately 5% or lower of total initial cell seeding number. This number is at least 7 times lower than results observed from experiments in static conditions, which emphasized the higher constraints in terms of nutrients and oxygen found in large and thick pcECM slabs.

Direct injection is one of the most common seeding techniques in 3D tissue engineering, which have been reported in literature^{488 490 491}. However, it has not always demonstrated the most suitable capabilities in term of cell distribution and viability⁴⁹², which are crucial for recellularization of thick scaffolds. For instance, Thevenot *et al.*⁴⁸⁸ have compared different seeding methods, including direct injection technique, of porous poly(lactide-co-glycolide) (PLGA) scaffolds. Even though the size of the scaffolds (5mm diameter, 3mm thickness), cell repopulation obtained by direct injection was poorly distributed and obtained lower viability than the other methods. Results obtained in this study corroborated with those reported findings. For all samples, H&E cross sectional stains revealed that cells were visible more in the form of isolated “cell pocket” areas (Fig. 35C) and were not uniformly spread throughout the entire cross section of the pcECM. Unfortunately, those cell areas were rarely observed with only none to one single cell pocket per cross section evaluated in at least 4 cross sections taken from four different locations along the pcECM slabs (data not shown). Several parameters could cause this weak ‘pocket-like’ cell presence reported by both cell attachment study and H&E stains. First of all, it is possible that the pcECM being a non-isotropic scaffold may possess areas whereby cell attachment is preferred due to particular constellation of structural proteins, glycosaminoglycans and growth factors within those pockets. Still, under static conditions, cells exhibited good attachment to the pcECM surface and this

“pocket phenomena” was not observed. As the pcECM slices used for static studies were randomly cut from similarly thick pcECM slabs, it seems unlikely that such a preferred matrix composition did not manifest itself under static cultures as well. Therefore, other factors related to the seeding methodology and to possible oxygen and nutrient deprivation may be more relevant. Relating to the injection methodology, it is possible that needle injection is very inefficient in terms of achieving a homogenous seeding of the entire pcECM slab (seeding punctures were performed randomly in the scaffold and were not controlled in any way). Furthermore, cells might have failed to survive during the attachment period or during the 24h-hrs perfusion following it. The pcECM environment has already demonstrated low penetration capabilities for cell attachment and growth in static conditions (see 8.1.2), suggesting that cells might be limited by oxygen and nutrient supply during the first initial hours of attachment within the bulk of the pcECM slab model. In addition, it is conceivable that some areas in the pcECM slab were insufficiently supplied with oxygen and nutrients due to lack of proper supply channels preservation. Indeed, our group has already reported that the pcECM vasculature was shown to preserve the main conduits but small capillaries were mostly destroyed during the decellularization procedure²⁸⁶, which could leave some areas within the pcECM less perfused. This finding could have direct consequence on the survival rate of those “cell pockets”. The ones closer to the main vascular conduits (and thus closer to medium perfusion) would have better chance of survival than the ones located further away. Last, it should be noted that unlike the physiological setting, these studies were performed without additional oxygen carriers. Thus the gradients of oxygen depletion caused by cellular consumption in close proximity to the supply arteries may result in very low oxygen levels throughout the remaining areas of the matrix as reported in 3D tissue engineered constructs⁴⁹³.

Nevertheless, within those surviving pockets cell morphology, the density measurements obtained by automated calculation using ImageJ software revealed some promising results. Morphologically, cells seemed to be aligned along the fibers suggesting functional attachment as opposed to physically trapped cells (Fig. 35C), which morphology is expected to be round. Cell counts from three different regions of “pocket” areas in H&E stained cross sections were averaged and compared to human cardiac tissue cell density (Fig. 36A-C). A cell density average of $4,305 \pm 757$ cells/mm² was obtained (Fig. 36D). Considering a cross sectional thickness of 10 μ m (0.01 mm), this number was further converted to 4.3×10^8 cells/cm³, which is comparable to the total physiological cell density in the human heart reported in the literature ($\sim 10^8$ cells/cm³)⁴⁹⁴, whereas cardiomyocyte density is estimated around 2×10^7 cells/cm³⁴⁹⁵. This result confirmed the capability of pcECM slab to support physiological cardiac cell densities. The cell density estimated was higher in dynamic conditions than in static conditions (8.1.1).

To date, no study has ever reported engineered constructs with physiologically relevant dimensions to replace an infarcted area and recellularized with similar cardiac cell densities found in the human heart. For instance, Radisic *et al.*⁴⁹⁶ have demonstrated high-density recellularized collagen sponges ($\sim 10^8$ cells/cm³) with neonatal rat CMs by using Matrigel® as a vehicle for rapid cell delivery. Even though those collagen sponges possessed considerable thickness (1.5 mm) that necessarily required a bioreactor apparatus to sustain cell viability, their constructs was far from being sufficient to adapt a replacement of an entire infarcted area in the human model. In this study, cells were not homogeneously distributed throughout the entire pcECM slab but results have demonstrated presence of viable high-density cell areas similar to physiological cardiac cell densities within the pcECM seeded by the single time point injection method. These results confirmed the applicability of such pcECM scaffold and gives, for the first time,

exciting prospects to the successful engineering of a thick cardiac construct with relevant dimensions for medical applications.

Fig. 36 Maximum cell density from pcECM slab by automated calculation using ImageJ software

(A) Initial H&E cross section of a high dense “cell pocket” with arrows pointing to elongated nuclei morphology which supports the notion of functional cell attachment. (B) Corresponding image after processing to obtain a binary image where cell nuclei were selected and highlighted by using a color threshold. (C) Three regions were selected and analyzed for particle counting. (D) Parameters table of each region (including total counts and dimensions per region) to finally obtain a cell density average of $4,305 \pm 757$ cells/mm².

8.3.2 Multi time-point injection seeding method

Cell injection seeding method has demonstrated in 8.3.1 the presence of high cell density areas randomly distributed within the pcECM slab. Assuming cell pockets do survive the initial seeding, they may subsequently spread throughout the matrix given proper dynamic culture conditions to repopulate increasingly larger areas. This would be expected though to be time dependent and so it is evident that increasing the initial attachment is vital to shorten the dynamic cultivation period. To elucidate whether the pocket areas were a result of some particular ECM composition within those structures or the result of oxygen and nutrient gradients, we performed a repeated injection seeding study. It is well established that with time the structural composition of reseeded ECM changes due to remodeling⁴⁹⁷, secretion of growth factors⁴⁹⁸ and glycoasaminoglycans⁴⁹⁹ by the reseeded cells. PcECM pretreatments seeded with human bmMSCs were also shown by other members of our group (data not shown) to support attachment and proliferation of subsequent reseeded cells. We therefore hypothesized that repeated injection seeding would enable to distinguish between attachment foci dependent pocket formation and nutrient deprivation pocket formation. An increase in attachment densities through time would be expected to reflect pcECM remodeling by the cells while a decrease to steady state values after each seeding cycle would suggest oxygen and nutrient deprivation mechanism.

Thick pcECM slabs were seeded using multi-time point injections (day0, day7 and day 14) with 10×10^6 bmMSCs (2×10^6 cell/ml). Following 1.5 hrs attachment incubation time, dynamic cultivation commenced and cell viability was monitored for over 17 days. Results indicated that pcECM slabs throughout those 17 days of culture did not show any significant increase of attached cell quantities. Initial cell attachment number reached $(4.2 \pm 0.4) \times 10^5$ bmMSCs at day 0 (Fig. 37A). After each cell injection, cell number

increased immediately but declined subsequently after 3-5 days, neutralizing the cell increase obtained right after injection. Total cell number reached a maximum of $(5.2 \pm 0.5) \times 10^5$ bmMSCs (day 15) and minimum of $(1.9 \pm 0.9) \times 10^5$ bmMSCs (day 13). Thus, despite the multiple cell injections performed, the overall bmMSC quantity did not increase and was reduced back to a constant steady state value between $2\text{--}4 \times 10^5$ cells.

Fig. 37 Cell viability of multi-injected pcECM slab

In order to raise the number of those cell “pockets”, a thick pcECM slab was multi-seeded with 10×10^6 bmMSCs (2×10^6 cell/ml) every 7 days (day0, day7 and day 14) by needle injection with 1.5 hrs attachment incubation time. Cell viability measurements were performed in dynamic conditions before and after each cell injection. (A) Cell viability of multi-injected pcECM slab.

In 8.3.1, single time-point cell injection seeding method was shown to result in the formation of high cell density areas (“pockets”), which remained small and randomly distributed within the pcECM slab. However, the current results (Fig. 37A) cultured in dynamic conditions over 15 days has demonstrated no cell proliferation, suggesting cells might have entered quiescent state or a homeostasis between proliferation and cell death. This effect was also observed under static culture conditions as described in section 8.1.2. This homeostatic state may be at least in part a result of metabolism i.e. limited oxygen

and nutrient supply as otherwise cell remodeling and secretion of ECM should have led to an increase in cell supporting capacities through repeated seeding. Nevertheless, whether additional pcECM specific signaling may also contribute to cell quiescence (and hence to less ECM remodeling and secretion), remains unclear at the moment and would be the scope of future studies.

8.3.3 Static pre-seeding before dynamic cultivation method

Under dynamic culture conditions the cultivated and reseeded pcECM slabs are fed by perfusion while being immersed in culture media inside a custom-built tissue culture bath (Fig. 8). From the results obtained using the injection seeded methodology (sections 8.3.1 and 8.3.2), we can assume a crucial role to oxygen and nutrient gradients in the vicinity of culture media sources, which in our case include the pcECM vasculature and the surrounding bath. As the culture media filling the bath originates from the same perfusion catheters and leaks to the bath from the pcECM slab, we hypothesized that those gradients would have higher concentrations the closer we get to the supply channels. This is particularly reasonable as the oxygen saturation (>95% oxygenation) occurs by an oxygenator located in close proximity to the bioreactor inlet. Thus, these oxygen gradients were hypothesized to dictate cell migration towards the feeding vasculature. Therefore, an alternative approach to the injection-based methodologies was developed, which involved a two-step sequential combination of both static and dynamic cell culture conditions. In previous static cultures, limited cell penetration depth was demonstrated (up to 100µm from the surface, section 8.1.1). Here, it was speculated that once a homeostatic steady state is achieved (also modeled in 8.1.1) switching from static to dynamic cultivation may elicit cell migration towards the feeding blood vessels, commonly located along the epicardial areas ~10mm away from the endocardial reseeded surface. Using histological cross sections, it was expected to see an improvement in cell

penetration depth resulting from such strategy, which would probably be also concomitant with an increase in cell proliferation. Thus, bmMSCs were pre-seeded on the endocardial surface of a nitrocellulose (NC)-coated (section 8.1.2.1) pcECM slab and cultured in static conditions for 4 weeks to reach steady state homeostatic cell density values. The scaffold was subsequently transferred to the bioreactor for additional 2 weeks under dynamic perfusion. Cell viability was measured using AlamarBlue™ and cell infiltration was assessed by cross-section histology and immunofluorescence staining at the endpoint of experiment.

During the pre-cultivation period under static conditions, cell viability increased from 0.9×10^6 to 3.85×10^6 total cell number at day 13 and remained constant around this homeostatic value for the following 2 weeks. At day 30, the pcECM slab was transferred to dynamic conditions where cell viability significantly increased ($p < 0.05$) by over 4 fold reaching $(13.31 \pm 0.22) \times 10^6$ cell number 44 days post seeding attributed to dynamic medium perfusion during that time (Fig. 38A). Nuclei fluorescence staining (Fig. 39A) and Masson trichrome staining (Fig. 39B) of pcECM cross sections revealed a densely populated initial 100 μm from the endocardial surface followed by what appeared to be migrating cells which have infiltrated up to 1.2 mm deep inside the scaffold. BmMSC identification was verified by CD44 immunofluorescence staining (Fig. 40) and was confirmed positive. In addition, bmMSCs were found CD-31 positive (Fig. 41) similarly as under static conditions (section 8.2.1) confirming potential endothelial transdifferentiation effects both under static or dynamic conditions. Furthermore, to determine if the static pre-culturing step was involved in triggering cell proliferation in dynamic conditions, a pcECM slab was similarly seeded (and coated with NC) but was directly perfused in the bioreactor, skipping the static pre-culturing step. Cell viability exhibited no increase over 14 days in the bioreactor (Fig. 38A), compared to the 4-fold

increase for the pre-seeded scaffold. This strongly emphasizes the importance of the static pre-culturing period to support cell proliferation under dynamic conditions in pECM slabs. It is possible that a minimal cell density is required before cell proliferation could occur in dynamic conditions. The only difference between both pcECM slabs (with and without pre-culturing step) was only cell density which differed at the start of perfusion ($\sim 4 \times 10^6$ vs 1×10^6 cells, respectively). In addition, bmMSCs might require time to express the correct genes to enable survival and growth, which is probably not favored if perfusing the 3D scaffold right away. In traditional 2D culture conditions, cell-secreted and medium factors mix and diffuse in the medium freely, rapidly reaching equilibrium⁵⁰⁰. However, in 3D environment, the scaffold slows down the diffusion of soluble factors and delays cellular reactions triggered by those diffused components. Structural features of the scaffold (pore size, interconnectivity, scaffold dimension) as well as cell density were demonstrated to modulate the diffusion of soluble factors⁵⁰¹.

Fig. 38 Cell viability of statically pre-seeded pcECM slab

A two-step combination of both static and dynamic cell culture conditions was attempted to recellularize thick pcECM slabs. (A) Cell viability measurements obtained during the pre-cultivation period in static conditions followed by the period in dynamic conditions (PRE-SEEDING). For comparison, a NC-coated pECM slab was similarly seeded but transferred directly in the bioreactor system (DYNAMIC ONLY). (B) Illustration of pcECM slab after AlamarBlue measurement. Because AlamarBlue is based on reduction of resazurin blue dye to resorufin, which is pink and fluorescent, cell areas on the pcECM slab appeared pink and easily visible by the naked eye.

Fig. 39 Histological cross sections of statically pre-seeded pcECM slab by nuclei fluorescent counterstaining (DAPI, blue) adjacent to ECM auto-fluorescent (red)

(A) and Masson Trichrome staining with control (low right side image) (B). Both staining methods have revealed cell infiltration up to 1 mm deep inside the scaffold. Populated areas have demonstrated high cell density.

Fig. 40 Immunofluorescence staining of CD44 (FITC) on cross sections of bmMSC-seeded pcECM slab

PcECM background (Red), nuclei staining (Blue), CD44 staining (FITC) were taken by confocal microscopy. CD44 staining was positive, verifying the presence of bmMSCs on seeded pcECM slab. Images of CD44 and nuclei staining were merged to confirm the overlapping of both stainings.

Fig. 41 Immunofluorescence staining of CD31 (DL557) on cross sections of bmMSC-seeded pcECM slab

PcECM background (BKG, green), nuclei staining (Hoescht, blue), CD31 staining (DL557, red) were taken by confocal microscopy. CD31 staining was positive, verifying similar effects under dynamic conditions as observed under static conditions of potential bmMSC transdifferentiation towards endothelial-like cells.

Furthermore, to confirm that the improving effects on cell proliferation and migration were indeed triggered by the bioreactor perfusion, a NC-coated pcECM slab was similarly seeded with bmMSCs and subsequently cultured for 3 weeks in static conditions but with no subsequent dynamic perfusion. Cell density has reached maximal surface confluency after 3 weeks in static culture (Fig. 42A) as cell viability measured Alamar Blue™ remained constant (similarly as for the static pre-seeded and dynamically cultured pcECM slab). The entire cECM slab was fixed, stained with Hoescht and observed under fluorescence microscopy (Fig. 43A). Results demonstrated high and dense cell density throughout the pcECM slab surface, confirming that cell confluency has reached its maximum. Cross sections were further characterized by confocal microscopy (Fig. 43B-C). Similarly as in static cultured pcECMs, cells have mainly confined as a monolayer on the surface of the pcECM slab, confirming the beneficial effects of the bioreactor perfusion.

Fig. 42 Cell viability of NC-coated pcECM slab cultured only under static conditions

(A) Cell viability of NC-coated pcECM slab cultured only in static condition. It was similarly seeded with bmMSCs in the rectangular seeding chamber (B) and subsequently cultured for 3 weeks in static conditions but with no subsequent dynamic perfusion. Cell density has reached maximal surface confluency after 3 weeks in static culture as cell viability measured AlamarBlue™ remained constant (similarly as for the previous pcECM slab).

Fig. 43 Nuclei fluorescence staining of surface and cross sections of pcECM (static cultured only) by binocular and confocal microscopy

(A) Entire pcECM slab was stained with Hoescht, observed under fluorescence microscopy and compared to control pcECM (no cells). Results demonstrated high and dense cell density throughout the pcECM slab surface, confirming the cell viability results that cell confluency has reached its maximum. Surface cell density (B) and cross sections (C) were further characterized by confocal microscopy. Similarly as in static cultured pcECMs in section 8.1.1, cells have mainly confined as a monolayer on the surface of the pcECM slab.

Coming back to our static pre-seeded and dynamically cultivated pcECM, cell density across the histological cross sections was further estimated by automated calculation using ImageJ software similarly as in 8.3.1. Cell counts of four rectangular regions (taken at 63x magnification) from three different distances to the pcECM surface (0-100, 100-500 and 500-1000 μm , Fig. 44A-B) were averaged and compared to human cardiac tissue cell density. Cell densities reached $6.6 \pm 2.28 \times 10^8$, $4.08 \pm 1.54 \times 10^8$, $1.03 \pm 0.11 \times 10^8$ cells/ cm^3 for 0-100, 100-500 and 500-1000 μm depth respectively (Fig. 44C). They were found comparable to the total physiological cell density in the human heart as reported in the literature⁴⁹⁴ ($\sim 10^8$ cells/ cm^3). Previous attempts to quantify by image analyses at 20x tile scans were underestimating and not sufficiently accurate (annex 11.2). Therefore image analyses were performed based on 63x image analyses of $n=4$ regions per distance interval (0-100, 100-500 and 500-1000 μm) repeated on at least 3 sections taken from our recellularized construct.

Fig. 44 Highly cell-populated pcECM area for cell density estimation by automated calculation using ImageJ software

(A) A highly cell-populated area from pcECM cross section was stained with DAPI (tile scan at 20x). (B) DAPI nuclei-stained images were further taken at 63x by confocal microscopy from three different distances to the pcECM surface, 0-100, 100-500 and 500-1000 µm. Their corresponding binary images with counted illustration images are shown. (C) Estimated cell densities obtained by automated counting using ImageJ software were found comparable to total physiological cell density in the human heart (10^8 cells/cm³) as previously reported in literature.

Very few studies have reported combining both static and dynamic culturing for recellularization in tissue engineering⁵⁰². Most have compared separately static against dynamic conditions using various seeding methods and bioreactor systems. In general, dynamic perfusion in 3D scaffolds has shown to enhance cell migration and proliferation as compared to static culturing by providing oxygen and nutrients throughout the entire 3D scaffolds. Therefore, the need to combine both culturing conditions has rarely

occurred. Previous seeding attempts, by me and others in my group, to achieve a robust and homogeneous recellularization of thick pcECMs slabs yielded mostly unsatisfactory results. Centrifugation, dynamic seeding, single and multiple time-point injection seeding methods have all demonstrated poor cell penetration and proliferation. The two step methodology developed herein offers for the first time a more reproducible and easy to use methodology which is based on the system intrinsic capacity to form steady-state relationship of cell densities coating the entire endocardial surface of the slab. Switching to dynamic cultivation following homeostasis resulted in more or less even cellular presence, which appeared to be migrating towards the epicardial surface. To the best of our knowledge, this is the first time such a complex seeding methodology is suggested based on cell intrinsic migration following nutrient / oxygen gradients. This also serves as a proof of concept for the importance of oxygen and nutrient gradients in this as well as other similar perfusion systems. Furthermore, by using this uncommon combination of culturing methods, it confirmed the complexity of our pcECM slabs and underlines the need to develop new and creative strategies to recellularize extra thick constructs, thicker than the ones usually reported to date (1-2 mm).

Altogether, these results suggest that our main hypothesis, that switching from static to dynamic cultivation could elicit cell migration towards the feeding blood vessels (Fig. 45A), was validated. A significant cell growth was observed in dynamic conditions when the pcECM slab was pre-seeded on the surface and cultured for a 4-week period prior dynamic culturing. As the surface reached near maximal cell density, it was conceived that cell migration had no other alternative than to progress deep inside the scaffold. It is important to note that the pcECM endocardial side was facing up for the pre-seeded pcECM during the entire dynamic perfusion. In consequence, medium was transported from the main vasculature towards the surface (Fig. 45B). As cells were located on the

pcECM surface, cell infiltration was probably promoted by preferential cellular migration towards freshly perfused medium (Fig. 45C). This hypothesis suggests that macro-dimensional parameters like scaffold orientation and direction of medium diffusion might play an important role when recellularizing a thick scaffold with relevant dimensions as in human cardiac tissue.

Fig. 45 Schematic illustration of hypothetical mechanism of cell migration in pcECM slab seeded by two-step seeding methodology

(A) Schematic of two-step seeding methodology. PcECM endocardial surface was facing during the entire dynamic perfusion (B). In consequence, medium was transported from the main vasculature towards the surface, resulting in an upward flow direction. As cells were located on the pcECM surface, cell infiltration was probably promoted by preferential cellular migration towards freshly perfused medium (C).

9 Conclusion

In this study, we have reported the suitability of such decellularized porcine extracellular matrix (pcECM) scaffold to support cell survival and cell growth in a single or co-culture system using human bone marrow mesenchymal stem cells (bmMSCs) and human cardiac ventricular fibroblasts (cvFB) towards the generation of cardiac construct. In addition, the feasibility to recellularize at some extent thick pcECM slab was demonstrated using a custom-made bioreactor.

PcECM treatments with native ECM proteins (laminin, fibronectin or gelatin) or synthetic polymers (polycaprolactone or nitrocellulose) have demonstrated either no or little improvement in term of cell attachment and proliferation, except for nitrocellulose treatment which enhanced cell viability by 3-fold compared to non-treated pcECMs. Overall, those treatments have not extensively modified cell support capabilities, demonstrating that non-treated pcECM surface was sufficient to support cell viability.

Cell viability results of cvFB/bmMSC co-cultures have demonstrated no significant difference comparing different cell ratios, which suggests no or little interactions between both cell types in term of cell attachment and proliferation. In consequence, cvFBs have not overwhelmed the co-culture systems, which is important to avoid a cellular response similar to fibrosis. This result was further confirmed by collagen metabolism study, which demonstrated reduced collagen synthesis after 18 days. In addition, cvFB/bmMSC co-cultures have demonstrated that only in the presence of cvFB did bmMSC manage to form Connexin43 (Cx43) gap junctions, which could be beneficial to support electrical

conduction in any future application.

Verifying bmMSC integrity, common MSC surface markers (i.e. CD29, CD44, CD73, CD90 and CD105) were shown positively expressed at >90% as demonstrated by flow cytometry after 4-week cultivation period on pcECM scaffolds, suggesting lineage integrity for MSC was preserved. However, CD31 (PECAM-1), a common negative marker for bmMSC but more importantly a positive marker for endothelial cells, has shown expression at 55% after similar amount of time, which may imply pcECM induced MSC to endothelial transdifferentiation. This suggests that pcECM scaffold might possess capabilities to support not only endothelial transdifferentiation but also endothelialization towards in vivo angiogenesis. Additional studies at the mRNA level were performed and confirmed the conclusions obtained from immunostaining (by FACS and confocal imaging) that genes specific for endothelial characterization were upregulated in bmMSCs seeded on pcECMs. In addition, results obtained from RNA expression from cardiac specific genes suggest that pcECM environment does not influence significantly bmMSC fate towards cardiac differentiation but may provide a basic initial platform (as pcECM is originally cardiac ECM), more suitable than synthetic scaffolds. This differentiation process may require external stimulations (i.e. factors or physical stimulations).

To characterize the remodeling activity of cvFBs cultivated on pcECMs, RNA expression levels of genes related to ECM and adhesion molecules were conducted and the results obtained suggest enhanced functional cvFB activity after 20 days cultivation on pcECMs as demonstrated by the increased fold regulation values for ECM- and adhesion molecules related genes, particularly those genes related to proteases (MMP) and cell-matrix adhesion molecules. This upregulated activity suggested enhanced cell-

matrix interactions and could be favourable to promote cell integration in the tissue constructs.

Finally, a two-step cultivation strategy in static and dynamic conditions demonstrated the importance of oxygen / nutrient gradients in our and probably other similar perfusion systems. Furthermore, our results have demonstrated here for the first time (to the best of our knowledge) how these gradients can be used to obtain better cellular infiltration up to 1.2 mm deep inside the scaffold. This also serves as a proof of concept to the feasibility of thick construct recellularization with human physiological relevant densities, by using inherent vasculature and such bioreactor platforms.

10 Future work

PcECM scaffold has demonstrated some interesting capabilities in transdifferentiating human bone marrow mesenchymal stem cells (bmMSCs) into endothelial-like cells. Further work could investigate the mechanisms regulating such transformation. There are several angiogenic factors, like vascular endothelial growth factor (VEGF), which could be present on the pcECM surface even after decellularization and could potentially trigger the transdifferentiation phenomena observed. In addition, spatial arrangement of transdifferentiated cells could be observed to detect any formation of new angiogenic structures (like tubular lumens).

To further assess the potential supporting role of fibroblast, the electrical impact of co-culturing human cardiac ventricular fibroblasts (cvFBs) with bmMSCs can be investigated. Because connexin43 gap junctions were significantly higher expressed in the presence of cvFBs, electrical resistivity and conduction can be compared between different ratios of cvFB/bmMSC using commercially available multielectrode arrays.

Finally, there are several strategies to consider in order to extend cell infiltration into pcECM slab. First of all, longer culture time for the dynamic perfusion step could increase cell density and cell infiltration (only 2 weeks were evaluated in this study). Usage of oxygen carriers could also improve the oxygen support for cell proliferation throughout the entire pcECM slab as well as the co-culture with endothelial cells, which may enable better vascular network formation to support the growing tissue construct.

11 Annexes

11.1 FACS characterization of HUVECs

Human umbilical vein endothelial cells (HUVECs) were not part of this thesis focus, but as they were ready available in our laboratory, they were also tested along MSCs and cvFBs against the studied markers (Fig. 46). Overall, only CD31 and vWF were shown to be specific to HUVECs only. HUVECS should be negative for α -SMA. It is not fully understood why we obtained positive α -SMA expression for HUVECs, though they can transdifferentiate towards smooth muscle cells with various treatments^{503 504}. These results are further supported by other studies⁵⁰⁵ claiming that HUVECs actually do express alpha-SMA which may be attributed to their less committed state.

Fig. 46 FACS characterization of HUVECs

Human umbilical vein endothelial cells (HUVECs) were thoroughly characterized by FACS from tissue culture plate for various positive (DDR2, α -SMA), negative (CD31, vWF) markers, and bmMSC positive markers (CD105, CD90, CD73 and CD44). CD44 and CD105 graphs for HUVECS: courtesy of Wang Yao.

11.2 Estimation of cell density at 20x using automated calculation with ImageJ software

Cell density was initially estimated using a tile-scanned nuclei-stained image taken at 20x magnification by confocal microscopy (Fig. 47). Because the nuclei staining looked more like polygons instead of having a normal round shape, the cell density was re-estimated but at 63x magnification (Fig. 44). It was observed that the calculation method with 20x magnification was slightly underestimating the cell density number by 4-fold compared to the method with 63x magnification.

Fig. 47 Areas selected for cell density estimation by automated calculation using ImageJ software

From the DAPI image source (A), a dual-colored image was obtained and three to four rectangular regions were selected from three different distances to the pcECM surface, 0-100, 100-500 and 500-1000 µm (B). On the right panel (C), examples of one region per distance group with its corresponding counted illustration are shown.

11.3 Gene array lists

As provided by the manufacturer website, Qiagen (DL).

11.3.1 Gene array related to endothelial cell biology

Position	Unigene	GeneBank	Symbol	Description	Gene Name
A01	Hs.654434	NM_000789	ACE	Angiotensin I converting enzyme	ACE1, CD143, DCP, DCP1, MGC26566, MVCD3
A02	Hs.404914	NM_003183	ADAM17	ADAM metalloproteinase domain 17	ADAM18, CD156B, CSVP, MGC71942, TACE
A03	Hs.19383	NM_000029	AGT	Angiotensinogen	ANHU, FLJ92595, FLJ97926, SERPINA8
A04	Hs.728754	NM_031850	AGTR1	Angiotensin II receptor, type 1	AG2S, AGTR1A, AGTR1B, AT1, AT1B, AT1R, AT2R1
A05	Hs.89499	NM_000698	ALOX5	Arachidonate 5-lipoxygenase	5-LO, 5-LOX, 5LPG, LOG5, MGC163204
A06	Hs.369675	NM_001146	ANGPT1	Angiopoietin 1	AGP1, AGPT, ANG1
A07	Hs.480653	NM_001154	ANXA5	Annexin A5	ANX5, ENX2, PP4
A08	Hs.654439	NM_000041	APOE	Apolipoprotein E	AD2, LDLCQ5, LPG, MGC1571
A09	Hs.624291	NM_004324	BAX	BCL2-associated X protein	BCL2L4
A10	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2	Bcl-2
A11	Hs.516966	NM_138578	BCL2L1	BCL2-like 1	BCL-XL, S, BCL2L, BCLX, BCLXL, BCLXS, Bcl-X
A12	Hs.37058	NM_001741	CALCA	Calcitonin-related polypeptide alpha	CALC1, CGRP, CGRP-I, CGRP1, CT, KC, MGC126648
B01	Hs.2490	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase	ICE, IL1BC, P45
B02	Hs.141125	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase	CPP32, CPP32B, SCA-1
B03	Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22kDa	BSCL3, CGL3, MSTP085, VIP21
B04	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2	GDCF-2, HC11, HSMCR30, MCAF, MCP-1, MCP1,
B05	Hs.514821	NM_002985	CCL5	Chemokine (C-C motif) ligand 5	D17S136E, MGC17164, RANTES, SCYA5, SISd, TCP228
B06	Hs.76206	NM_001795	CDH5	Cadherin 5, type 2 (vascular endothelium)	7B4, CD144, FLJ17376
B07	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator	CASH, CASP8AP1, CLARP, Casper, FLAME, FLAME-1, FLAME1, FLIP,
B08	Hs.517356	NM_030582	COL18A1	Collagen, type XVIII, alpha 1	FLJ27325, FLJ34914, KNO, KNO1, KS, MGC74745
B09	Hs.531668	NM_002996	CX3CL1	Chemokine (C-X3-C	ABCD-3, C3Xkine, CXC3,

				motif) ligand 1	CXC3C, NTN, NTT,
B10	Hs.511899	NM_001955	EDN1	Endothelin 1	ET1, HDLCQ7, PPET1
B11	Hs.1407	NM_001956	EDN2	Endothelin 2	ET2, PPET2
B12	Hs.183713	NM_001957	EDNRA	Endothelin receptor type A	ETA, ETAR, ETRA
C01	Hs.76753	NM_000118	ENG	Endoglin	CD105, END, FLJ41744, HHT1, ORW, ORW1
C02	Hs.482562	NM_001992	F2R	Coagulation factor II (thrombin) receptor	CF2R, HTR, PAR-1, PAR1, TR
C03	Hs.62192	NM_001993	F3	Coagulation factor III (thromboplastin, tissue factor)	CD142, FLJ17960, TF, TFA
C04	Hs.244139	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)	ALPS1A, APO-1, APT1, CD95, FAS1, FASTM, TNFRSF6
C05	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)	APT1LG1, CD178, CD95-L, CD95L, FASL, TNFSF6
C06	Hs.483635	NM_000800	FGF1	Fibroblast growth factor 1 (acidic)	AFGF, ECGF, ECGF-beta, ECGFA, ECGFB, FGF-alpha
C07	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)	BFGF, FGFB, HBGF-2
C08	Hs.654360	NM_002019	FLT1	Fms-related tyrosine kinase 1	FLT, VEGFR1
C09	Hs.203717	NM_002026	FN1	Fibronectin 1	CIG, DKFZp686F10164, DKFZp686H0342, DKFZp686I1370
C10	Hs.597216	NM_001530	HIF1A	Hypoxia inducible factor 1, alpha subunit	HIF-1alpha, HIF1, HIF1-ALPHA, MOP1, PASD8, bHLHe78
C11	Hs.517581	NM_002133	HMOX1	Heme oxygenase (decycling) 1	HO-1, HSP32, bK286B10
C12	Hs.643447	NM_000201	ICAM1	Intercellular adhesion molecule 1	BB2, CD54, P3.58
D01	Hs.467304	NM_000641	IL11	Interleukin 11	AGIF, IL-11
D02	Hs.126256	NM_000576	IL1B	Interleukin 1, beta	IL-1, IL1-BETA, IL1F2
D03	Hs.694	NM_000588	IL3	Interleukin 3 (colony-stimulating factor, multiple)	IL-3, MCGF, MGC79398, MGC79399, MULTI-CSF
D04	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)	BSF2, HGF, HSF, IFNB2, IL-6
D05	Hs.591873	NM_000880	IL7	Interleukin 7	IL-7
D06	Hs.505654	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	CD49e, FNRA, VLA5A
D07	Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	CD51, DKFZp686A08142, MSK8, VNRA
D08	Hs.643813	NM_002211	ITGB1	Integrin, beta 1	CD29, FNRB, GPIIA, MDF2, MSK12, VLA-BETA, VLAB
D09	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein	CD61, GP3A, GPIIIa

				IIIa, antigen CD61)	
D10	Hs.479756	NM_002253	KDR	Kinase insert domain receptor	CD309, FLK1, VEGFR, VEGFR2
D11	Hs.479754	NM_000222	KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	C-Kit, CD117, PBT, SCFR
D12	Hs.171995	NM_001648	KLK3	Kallikrein-related peptidase 3	APS, KLK2A1, PSA, hK3
E01	Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1	CLG, CLGN
E02	Hs.513617	NM_004530	MMP2	Matrix metalloproteinase 2	CLG4, CLG4A, MMP-II, MONA, TBE-1
E03	Hs.297413	NM_004994	MMP9	Matrix metalloproteinase 9	CLG4B, GELB, MANDP2, MMP-9
E04	Hs.707978	NM_000603	NOS3	Nitric oxide synthase 3 (endothelial cell)	ECNOS, eNOS
E05	Hs.219140	NM_002521	NPPB	Natriuretic peptide B	BNP
E06	Hs.490330	NM_000906	NPR1	Natriuretic peptide receptor A/guanylate cyclase A	ANPRA, ANPa, GUC2A, GUCY2A, NPRA
E07	Hs.592605	NM_002538	OCLN	Occludin	BLCPMG, FLJ08163, FLJ18079, FLJ77961, FLJ94056, MGC34277
E08	Hs.74615	NM_006206	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	CD140A, MGC74795, PDGFR2, RHEPDGFRA
E09	Hs.514412	NM_000442	PECAM1	Platelet/endothelial cell adhesion molecule	CD31, FLJ34100, FLJ58394, PECAM-1
E10	Hs.81564	NM_002619	PF4	Platelet factor 4	CXCL4, MGC138298, SCYB4
E11	Hs.252820	NM_002632	PGF	Placental growth factor	D12S1900, PGFL, PLGF, PIGF-2, SHGC-10760
E12	Hs.491582	NM_000930	PLAT	Plasminogen activator, tissue	DKFZp686I03148, T-PA, TPA
F01	Hs.77274	NM_002658	PLAU	Plasminogen activator, urokinase	ATF, UPA, URK, u-PA
F02	Hs.143436	NM_000301	PLG	Plasminogen	DKFZp779M0222
F03	Hs.647450	NM_006404	PROCR	Protein C receptor, endothelial	CCCA, CCD41, CD201, EPCR, MGC23024, bA4204.2
F04	Hs.302085	NM_000961	PTGIS	Prostaglandin I2 (prostacyclin) synthase	CYP8, CYP8A1, MGC126858, MGC126860, PGIS, PTGI
F05	Hs.196384	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2	COX-2, COX2, GRIPGHS, PGG, HS, PGHS-2, PHS-2, hCox-2
F06	Hs.395482	NM_005607	PTK2	PTK2 protein tyrosine kinase 2	FADK, FAK, FAK1, FRNK, pp125FAK
F07	Hs.89546	NM_000450	SELE	Selectin E	CD62E, ELAM, ELAM1, ESEL, LECAM2
F08	Hs.728756	NM_000655	SELL	Selectin L	CD62L, LAM1, LECAM1, LEU8, LNHR, LSEL,

					LYAM1, PLNHR, TQ1
F09	Hs.591014	NM_003006	SELPLG	Selectin P ligand	CD162, CLA, PSGL-1, PSGL1
F10	Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E	PAI, PAI-1, PAI1, PLANH1
F11	Hs.443914	NM_000454	SOD1	Superoxide dismutase 1, soluble	ALS, ALS1, IPOA, SOD, hSod1, homodimer
F12	Hs.68061	NM_021972	SPHK1	Sphingosine kinase 1	SPHK
G01	Hs.89640	NM_000459	TEK	TEK tyrosine kinase, endothelial	CD202B, TIE-2, TIE2, VMCM, VMCM1
G02	Hs.516578	NM_006287	TFPI	Tissue factor pathway inhibitor	EPI, LACI, TFI, TFPI1
G03	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	CED, DPD1, LAP, TGFB, TGFbeta
G04	Hs.2030	NM_000361	THBD	Thrombomodulin	AHUS6, BDCA3, CD141, THRM, TM
G05	Hs.164226	NM_003246	THBS1	Thrombospondin 1	THBS, THBS-1, TSP, TSP-1, TSP1
G06	Hs.522632	NM_003254	TIMP1	TIMP metalloproteinase inhibitor 1	CLGI, EPA, EPO, FLJ90373, HCI, TIMP
G07	Hs.241570	NM_000594	TNF	Tumor necrosis factor	DIF, TNF-alpha, TNFA, TNFSF2
G08	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	APO2L, Apo-2L, CD253, TL2, TRAIL
G09	Hs.592212	NM_001953	TYMP	Thymidine phosphorylase	ECGF, ECGF1, MEDPS1, MNGIE, MTDPS1, PDECGF, TP, hPD-ECGF
G10	Hs.109225	NM_001078	VCAM1	Vascular cell adhesion molecule 1	CD106, DKFZp779G2333, INCAM-100, MGC99561
G11	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A	MGC70609, MVCD1, VEGF, VPF
G12	Hs.440848	NM_000552	VWF	Von Willebrand factor	F8VWF, VWD

11.3.2 Gene array related to cardiomyocyte genes

Unigene	GeneBank	Symbol	Description	Gene Name
Hs.498178	NM_001103	ACTN2	Actinin, alpha 2	CMD1AA
Hs.99913	NM_000684	ADRB1	Adrenergic, beta-1-, receptor	ADRB1R, B1AR, BETA1AR, RHR
Hs.334347	NM_001824	CKM	Creatine kinase, muscle	CKMM, M-CK
Hs.594952	NM_001927	DES	Desmin	CMD1I, CSM1, CSM2, FLJ12025, FLJ39719, FLJ41013, FLJ41793
Hs.243987	NM_002052	GATA4	GATA binding protein 4	MGC126629
Hs.388245	NM_021973	HAND2	Heart and neural crest derivatives expressed 2	DHAND2, FLJ16260, Hed, MGC125303, MGC125304, Thing2, bHLHa26, dHand
Hs.95162	NM_000218	KCNQ1	Potassium voltage-	ATFB1, ATFB3, FLJ26167,

			gated channel, KQT-like subfamily, member 1	JLNS1, KCNA8, KCNA9, KVLQT1, Kv1.9, Kv7.1, LQT, LQT1, RWS, SQT2, WRS
Hs.517586	NM_005368	MB	Myoglobin	MGC13548, PVALB
Hs.929	NM_000257	MYH7	Myosin, heavy chain 7, cardiac muscle, beta	CMD1S, CMH1, DKFZp451F047, MGC138376, MGC138378, MPD1, MYHCB, SPMD, SPMM
Hs.75535	NM_000432	MYL2	Myosin, light chain 2, regulatory, cardiac, slow	CMH10, DKFZp779C0562, MLC2
Hs.517939	NM_000258	MYL3	Myosin, light chain 3, alkali; ventricular, skeletal, slow	CMH8, MLC1SB, MLC1V, VLC1
Hs.75636	NM_021223	MYL7	Myosin, light chain 7, regulatory	MYL2A, MYLC2A
Hs.54473	NM_004387	NKX2-5	NK2 homeobox 5	CHNG5, CSX, CSX1, FLJ52202, FLJ97166, FLJ97195, FLJ97197, FLJ99536, NKX2.5, NKX2E, NKX4-1
Hs.75640	NM_006172	NPPA	Natriuretic peptide A	ANF, ANP, ATFB6, CDD-ANF, PND
Hs.170839	NM_002667	PLN	Phospholamban	CMD1P, PLB
Hs.109514	NM_001035	RYR2	Ryanodine receptor 2 (cardiac)	ARVC2, ARVD2, RYR-2, VTSIP
Hs.728911	NM_021097	SLC8A1	Solute carrier family 8 (sodium/calcium exchanger), member 1	DKFZp779F0871, FLJ37694, FLJ43417, MGC119581, NCX1
Hs.644596	NM_000363	TNNI3	Troponin I type 3 (cardiac)	CMD1FF, CMD2A, CMH7, MGC116817, RCM1, TNNC1, cTnI
Hs.533613	NM_000364	TNNT2	Troponin T type 2 (cardiac)	CMH2, CMPD2, LVNC6, MGC3889, RCM3, TnTC, cTnT
Hs.591847	NM_000662	NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	AAC1, MNAT, NAT-1, NATI
Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, MGC88685

11.3.3 Gene array related to extracellular matrix and adhesion molecules

Position	Unigene	GeneBank	Symbol	Description	Gene Name
A01	Hs.643357	NM_006988	ADAMTS1	ADAM metalloproteinase with thrombospondin type 1 motif, 1	C3-C5, KIAA1346, METH1
A02	Hs.131433	NM_139025	ADAMTS13	ADAM metalloproteinase with thrombospondin type 1 motif, 13	C9orf8,
A03	Hs.271605	NM_007037	ADAMTS8	ADAM metalloproteinase with thrombospondin type 1 motif, 8	ADAM-TS8, FLJ41712, METH2
A04	Hs.502328	NM_000610	CD44	CD44 molecule (Indian blood group)	CDW44, CSPG8, ECMR-III, HCELL

A05	Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Arc-1, CD324, CDHE, ECAD, LCAM, UVO
A06	Hs.476092	NM_003278	CLEC3B	C-type lectin domain family 3, member B	DKFZp686H17246, TN, TNA
A07	Hs.143434	NM_001843	CNTN1	Contactin 1	F3, GP135
A08	Hs.523446	NM_080629	COL11A1	Collagen, type XI, alpha 1	CO11A1, COLL6, STL2
A09	Hs.101302	NM_004370	COL12A1	Collagen, type XII, alpha 1	BA209D8.1
A10	Hs.409662	NM_021110	COL14A1	Collagen, type XIV, alpha 1	UND
A11	Hs.409034	NM_001855	COL15A1	Collagen, type XV, alpha 1	FLJ38566
A12	Hs.368921	NM_001856	COL16A1	Collagen, type XVI, alpha 1	447AA
B01	Hs.172928	NM_000088	COL1A1	Collagen, type I, alpha 1	OI4
B02	Hs.508716	NM_001846	COL4A2	Collagen, type IV, alpha 2	DKFZp686I14213, FLJ22259
B03	Hs.210283	NM_000093	COL5A1	Collagen, type V, alpha 1	-
B04	Hs.474053	NM_001848	COL6A1	Collagen, type VI, alpha 1	OPLL
B05	Hs.420269	NM_001849	COL6A2	Collagen, type VI, alpha 2	DKFZp586E1322, FLJ46862, PP3610
B06	Hs.476218	NM_000094	COL7A1	Collagen, type VII, alpha 1	EBD1, EBDCT, EBR1
B07	Hs.654548	NM_001850	COL8A1	Collagen, type VIII, alpha 1	C3orf7, MGC9568
B08	Hs.591346	NM_001901	CTGF	Connective tissue growth factor	CCN2, HCS24
B09	Hs.534797	NM_001903	CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	CAP102, FLJ36832, FLJ52416
B10	Hs.476018	NM_001904	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB, DKFZp686D02253
B11	Hs.166011	NM_001331	CTNND1	Catenin (cadherin-associated protein), delta 1	CAS, CTNND, KIAA0384
B12	Hs.314543	NM_001332	CTNND2	Catenin (cadherin-associated protein)	GT24, NPRAP
C01	Hs.81071	NM_004425	ECM1	Extracellular matrix protein 1	-
C02	Hs.203717	NM_002026	FN1	Fibronectin 1	CIG, FN, FNZ, GFND, GFND2, LETS, MSF
C03	Hs.57697	NM_001523	HAS1	Hyaluronan synthase 1	HAS
C04	Hs.643447	NM_000201	ICAM1	Intercellular adhesion molecule 1	BB2, CD54, P3.58
C05	Hs.644352	NM_181501	ITGA1	Integrin, alpha 1	CD49a, VLA1
C06	Hs.482077	NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	BR, CD49B, GPIa, VLA-2, VLAA2
C07	Hs.265829	NM_002204	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	CD49C, FLJ34631, FLJ34704, GAP-B3, GAPB3
C08	Hs.694732	NM_000885	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	CD49D, IA4, MGC90518
C09	Hs.505654	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	CD49e, FNRA, VLA5A
C10	Hs.133397	NM_000210	ITGA6	Integrin, alpha 6	CD49f, DKFZp686J01244
C11	Hs.524484	NM_002206	ITGA7	Integrin, alpha 7	FLJ25220
C12	Hs.171311	NM_003638	ITGA8	Integrin, alpha 8	-

D01	Hs.174103	NM_002209	ITGAL	Integrin, alpha L (antigen CD11A (p180))	CD11A, LFA-1, LFA1A
D02	Hs.172631	NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	CD11B, CR3A, MAC-1, MAC1A
D03	Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	CD51, DKFZp686A08142, MSK8, VNRA
D04	Hs.643813	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	CD29, FNRB, GPIIA, MDF2, MSK12, VLA-BETA, VLAB
D05	Hs.375957	NM_000211	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	CD18, LAD, LCAMB, LFA-1, MAC-1, MF17, MF17
D06	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa)	CD61, GP3A, GPIIIa
D07	Hs.632226	NM_000213	ITGB4	Integrin, beta 4	CD104
D08	Hs.536663	NM_002213	ITGB5	Integrin, beta 5	FLJ26658
D09	Hs.521869	NM_000216	KAL1	Kallmann syndrome 1 sequence	ADMLX, HHA, KAL, KALIG-1
D10	Hs.270364	NM_005559	LAMA1	Laminin, alpha 1	LAMA, S-LAM-alpha
D11	Hs.200841	NM_000426	LAMA2	Laminin, alpha 2	LAMM
D12	Hs.436367	NM_000227	LAMA3	Laminin, alpha 3	BM600, E170, LAMNA, LOCS, lama3a
E01	Hs.650585	NM_002291	LAMB1	Laminin, beta 1	CLM, MGC142015
E02	Hs.497636	NM_000228	LAMB3	Laminin, beta 3	BM600-125KDA
E03	Hs.609663	NM_002293	LAMC1	Laminin, gamma 1 (formerly LAMB2)	LAMB2, MGC87297
E04	Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	CLG, CLGN
E05	Hs.2258	NM_002425	MMP10	Matrix metalloproteinase 10 (stromelysin 2)	SL-2, STMY2
E06	Hs.143751	NM_005940	MMP11	Matrix metalloproteinase 11 (stromelysin 3)	SL-3, ST3, STMY3
E07	Hs.1695	NM_002426	MMP12	Matrix metalloproteinase 12 (macrophage elastase)	HME, ME, MGC138506, MME, MMP-12
E08	Hs.2936	NM_002427	MMP13	Matrix metalloproteinase 13 (collagenase 3)	CLG3, MANDP1
E09	Hs.2399	NM_004995	MMP14	Matrix metalloproteinase 14 (membrane-inserted)	1, MMP-14, MMP-X1
E10	Hs.80343	NM_002428	MMP15	Matrix metalloproteinase 15 (membrane-inserted)	MT2-MMP, MTMMP2, SMCP-2
E11	Hs.546267	NM_005941	MMP16	Matrix metalloproteinase 16 (membrane-inserted)	C8orf57, DKFZp761D112
E12	Hs.513617	NM_004530	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4, CLG4A, MMP-II, MONA, TBE-1
F01	Hs.375129	NM_002422	MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	CHDS6, MGC126102,

					MGC126103
F02	Hs.2256	NM_002423	MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	MMP-7, MPSL1, PUMP-1
F03	Hs.161839	NM_002424	MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	CLG1, HNC, MMP-8, PMNL-CL
F04	Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	CLG4B, GELB, MANDP2, MMP-9
F05	Hs.503878	NM_000615	NCAM1	Neural cell adhesion molecule 1	CD56, MSK39, NCAM
F06	Hs.514412	NM_000442	PECAM1	Platelet/endothelial cell adhesion molecule	CD31, FLJ34100, FLJ58394, PECAM-1
F07	Hs.89546	NM_000450	SELE	Selectin E	CD62E, ELAM, ELAM1, ESEL, LECAM2
F08	Hs.728756	NM_000655	SELL	Selectin L	CD62L, LAM1, LECAM1, LEU8
F09	Hs.73800	NM_003005	SELP	Selectin P (granule membrane protein 140kDa, antigen CD62)	CD62, CD62P, FLJ45155
F10	Hs.371199	NM_003919	SGCE	Sarcoglycan, epsilon	DYT11, ESG
F11	Hs.111779	NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	ON
F12	Hs.185597	NM_003119	SPG7	Spastic paraplegia 7 (pure and complicated autosomal recessive)	CAR, CMAR, FLJ37308, MGC126331
G01	Hs.313	NM_000582	SPP1	Secreted phosphoprotein 1	BNSP, BSPI, ETA-1, MGC110940, OPN
G02	Hs.369397	NM_000358	TGFBI	Transforming growth factor, beta-induced, 68kDa	BIGH3, CDB1, CDG2, CDGG1
G03	Hs.164226	NM_003246	THBS1	Thrombospondin 1	THBS, THBS-1, TSP, TSP-1, TSP1
G04	Hs.371147	NM_003247	THBS2	Thrombospondin 2	TSP2
G05	Hs.169875	NM_007112	THBS3	Thrombospondin 3	MGC119564, MGC119565, TSP3
G06	Hs.522632	NM_003254	TIMP1	TIMP metallopeptidase inhibitor 1	CLGI, EPA, EPO, FLJ90373, HCI, TIMP
G07	Hs.633514	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2	CSC-21K
G08	Hs.644633	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3	HSMRK222, K222, K222TA2, SFD
G09	Hs.143250	NM_002160	TNC	Tenascin C	150-225, GMEM, GP, HXB
G10	Hs.109225	NM_001078	VCAM1	Vascular cell adhesion molecule 1	CD106, DKFZp779G2333, INCAM-100, MGC99561
G11	Hs.643801	NM_004385	VCAN	Versican	CSPG2
G12	Hs.2257	NM_000638	VTN	Vitronectin	V75, VN, VNT

11.4 Publications

List of publications attached:

1. Nguyen EB, Zilla P, Bezuidenhout D, *Nitric oxide release from polydimethylsiloxane-based polyurethanes*, J Appl Biomater Funct Mater. 2014, [ahead of print]
2. Steele TW, Huang CL, Nguyen E, Sarig U, Kumar S, Widjaja E, Loo JS, Machluf M, Boey F, Vukadinovic Z, Hilfiker A, Venkatraman SS, *Collagen-cellulose composite thin films that mimic soft-tissue and allow stem-cell orientation*, J Mater Sci Mater Med. 2013 Aug;24(8):2013-27
3. Bronshtein T, Au-Yeung GC, Sarig U, Nguyen EB, Mhaisalkar PS, Boey FY, Venkatraman SS, Machluf M, *A mathematical model for analyzing the elasticity, viscosity, and failure of soft tissue: comparison of native and decellularized porcine cardiac extracellular matrix for tissue engineering*, Tissue Eng Part C Methods. 2013 Aug;19(8):620-30
4. Wang Y, Bronshtein T, Sarig U, Nguyen EB, Boey FY, Venkatraman SS, Machluf M, *A mathematical model predicting the coculture dynamics of endothelial and mesenchymal stem cells for tissue regeneration*, Tissue Eng Part A. 2013 May;19(9-10):1155-64
5. Sarig U, Nguyen EB, Dahan N, Bronshtein T, Sarig H, Buring M, Scheper T, Boey YCF, Venkatraman SS and Machluf M, *Pushing the limits in heart tissue engineering: dynamic culturing and ex vivo construction of thick vascularized constructs*, under revision

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