Extracellular Matrix: A Cardiac Regenerative Niche

Dissertation for Master Degree in Biomedical Research

Host Institution

INEB – Instituto de Engenharia Biomédica

Rua do Campo Alegre 823

4150-180, Porto

Portugal

www.ineb.up.pt

Supervisor: Diana S. Nascimento, PhD [1]

Co-supervisor: Mário A. Barbosa, PhD[1,2]

Co-supervisor: João Malva, PhD [3]

Affiliations:

[1] INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal;

[2] ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal;

[3] FMUC - Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal.

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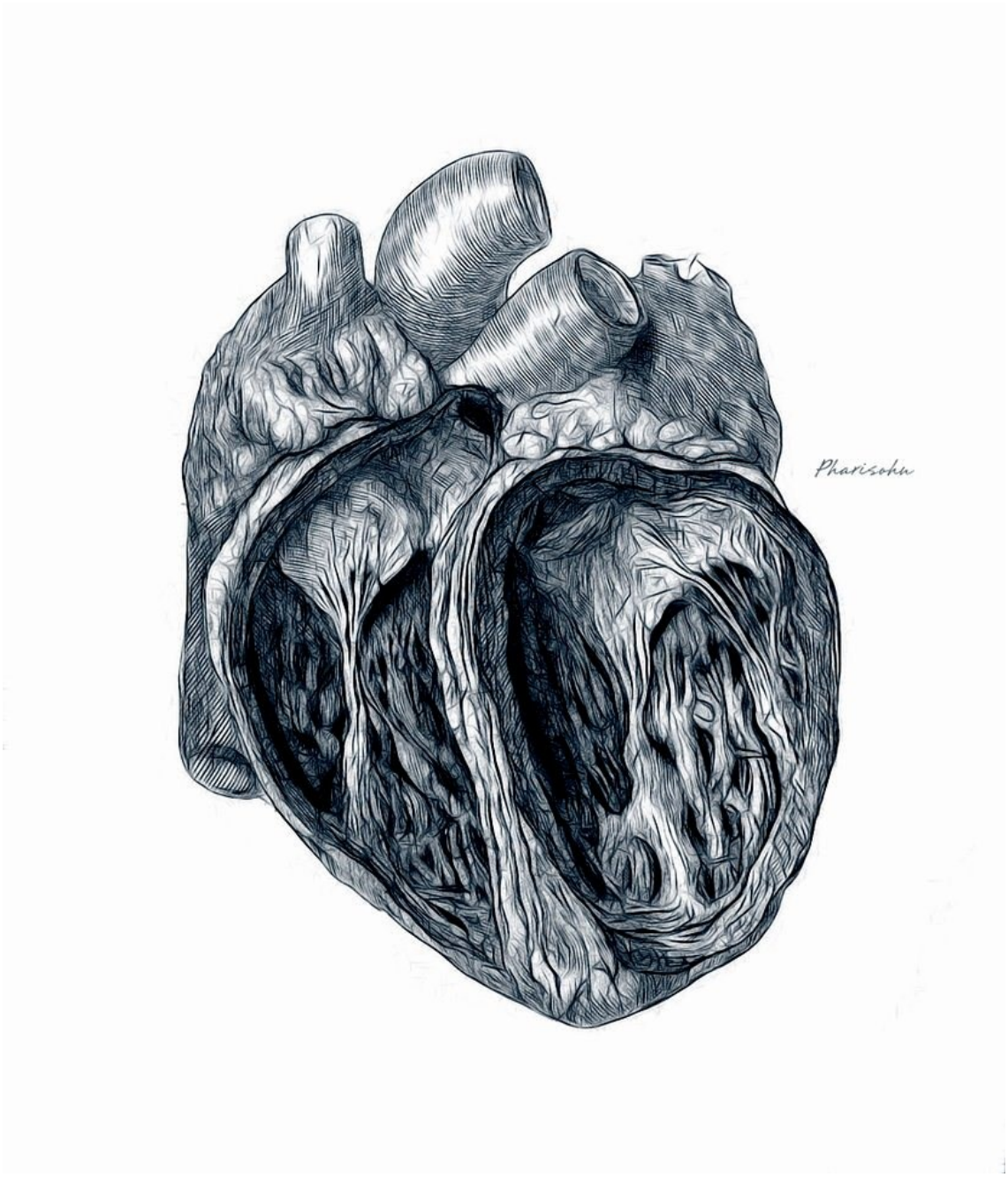

"I will remove from you your heart of stone and give you a heart of flesh"

Ezekiel 36:26

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Abstract

The cardiac extracellular matrix (ECM) is a three dimensional (3-D) structure constituted by different families of proteins of structural and cell regulatory nature. The latter translate critical functional information and undertake highly specific roles that provide for the cells/tissue homeostasis. In order for the tissues and organs to be formed, the ECM undergoes dramatic changes throughout development. Moreover, it is further engaged in tissue remodeling under pathological conditions. Thus, the ECM is a dynamic unity that is simultaneously a physical fraction and a regulatory agent of the microenvironment. In the heart, the ECM undergoes profound changes in response to injury. Whereas in the adult stages the heart develops non-functional scar tissue after wounding with subsequent dysrhythmias eventually leading to heart failure, a regeneration-like pattern was recently described for the fetal/neonatal organ.

An increasing number of patients with heart disorders in western countries and the poor availability of the hearts for transplantation are driving the researchers' and medical communities towards the development of novel therapies to restore the function of the damage myocardium. In recent years, decellularized native ECM has been proposed as a basis in tissue engineering applications for regenerative medicine. This was already successfully illustrated not only in preclinical studies but also in other organs clinical application.

In accordance with the above described observation that the capacity for cardiac tissue healing differs throughout the mammal's ontogeny we have initiated a pioneering study to identify the regenerative-potential information contained in the fetal (E18) cardiac ECM as allegedly opposed to the adult matrix. The ultimate goal is to dissect how different ontogenic-specific ECM components could improve adult heart regeneration after injury. Full elucidation of the capacity of this dynamic structure, *i.e.* the cardiac ECM, to change and meet distinct physiologic and pathological conditions during the heart' lifespan will bring out the knowledge critical for creating effective and patients safe natural cardiac scaffolds for clinical applications.

In the herein work it was implemented for the first time an efficient protocol for parallel decellularization of the fetal and the adult heart tissue, with preserved structure and

minimal loss of the ECM contents. Preliminary biochemical and physical characterization of the fetal- and adult- derived ECM was initiated demonstrating the achievement of porous bioscaffolds that keep key ECM elements of the basal lamina, such as laminin, fibronectin and type IV collagen. The biocompatibility studies performed in *in vitro* culture by using a cardiac progenitor cell line demonstrated that fetal (E18) decellularized matrices induced more cell migration and adhesion when compared to the adult counterpart. Studies are underway to further assess the biochemical composition of the recellularized matrices and the cells phenotype.

Key words: Extracellular matrix; Decellularization; Fetal (E18) and adult heart; Ontogeny; Regeneration.

Resumo

A matriz extracelular (ECM) cardíaca é uma rede tridimensional constituída por diferentes moléculas com funções estruturais, sinalização e acções preponderantes na homeostasia celular e tecidular. Adicionalmente a ECM possui um papel activo no desenvolvimento e na remodelação do tecido em condições patológicas. Após enfarte do miocárdio a ECM sofre alterações profundas na sua estrutura e composição. No entanto, no coração adulto este processo resulta na formação de uma cicatriz não funcional contrariamente ao coração fetal/neonatal no qual há restabelecimento da estrutura tecidular nativa e subsequentemente da função do órgão.

O aumento de pessoas afectadas por doenças cardiovasculares e o número reduzido de corações disponíveis para transplante serve de estímulo para a investigação de novas terapias que permitam a recuperação funcional do miocárdio após lesão. Na última década, a aplicação de matrizes descelularizadas em estudos pre-clínicos e clínicos de engenharia de tecidos demonstrou resultados muito promissores na recuperação de diferentes tecidos após lesão. Assim iniciamos um estudo pioneiro utilizando matrizes descelularizadas de coração fetal (E18) e de adulto de forma a clarificar como alterações na ECM durante a ontogenia do coração se relacionam com a regeneração cardíaca (fetal), ou com a formação de tecido cicatricial (adulto) após lesão do miocárdio. O principal objectivo é dissecar como diferentes componentes específicos da ECM podem promover a regeneração do coração adulto. Adicionalmente, esta abordagem irá permitir a criação de matrizes cardíacas naturais para avaliação pré-clínica em modelos experimentais de enfarte do miocárdio.

O presente trabalho descreve pela primeira vez a implementação paralela de um protocolo de descelularização para tecido cardíaco fetal (E18) e adulto com preservação da composição e estrutura da ECM nativa. A caracterização física e bioquímica preliminar das matrizes descelularizadas demonstrou que estas apresentam uma estrutura porosa com manutenção da vasculatura e de importantes elementos da membrana basal, nomeadamente, a laminina, fibronectina e colagénio tipo IV. Estudos de biocompatibilidade utilizando iCPCSca-1, uma linha celular modelo de progenitores

cardíacos, demonstraram que as matrizes descelularizadas fetais promovem uma maior migração e adesão celular em comparação com o tecido adulto. Outras experiências estão agora a ser completadas com o objectivo de caracterizar a composição bioquímica das matrizes recelularizadas assim como o fenótipo das células.

Palavras-chave: Matriz Extracelular; Descelularização; Coração fetal (E18) e adulto; Regeneração; Ontogenia.

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Abbreviations

AV - AtrioVentricular junction

BSE - Back-Scattered Electrons detector

CADUCEUS - CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction

CHD - Coronary Heart Disease

c-Kit cells - Cardiac-Kit Expressing Cells

CPC - Cardiac Progenitor Cells

CSPGs - Chondroitin Sulfate Proteoglycans

CVD - Cardiac Vascular Diseases

DAPI - 4',6-diamidino-2-phenylindole

DMA - Dynamic Mechanical Analysis

ECM - Extracellular Matrix

EMT - Epithelial–Mesenchymal Transition

E18 - Eighteen days of gestation

FGFs - Fibroblast Growth Factors

GAGs - Glycosaminoglycans

Gpc3 - Glypican-3

HA - Hyaluronic Acid

HBEGF - Heparin-Binding EGF-like Growth Factor

HE - Hematoxylin and Eosin stain

HIER - Heat-Induced Epitope Retrieval

HSPGs - Heparan Sulfate Proteoglycans

Isl-1 - LIM-homeodomain transcription factor islet-1

Isl-1 cells - Cardiac Isl-1 Expressing Cells

LV - Left Ventricle

MI - Myocardial Infarction

MMPs - Matrix Metalloproteinases

PBS - Phosphate Buffer Saline

PGs - Proteoglycans

PrER - Enzymatic Epitope Retrieval

RGD - 'arginine, glycine, aspartic acid'

RT – Room temperature

Sca-1 - Stem Cell Antigen-1

Sca-1 cells - Cardiac Sca-1 Expressing Cells

SCIPIO - Stem Cell Infusion in Patients with Ischemic cardiOmyopathy

SDF-1 - Stromal cell-Derived Factor-1

SDS - Sodium Dodecyl Sulfate

SEM - Scanning Electron Microscopy

sGAG - Sulfated Glycosaminoglycans

TEM - Transmitted Electron Microscopy

TGF-β1 - Transforming Growth Factor beta 1

TIMPs - inhibitors of metalloproteinases

VEGF - Vascular Endothelial Growth Factor

WHO- World Health Organization

2- D - Two Dimensional

3-D - Three Dimensional

Introduction

Cardiac vascular diseases (CVD), namely, coronary heart disease (CHD), stroke, heart failure, hypertrophic cardiomyopathy, heart infections, among others, represent the leading cause of morbidity and mortality worldwide. The World Health Organization (WHO) estimates that in 2030 almost 23.6 million people will die from CVD, reaching epidemic values. Although the prevalence of these diseases affected mostly "rich" countries, the trend is shifting and the incidence of CVD is increasing also in middle-income and low-income countries [1]. CVD causes significant reduction in quality of life, incapacity, and lifelong dependence on health services and medications [2]. Among the CHD, myocardial infarction (MI) is a very limiting condition leading to a sequence of dramatic events that results in cardiomyocyte death, ECM misbalance and subsequent non-functional scar tissue formation [3]. Over the years, potential strategies have been designed to control and reverse the damages in order to achieve clinical relevant functional recovery. The standard treatments have largely been pharmacological-based approaches or heart transplantation itself. Although very effective heart transplantation is limited by the low number of donors, graft rejection, side effects associated to the immunosuppression therapy and concurrent development of other pathologies [4]. Thus great effort has been placed by the cardiovascular research community on surpassing the limits of the standard treatments available and on developing novel therapies for the heart. Noteworthy, although cell-based and tissue engineering approaches have been vastly explored to the extent of reaching the clinical setting, long-lasting cardiac functional restoration still remains to accomplish.

1. Cardiac regeneration

Regeneration might be defined as the result of the intrinsic ability to restore a lost or damaged tissue by the *de novo* synthesis of functional tissue at the original anatomical site, recapitulating to a varying extent the embryonic program that produced the original tissue [5, 6]. In mammals, the capacity to regenerate and reestablish functional tissue following injury is verified only in some organs, such as the liver, skin, and skeletal

muscle and precisely tuned according to the inflicted injury type/area and the organism' ontogenic stage. This innate regenerative ability is largely associated with the activation of resident and/or recruitment lineage-restricted stem cells that do not complete their differentiation program during ontogenesis. These resident stem cells are capable to respond to physiological cell turnover necessity or an injury, resuming cell cycle and committing into a tissue-specific differentiation program [7]. This stem-cell functionality is heterogeneously distributed amongst tissues and appears to best correlate with naturally high cellular turnover capacity (Figure 1). Conversely, the heart, an organ majorly constituted by fully differentiated cardiac myocytes with very modest renewal throughout life, displays limited regeneration and is unable to recover function following extensive tissue loss.

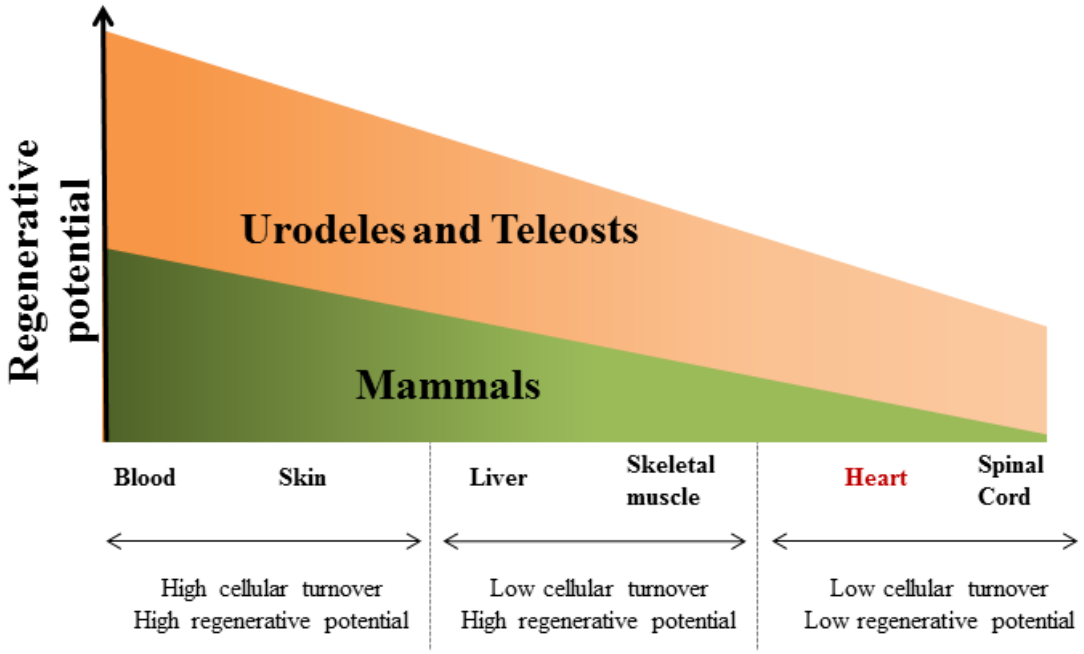

Figure 1. Schematic overview of the spectrum of regenerative capacity in mammals *vs*. lower vertebrates. Many mammalian tissues like blood, skin, liver, skeletal muscle, displays a prominent stem-cell compartment and, by definition, have higher regenerative capacity. However, mammalian CNS structures and heart display lower cell turnover as well as lower regenerative potential. How and why the regenerative capacity of these tissues has been unequally retained during evolution is unclear. Adapted from [8, 9].

Indeed, studies in human hearts previously exposed to the radioactivity generated by the testing of nuclear weapons during the Cold War, showed that, by measuring of ^{14}C incorporation in the DNA of proliferating cells, approximately 45% of whole cardiomyocytes are renewed throughout a lifetime [10, 11].Moreover, in the last decade

panoply of multipotent cardiac progenitor cells (CPC) has been reported [12-24]. CPC are scarcely interspersed along the myocardial tissue and defined as multipotent cells able to differentiate into cardiomyocytes and the vascular smooth muscle and endothelial cells. As a result of the lack of definitive marker(s) to determine the CPC potential, different subsets of CPC are usually isolated based on the expression of the cell surface antigens, *e.g.* stem cell antigen-1 (Sca-1) and c-Kit, and the LIM-homeodomain transcription factor islet-1 (Isl-1) [25]. A universal marker to identify the cardiac progenitor cells is still to be identified [26].

Cardiac Sca-1 expressing cells (Sca-1 cells) *in vitro* were shown to differentiate in cardiomyocytes-like cells and when transplanted into MI animal model contributed to an amelioration of heart function [27]. Sca-1 cells derived from cardiospheres were already tested in a human clinical trial, named CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction (CADUCEUS), demonstrating an increase in the viable myocardium and a decrease in scar formation at the first year posttransplantation preliminary assessment.

Cardiac-Kit expressing cells (c-Kit cells) are the best characterized CPC, but, their *in vivo* capacity to differentiate in cardiomyocytes is still a matter of debate [27]. Recent data suggest that following adult heart infarction c-Kit cells are recruited to the damage tissue, but fail to undergo myogenic differentiation, contrarily to neonatal hearts. This study illustrate that infarct environment don't limits c-Kit cells differentiation potential, but that this ability is critical depend of heart developmental state [27]. However, The Stem Cell Infusion in Patients with Ischemic cardiOmyopathy (SCIPIO) clinical trial revealed an improvement in left ventricular ejection fraction [28-30].

Cardiac Isl-1 expressing cells (Isl-1 cells) were the first cardiovascular progenitor to be identified during the embryogenesis and besides their ability to *in vitro* differentiate into cardiomyocytes, is lacking an active role of Isl-1 cells after ischemic injury [31]. Studies continue to be performed for a better comprehension of the origin of these cells, as well as, their behavior at the ischemic milieu.

Despite indication for postnatal mammalian myocardium as containing CPC, these cells represent a low *pool* in an adult heart. Moreover, preliminary assessment of the two recently established clinical trials have not been able to reveal significant long term regenerative ability. Several factors might be hypothesized to impair transplanted CPC

functions, such as, the adverse ischemic and inflammatory environments during injury or the misbalance of the ECM network destabilizing the proper cell niche.

2. Heart development and regeneration

From an evolutionary viewpoint, there is a regenerative-ability hierarchy in which invertebrates and several lower vertebrates appear to retain unique capabilities for restoring full organ/anatomical structures throughout life. Zebrafish and newts are able to regenerate their tissues and many organs, such as fin, optic nerve, spinal cord, and heart [6, 32]. To better understand why certain animals have extended regenerative potential while others do not, multiple *in vivo* studies have been initiated using prototypical organism models. The latter efforts are largely focused on highlighting the differences and similarities amongst species towards identification of the underlying molecular key players [33] (Figure 1).

Scar-free healing, although almost absent in adult mammals, is a common feature in early development and several studies support that fetal structure heal without scar-tissue formation [34, 35]. Recent work demonstrated that mice maintain a transient capacity for heart regeneration [36] which appears to disappear in the neonate over the first week after birth [37]. This regenerative ability is the result of a sequence of events already unveiled in the zebrafish that starts out with from formation of blood clot at the injury site, followed with inflammatory and fibroblast cells infiltration with consequent ECM deposition and cardiomyocyte proliferation. The loss of regenerative capacity (~day7) coincides with cardiomyocytes binucleation and cell cycle arrest. Additionally, a regenerative potential loss may as well relate to activation of a number of tumor suppressor pathways in cardiomyocytes. This would therefore represent a mechanism for cancer protection at the expense of regeneration [38]. Although this remains a largely elusive subject, all the above mentioned findings have challenged the scientific community to investigate the mechanisms for the switching off of the regenerative capability after birth. The evidence that fetal/neonatal mice heart retain a transient regenerative ability in comparison to the adult not only opens the way for obtaining novel therapeutic approaches as it also urge us with to perform more comprehensive studies in embryonic cardiac development. Heart development and maturation is a thigh regulated process of proliferation and differentiation of cardiovascular progenitors. The changes that limit the presence/proliferation of such cells after birth are yet to elucidate.

Recent findings suggested that embryonic fibroblasts are key player in the building-up of a cardiac niche favorable for cardiomyocytes proliferation, by high secretion of fibronectin, collagen and heparin-binding EGF-like growth factor (HBEGF) not observed in the adult fibroblasts [39]. It is timely to inquire which alteration(s) in the ECM are determinant for the shifting regenerative capacity that apparently occurs in the fetal/neonatal temporal frame. Previous studies already dated alterations on ECM elements expression and organization in heart after birth, indicating that ECM affects how cells "sees" and behave in the microenvironment [40].

3. Extracellular matrix of the heart

The ECM is a 3-D network composed by collagen, glycoproteins, elastins, growth factors, cytokines, proteases and proteoglycans which contain specific architectural and signaling functions that modulates tissue structure and cardiac pump function [40, 41]. The specific composition of the ECM and its macromolecules organization is critical to meet the functional requirements of each tissue. Thus, this scaffold is a dynamic structure, responding to environmental cues and tissue injury, changing their composition, organization and consequently the organ function. It is also recognized an active regulatory role for the ECM on the cells that contact t it, *e.g.* influence cell' survival, development, migration, proliferation, shape and function [40, 42].

Although the cardiac fibroblasts are chief producers of multiple ECM components, other non-cardiac cells in the heart produce ECM molecules related with their basement membrane, *e.g.* endothelial and smooth muscles cells. As ECM producers, fibroblasts allow the myocardial response to signals received of environment during heart genesis and pathologic situations [42].

The continual ECM turnover results from the tight balance on its synthesis *vs.* degradation, thus keeping the tissue homeostasis and integrity. Different molecules are involved on ECM homeostasis, namely the matrix metalloproteinases (MMPs), enzymes that degrade ECM constituents and inhibitors of metalloproteinases (TIMPs) that keep the MMPs under strict control [43, 44].

3.1. The ECM structure and components

The heart is a complex organ with dynamic functional properties that require an asymmetrical and helical architecture of specialized cells and ECM components for a normal pump function [45].

The cardiac ECM is composed by diverse families of molecules essential for tissue integrity and proper heart function. These molecules, such as, collagen and a variety of specific glycoproteins and proteoglycans are essential from the heart very beginnings and orchestrate the organ's morphogenesis and homeostasis, playing also a role during disease [40].

The ECM can be divided into two components, the basement membrane and the stromal matrix. The basement membrane is a thin network that give support to the cells, permits the interface between cells and the stromal matrix elements and more importantly offer functional input to modulate cellular function [46]. The stromal matrix is the bulk of the matrix, constituted mainly by fibrous molecules and provides the main structural support of the tissue [47] (Figure 2).

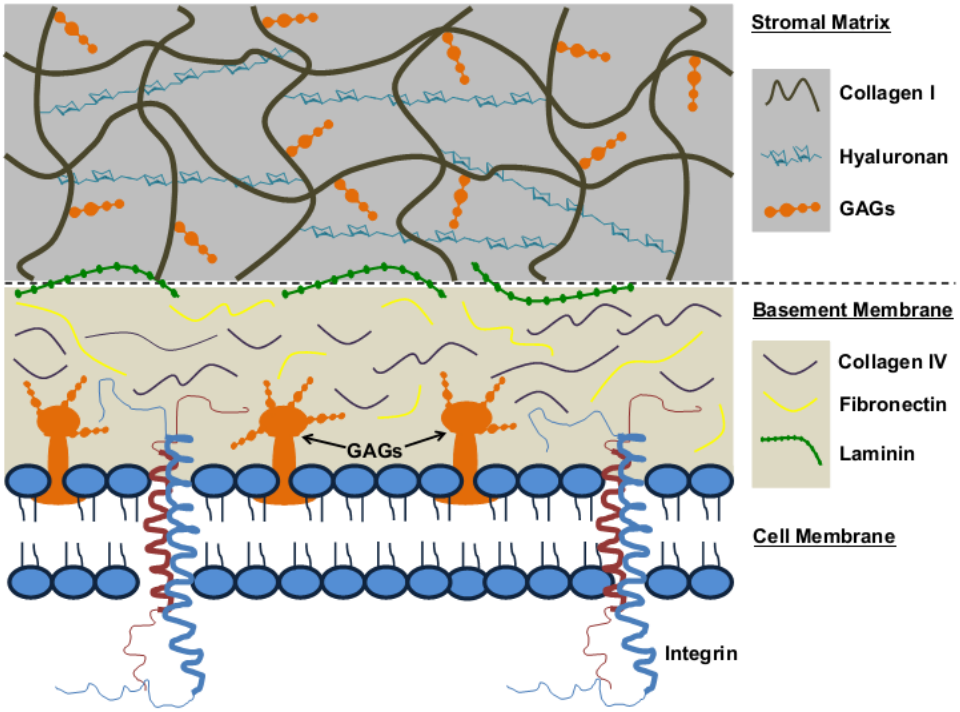

Figure 2. Extracellular matrix organization. Arrangement and localization of the ECM components within the basement membrane and stromal matrix. The scheme is not to scale. Adapted from [47].

Collagen

The collagens are a superfamily of macromolecules formed by three polypeptide chains, where one of them contains a characteristic triple helix. These macromolecules are characterized by their fibrous structure, slow synthesis, accumulation and turnover. In vertebrates approximately twenty eight different types of collagen are expressed and a significant number of them are transiently expressed during the heart development, as type II and VIII collagens [43, 48]. However, in the adult heart were identified five principle collagen types, namely, type I, III, IV, V, VI [49].

The expression of some collagen subtypes is time-specific during development and remodeling, depending on the required function of collagen expression. The distribution of the different collagens in the heart is not homogeneous and the organization and ratio between collagen types are preponderant in the regulation of stiffness, flexibility and contractility required in each heart region [50].

The collagen type I, III and V are the major constituents of cardiac connective tissue and are more insoluble than collagen in other tissues due the increased of cross-linking of the interstitial collagen network. The collagen fibrils make part of characteristic structures in the heart identified as weaves, coils, and struts [51] (Figure 3). These collagens create a network stress-tolerant in the mature myocardium dissipating the mechanical tension, but also, form a weave that sustain the adequate alignment of the cardiomyocytes during the contraction [40].

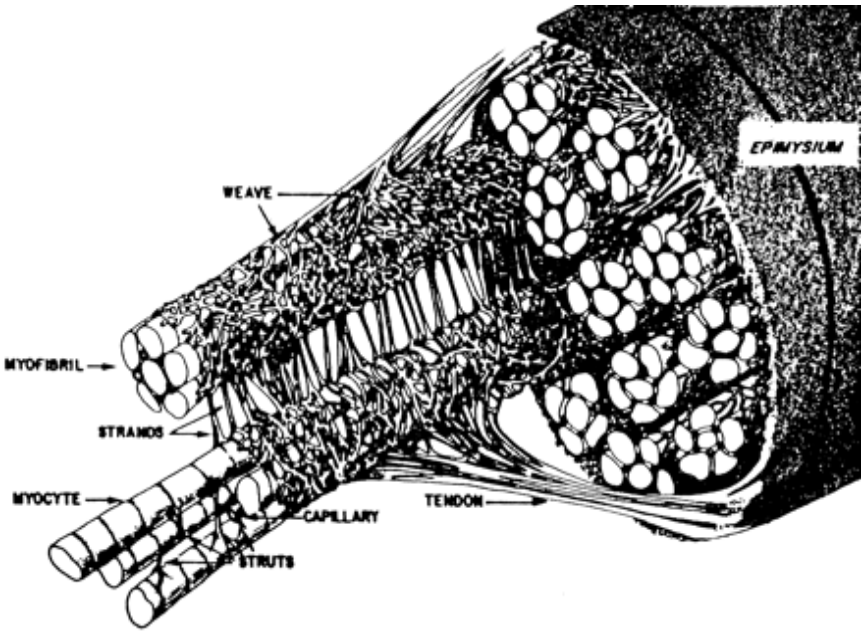

Figure 3. Schematic representation of the collagen matrix of the myocardium and its epimysial, perimysial and endomysial components. Image adapted from [52].

Type I collagen mainly contributes to rigidity and type III to elasticity of the myocardium, the abundance of these two types of collagen is higher in neonates in comparison with the adults (ratio type I collagen: type III collagen ~ 0,5) [53]. On the other hand, the type IV and VI collagen are elements of basal membrane that display in their constitution an 'arginine, glycine, aspartic acid' (RGD) sequence that allow the interaction with integrins and consequently with cells [54, 55].

All these collagens types create connections between each other, the type V coexists with type IV collagen in the basement membrane while both types V and VI collagens co-localize with collagen I and III fibrils in the interstitium. Type V retain a particular function that contrasts with the function of other collagens, because it demonstrates the ability to inhibit endothelial cell adhesion and proliferation [43]. Other interesting point is that after being secreted by cells this collagen seems to remain in the vicinity of the cell membrane, being designed as pericellular collagen [56].

The production of collagens is mainly performed by cardiac fibroblasts, which secrete also proteolytic enzymes that degrade the collagen network. Fibroblasts are therefore important players to maintain homeostasis of the collagen in the tissue. Deregulation of collagen synthesis or degradation have been demonstrated to lead to the development of heart diseases (Table 1) [52]. However, the mechanical impact of changes in collagen composition and collagen cross-linking is still to be convincingly dissected [57].

Elastin

Elastin is part of the group of ECM highly insoluble components and is one of the most important elements involved on tissue adaptation to mechanical stress. The elastin is encoded by one gene and is subject to alternative splicing, giving rise to various tropoelastin isoforms [58]. Elastin is normally found associated with other elements, namely microfibrils [59], helping in the maintenance of tissue structure [60]. In the case of the heart, elastin, although mainly associated with the vasculature, provides elasticity and resilient recoil to the myocardium and maintains its architecture against repeated expansion [57, 61].

Laminin

The laminins constitute a complex family of molecules composed by distinct isoforms involved in the production of heterotrimeric glycoproteins formed by three similar

chains (α, β, γ) [49]. As principal constituents of the basement membrane, their functions are associated with the adhesion and modulation of cell behavior, thus influencing cell migration, growth and differentiation [47]. Laminins can also contribute to tissue ECM organization, by acting as binding sites for other ECM components, like proteoglycans, integrins and collagen type IV [62, 63].

Table 1. ECM elements expression throughout heart lifespan and disease (↑↑ increase in expression, ↑ moderate increase in expression, ↔ expression maintenance, 0 expression not detected, ↓ expression reduction; *n.a.* data not available).

	ECM proteins	Fetal Myocardium	Neonatal Myocardium	Adult Myocardium	Pathology: Myocardium Infarction	References
Structural ECM	Collagen I	↑	↑	↑↑		[50, 62]
	Collagen III	↑	↑	↑↑		[50, 62]
	Collagen IV	↑	↑	↑↑		[50, 62]
	Collagen V	*n.a.*	*n.a.*	↑↑		[50, 62]
	Fibronectin	↑↑	↓	↑↑		[49, 50, 62]
	Laminin	↑	↔	↑		[49, 62]
	GAG	↑↑	*n.a.*	*n.a.*		[48, 64, 65]
Matricellular Proteins	Periostin	↑	↓	↑		[50, 62, 66]
	Osteopontin	↑	↓	↑		[50, 67, 68]
	Thrombospondin-1	*n.a.*	0	↑↑		[3, 62, 67]
	Thrombospondin-2	*n.a.*	0	↑↑		[62, 67]
	Tenascin-C	↑	0	↑		[3, 67, 69]
	Tenascin-X	*n.a.*	0	↑		[62, 67]
	Osteonectin/ SPARC	↑↑	*n.a.*	*n.a.*		[51, 68]
Matrix Metalloproteinases (MMPs)	MMP-1	↑	0	↑		[70]
	MMP-2	↑	↑	↑↑		[44, 70]
	MMP-3	↑	*n.a.*	↑		[44, 70]
	MMP-9	↑	*n.a.*	↑		[44, 70]
	MMP-13	↑	*n.a.*	↑		[44, 70]
	MMP-14	*n.a.*	*n.a.*	↑		[70, 71]
MMPs Inhibitors (TIMPs)	TIMP-2	↑	*n.a.*	↑,↔	(end-stage)	[44, 72, 73]
	TIMP-3	↑	↑	↓	(end-stage)	[44, 72, 73]
	TIMP-4	*n.a.*	*n.a.*	↓	(end-stage)	[72, 73]

The laminin is the first member of glycoproteins detected during the embryo development and is thought to be responsible for the coordination of the subsequent formation of other ECM elements [62].

A study performed by Price et al. demonstrated that the laminin network in the myocyte basement membrane is produced in a patchy manner, although it starts to be contiguous in the neonatal heart [74]. This rearrangement and seems to be important for the attachment of collagen on the myocyte surface. Therefore, following the neonatal period *de novo* attachments are impaired. Consequently, in pathological conditions that result on the destruction of these interactions, mechanical changes in the heart contraction can be produced [40, 74].

Although the laminin network suffers alterations in its organization during heart development they are constantly produced throughout the life (Table 1) [75].

Fibronectin

Fibronectin is a multi-domain protein that interacts with integrins, collagens and proteoglycans *via* RGD motif. Fibronectin is present in the basement membrane, but also in the stromal matrix [43]. This protein can suffer different alternative splices and give rise to different fibronectin variants that are differently expressed during heart development and adulthood [48]. The fibronectin contains on its structure multiples bindings sites for collagen, heparin fibrin, proteoglycans and for different cells types. It is synthetized by different resident cells of the cardiac tissue, such as endothelial cells, smooth muscle cells, fibroblasts and myocytes. Like other components present in the basal lamina, the fibronectin promotes cell adhesion, it is involved in the regulation of endothelial cells and orchestrate several events during heart development [75]. The lack of expression of this protein during development demonstrated to be embryo-lethal in animal knockouts [47]. Indeed, fibronectin is highly expressed during development and cardiac pathologies (Table 1).

Glycosaminoglycans (GAGs)

The GAGs are a family of molecules characterized by long unbranched poly-saccharides formed by dimers of repeating sugars. These molecules are divided in three main groups: heparin sulfate, chondroitin sulfate and hyaluronic acid. The majority of glycosaminoglycan elements are bounded to a core protein, *e.g.* glypican, perlecan or syndecan, and are therefore named proteoglycans (PGs). The hyaluronic acid is an exception and does not bind to any protein. Depending on the type of side-chain residues that bind to the core protein, GAGs are designated either heparan or chondroitin sulfate proteoglycans [47, 76].

Heparan Sulfate Proteoglycans

The heparan sulfate proteoglycans (HSPGs) are a group of proteoglycans whose side-chain is composed by heparan chains, or glucosamine and glucuronic or iduronic acid disaccharide units [47]. In mammals HSPGs can be found at the cell surface but also in

the basal lamina and stromal matrix. The organization of these molecules through the matrix is close-related with their function to regulate the interaction between cells and their microenvironment and consequently, control the cell fate [77]. This group of PGs confers high hydration to the tissue, because their structure is rich in hydrate domains, thus having an important impact on the tissue properties.

The HSPGs may act like a binding-protein for multiple growth factors and cytokines [47]. Indeed, it is able to anchor to the cell surface the stromal cell-derived factor-1 (SDF-1), an important progenitor cells mobilizing and homing factor [47].

The glypican-3, also known as Gpc3, is a membrane-bound HSPG expressed during development. The human mutation of *GPC3* gene is associated with Simpson-Golabi-Behmel syndrome, which leads to the development of a congenital heart disease, between others [48].

The syndecans are transmembrane molecules members of HSPGs with an important involvement during development, wound healing processes and tumor progression, thus influencing cell adhesion, migration, proliferation and differentiation [48, 78]. Studies performed in the rat heart demonstrated that their expression is altered throughout ontogeny, so high levels of syndecan3 and moderate levels of syndecan2 are found during development and low levels of syndecan1 is observed in the adult heart [48, 79] (Table 1).

Chondroitin Sulfate Proteoglycans

The chondroitin sulfate proteoglycans (CSPGs) are formed by uronic acid-N-acetyl-D-galactosamine disaccharide units [80] and define a diverse group since different core proteins and posttranslational modifications may be involved in the assembly of the macromolecule [81]. Similarly to HSPGs, CSPGs are an element of the connective tissue and can also be found at the surface of many cells types.

The CSPGs versican and aggrecan were identified during heart development (Table 1). A deficiency on the versican expression is associated with heart abnormalities in animal models [48, 82]. The core protein of versican is present in different isoforms which are expressed in a time-specific manner and have different functions during heart development [48]. The aggrecan is a CSPGs expressed mainly in the cartilage. In

addition, although found in the chick heart in the epicardium and in other heart structures its seems not to be expressed in the murine heart [47].

Hyaluronic acid

Hyaluronic acid (HA) or also named hyaluronan is the simplest GAG, composed by a polysaccharide chains comprising a repeating sequence of amino sugar and glucuronic acid disaccharide units. Contrarily to other GAGs, HA has high molecular weight, lacks sulfate groups and does not attach to core proteins. Although considered a common component of ECM it is the mostly abundant during development [48]. HA is abundantly found in the pericellular matrix, contributes to tissue hydration and is involved in innumerous processes including cell proliferation, cell motility, inflammation, development and regeneration [69, 83]. Like other elements of the ECM, HA has a structural role in different tissues however more importantly, is their involvement in multiple complex signaling pathways when compared with other ECM molecules [64].

Although HA is synthetized as a larger polymer, it may be degraded into smaller forms, endowed with different biological properties. High-molecular weight HA has the capacity to mediate the epithelial-mesenchymal transition during heart valve formation and low-molecular weight HA stimulates angiogenesis [47, 84].

3.2. Other components

Matricellular Proteins

Matricellular proteins are a portfolio of proteins with signaling functions that modulate cell behavior, participate in cell-matrix interactions, but do not hold a direct structural role. The expression of these proteins is high during embryogenesis, however, their levels decrease abruptly after birth and remain low during adult life (Table 1) [85]. These molecules appear however to be re-expressed after tissue injury indicating a role for matricellular proteins during the healing process [68, 86]. The signaling function of these proteins is mediated by direct binding to cell receptors and regulate the expression of other ECM proteins, cytokines and growth factors. The matricellular proteins osteopontin, thrombospondin-1/2, tenascin-C/X, periostin and osteonectin have already been shown to play key roles in heart homeostasis *vs.* pathology [51, 68].

Matrix metalloproteinases

The matrix metalloproteinases (MMPs) represent a group of endogenous proteolytic enzymes involved in ECM digestion on the majority of biological tissues. Their activities are involved in critical processes, as development, morphogenesis, reproduction, inflammation and in pathological remodeling in several disorders, including cardiovascular diseases (Table 1) [3, 44, 87]. Different MMPs are synthetized, but only six are continually expressed on heart for myocardial ECM turnover. The activity of this family of enzymes also regulates the cardiomyocytic response to the infarcted and non-infarcted environment.

Although MMPs are capable to degrade different ECM constituents, the enzymatic activity shows substrate-specificity and to act in an organized pattern during tissue remodeling [43]. Its expression and activation is a strictly controlled process thus maintaining a balanced ECM synthesis *vs.* degradation; changes to the steady-state are intimately associated with cardiovascular diseases.

Inhibitors of metalloproteinases

Conversely, MMPs activity is modulated by the inhibition exerted by specific inhibitors, *i.e.* TIMPs. The latter are normally expressed in the myocardium at different levels and consist in a group of distinct molecules with low molecular weight and two domains that retain an amino (N) and carboxyl (C) domain. The N domain is directly involved in a reversible inhibition process, binding non-covalently to the catalytic domain of MMPs in a 1:1 stoichiometry [43]. Although some degree of overlap/redundancy can be observed for TIMPs inhibitory activity, these molecules display a grade of specificity and affinity for the distinct MMPs. In the diseased heart, *e.g*. MI, TIMPs have been shown to play an important role in the myocardial remodeling reducing the adverse effects of intense matrix degradation by MMPs (Table 1) [72, 88]. Furthermore, TIMPs activity is not limited to MMPs inhibition being also involved in cellular growth, proliferation, and apoptosis, with effects independent of their influence on MMPs [44].

4. The heart ECM throughout ontogeny

The extracellular matrix plays a crucial role on regulating the dynamics of many cellular events through the organisms' lifespan [89]. Thus, since early embryogenesis and in a

specific spatiotemporal order, the ECM orchestrates cells' migration, reorganization and differentiation. For the heart, evidence for such phenomena has been obtained in different animal models as well as in human conditions*, e. g*. Congenital Heart Disease where changes were observed in genes encoding matrix proteins. The available data confirm that perturbations of expression or/and alteration in the signaling pathways of ECM components lead ultimately to cardiac malformations and to congenital heart diseases [48].

During embryogenesis the heart is the first functional organ to be formed, starting to beat around embryonic day 8 in the mouse. The developing embryonic organ thus sustains circulation of nutrients and waste when cells diffusion process starts to be inefficient. At this time point, the heart is a primitive tube-like structure, composed by two cellular monolayers, *i.e.* endocardium (inner cell layer) and myocardium (outer layer) separated by a rich ECM commonly referred as cardiac jelly [90, 91].

The cardiac jelly consist in a homogeneous gelatinous material composed by several molecules already identified as type I and type IV collagen, elastin, laminin, fibronectin although the hydration levels of the jelly are primarily controlled by GAGs.

Although several aspects of the cardiac jelly function are still elusive, *e.g*. the mechanisms involved on specific ECM molecules accumulation, it is known that it serves as a substratum for diffusion of growth factors and other molecules at the myocardium-endocardium interface. In addition to the cardiac jelly glue-like acting to give support to endocardium and myocardium, key roles in peristaltic pumping of the tubular heart [92] and modulation of the endocardial cushions formation at the atrioventricular (AV) junction have also been recognized [48].

The ECM composition is a main determinant in the process of epithelial–mesenchymal transition (EMT) observed at distinct stages of development, and in the response to, and subsequent development, of pathological conditions. Noteworthy, the EMT is a very important phenomenon in cardiac cushion morphogenesis and valve formation [48].

In the context of such events, the myocardium distends secreting localized amounts of ECM that induce the endocardium to invade the cardiac jelly thus forming endocardial cushions. The latter undergo a sequence of processes such as EMT, differentiation, growth and an intense remodeling (orchestrated by ECM elements present in the cardiac jelly) all of which affecting cardiac architecture and heart function [50]. From the concerted action of all those developmental events mature mitral, tricuspid, aortic and pulmonary valves, as well as the atrioventricular and outflow septa, are formed [93].

Furthermore, during the prenatal period the heart undergoes an increase on the ventricles wall thickness and tensile strength. Circumferential collagen deposition around the heart responds to the functional requirements after birth and the opening of the *ductus arteriosis*. After birth, occurs a peculiar reduction of some ECM elements, *e.g.* periostin [50], fibronectin [94]. Nevertheless, knowledge concerning the spatiotemporal distribution of ECM proteins is still incomplete at the present time [94]. As with any other major organ, a correct sequence of events during heart development is crucial for a healthy heart formation. Thus, when for some reason a particular event fails the expected events are fetal to early perinatal death or development of congenital diseases. Some of these diseases are syndromes associated with connective tissue defects, *e.g.* Loeys-Dietz syndrome that is the result of mutation in genes encoding collagen proteins [95]. A balanced composition of ECM elements is crucial for all the processes involved in heart formation.

The adult mammalian heart presents a network of different cells and matrix components that work together to maintain heart function in normal and pathological conditions. At this stage, the heart is an organ with a complex organization. The ventricular myocardium is composed by laminae of overlapping myocytes involved in a collagen network, endothelial cells and vascular smooth cells restricted largely to the coronary vasculature [96]. The heart also contains a reservoir of stem/progenitor and 'transient' circulating cells (monocytes and mast cells) spread throughout myocardium and interstitial space. The ECM retains a structure and composition that facilitate the mechanical, chemical and electrical properties required for the homeostasis of cardiac tissue. The main components of ECM, collagen type I and III, are produced and secreted by fibroblasts and vascular smooth muscle cells. Conversely, the myocytes and endothelial cells produce principally components of basal lamina, *e.g.* collagen type IV and laminin, but also, collagen VI and proteoglycans [49, 50]. A proper balance on synthesis *vs*. degradation of ECM is important and also assured by the appropriate production of the main proteases (MMPs) produced by cardiac fibroblasts. However, cardiac function declines with aging, like any other organ in the body. Studies in rats consistently showed that the aging process correlates to an increase in collagen [97] which has an impact on diastolic stiffness due to decreased ventricular elasticity [98]. The reason(s) underlying such increase is not known; it has been suggested that it may be associated with cardiomyocytes loss or, alternatively, with some degree of inhibition

on collagen degradation. After an insult, the heart suffers critical changes associated with intense cell loss and misbalance of the ECM and the adult organ unsuccessfully try to meet homeostatic conditions. One might question to which extent the remodeling process, that partly keeps the heart functioning and ultimately leads to non-functional scar tissue (repair), is also the result of an aborted recapitulation of the early fetal cardiogenic program.

At present it is recognized that the functional information contained in the ECM network modulates the cells' behavior. The latter premise has been explored in tissue engineering approaches in the last years through the development of acellular matrices, the so-called *bioscaffold*, for tissue replacement and repair in preclinical and clinical applications [99]. These matrices are produced by applying decellularization protocols, procedures where the cellular fraction of the tissues is removed remaining only the 3-D structure or natural tissue scaffolding. Matrices have recently been successfully used in the clinic for tissue-organ replacement, *e.g.* trachea replacement [100]; cardiac valves [101]; skin [102].

In 2008, in a proof-of-principle study, Doris Taylor Team performed a whole organ (heart) decellularization and showed the complete heart decellularization with maintenance of all the cardiac structures (ventricles, atrias, valves and the vascular system). The cardiac ECM demonstrated to be preserved and the cellular remnants efficiently removed. Furthermore, recellularization of the heart scaffold was also obtained after perfusion of endothelial cells and intramural injection of cardiac-derived cells in a bioreactor system. The cell colonized heart-construct displayed electrical and contractile functions at 8 days post-seeding. These results gave strength to the concept that the ECM has an essential role on restoring a functional histological unity that characterizes an organ [103].

5. Tissue and Organ Decellularization

5.1. Decellularized matrices

Due to its natural origin, the retention of the ECM components and 3-D organization of the tissues and the biospecific signals *(e.g.* growth factor and cytokines) anchored within the fibrous structure, the decellularized matrices have been intensively

investigated as to whether these structures may constitute systems for therapeutic use. There is already evidence of ECM-derived materials used to facilitate the reconstruction of different tissues both in preclinical studies and in human clinical applications [104]. These naturally biodegradable materials are enzymatically processed by the host tissue and, following degradation of ECM proteins such as collagen(s), glycoproteins and elastin among others, soluble bioactive peptides *(i.e.* matricryptins or matrikines) are released. The peptides were demonstrated to induce cell proliferation, migration, and differentiation and, a potential role in angiogenesis induction was also proposed [3, 50].
In this matter, one more beneficial attribute is that the application of acellular tissue-fragments not only overruns the allogeneic/ xenogeneic transplantation barriers as the ECM components per se also appear to be highly conserved amongst the species. Preliminary data from animal studies have provided encouraging results [98] and there are currently several products based on decellularized tissues for clinical application [99].
The potential of the decellularized tissue has been explored at the acellular scaffold and also at the hydrogel level [105]. As hydrogel, the ECM is an easy workable material that allows minimally invasive application. Some disadvantages on the latter formulation can stem from that the dehydrated material will not re-hydrate to its original architecture, thus suffering irreversible changes that affect the cells basic properties as compared to a much less altered non-lyophilized ECM. Encouragingly, skeletal myoblast progenitors cultured on lyophilized-and-reconstituted skeletal muscle ECM were shown to maintain their growth further exhibiting differentiation features [47].
The decellularization procedures for 3-D scaffolds were tested successfully in multiple organs, such as, heart [103], lung [106], liver [107], skin [102]. In the field, a current indication is to apply the decellularized matrices in the same type of organs from which the ECM is harvested. One such example is the heart, where the ECM presents an asymmetrical, helical architecture intimately linked to the heart pump function. Indeed, the ECM not only provides physical cues that modulate proliferation and cellular differentiation as it is has also an impact on the electromechanical activity of the myocardium [108].

5.2. Decellularization methodologies

The decellularization protocols are designed according to numerous factors taking in account the target-tissue characteristics (*e.g.* cellularity, density, lipid content and thickness) and the final application(s). In brief, various agents and methodologies have been tested with distinct effect on the ECM properties, thus showing that multiple parameters need to be addressed to achieve a biofunctional formulation [109].
An efficient removal of the cytoplasmic remnants, nuclear material and antigenic epitopes is required to avoid, or at least minimize, adverse immune response by allogeneic/xenogeneic recipients of the ECM scaffold material [104]. Chemical, biological and physical decellularization agents can be combined to create robust and effective decellularization protocols to achieve a delicate balance between cell removal and ECM structure and biological integrity [102, 110].

The principle of current decellularizing methods, *i.e.* its primary action and the respective alterations verified in the ECM are summarized in the Table 2. The complexity and length of the protocols are in accordance with the degree of geometric and biologic conservation desired for the acellular tissues (e.g. macrostructure, ultrastructure, matrix and basement membrane proteins, growth factors, etc.), especially for whole organs.

The introduction of the decellularization agents can follow distinct routes, and amongst the most popular is perfusion, and immersion with agitation.

Perfusion is the most used method, characterized by delivery of the agents throughout the vascular system thus maximizing the agents' distribution throughout the tissue. Moreover, it also provides for a more efficient removal of cellular material from the tissue and a high preservation of the tissue architecture. Related with the nature of this method, decellularization protocols can use low concentration of detergents and/or the decellularization of the tissue is accomplished at shorter time-periods. This approach has been used in several studies mainly for whole organ decellularization, such as of the kidney, liver, heart and lungs. Wainwright and colleagues scaled-up the perfusion system in order to accomplish efficient decellularization of whole porcine heart [111].

The immersion with agitation is an advantageous approach in tissues whose vasculature is compromised or not accessible, as heart valves [101], skeletal muscle/tendon [112], dermis [102], among others. Because the latter approach is based on the diffusion of

decellularization agents, the duration of this particular procedure depends upon specific tissue characteristics (the thicker is the tissue, the longer is the exposure time to decellularization agents), detergent properties and agitation intensity, due the decellularization process to occurs by diffusion.

Table 2. Selected agents and techniques for decellularizing tissue. Adapted from [92].

Agent	Technique	Mode of action	Effect on ECM
Chemical Agents	Acids and bases	**Acids:** Solubilizes cytoplasmic components of cells. **Bases:** Disrupts nucleic acids, tend to denature proteins.	May damage collagen, GAG, and growth factors
	Hypotonic and hypertonic solutions	Cell lysis by osmotic shock, disrupt DNA-protein interactions	Effectively lyses cells, but does not effectively remove cellular residues
	Non-ionic detergents (eg.Triton X-100)	Disrupt DNA-protein interactions, disrupt lipid-lipid and lipid-protein interactions and to a lesser degree protein-protein interactions.	Some disruption of ultrastructure and removal of GAG, less effective than SDS.
	Ionic detergents (eg.SDS)	Solubilize cell and nucleic membranes, tend to denature proteins.	Tends to disrupt ultrastructure, removes GAG and growth factors and damages collagen.
	Solvents (Alcohols and acetone)	Cell lysis by dehydration, solubilize and remove lipids.	Crosslinks and precipitates proteins, including collagen.
Biological Agents	Enzymes	**Nucleases:** Catalyze the hydrolysis of ribonucleotide and deoxyribonucleotide chains. **Trypsin:** Cleaves peptide bonds on the C-side of Arg and Lys.	**Nucleases:** Difficult to remove from the tissue, could invoke an immune response. **Trypsin:** Can disrupt ECM ultrastructure, removes ECM constituents.
	Non-enzymatic (Chelating Agents, eg. EDTA, EGTA)	Chelating agents bind metallic ions, thereby disrupting cell adhesion to ECM.	Ineffective when used alone.
Physical and Miscellaneous Agent	Temperature	Intracellular ice crystal disrupts cell membrane.	Ice crystal formation can disrupt or fracture ECM.
	Direct application of force	Removal of tissue eliminates cells and force can burst remaining cells.	Force can directly damage ECM.
	Pressure	Pressure can burst cells and aid in removal of cellular material.	Pressure can disrupt ECM.
	Electroporation	Pulsed electrical fields disrupt cell membranes.	Electrical field oscillation can disrupt ECM.

5.3. Decellularization efficiency

Different methodologies can be applied to analyze the decellularization efficiency, which are largely based on histological evaluation and quantitative methods of molecular biology. The Hematoxylin and Eosin (HE) is a histological stain used as first qualitative approach to analyze the presence of cytoplasmic material and nuclei in the tissues. Other stains, such as Masson's Trichrome, Movat's pentachrome, Alcian Blue or Safranin O can be also used to evaluate the presence of cellular remnants and several

of the ECM distinct molecules. On the other hand, immunohistochemical or immunofluorescence methods are used to identify and analyze the patterns of specific ECM components or intracellular proteins in the tissue (*e.g.* Laminin, Cardiac Troponin T, Vimentin, etc.), due their specificity and sensitivity. The presence of the nuclear material in the acellular tissue can be accessed staining the DNA with 4',6-diamidino-2-phenylindole (DAPI) or Hoechst, two probes that bind to the DNA chains and that can be detected by fluorescence microscope. Another technique in use is the PicoGreen assay, very sensitive to the DNA and that allow the quantitation of the DNA retained in the matrices [110]. Other important aspect after decellularization is the evaluation of alterations in the ECM components using histological techniques already mentioned, and also quantitative methods, *e.g.* Sircol assay for collagens quantifications and Blyscan assay to quantify GAGs. Mechanical tests can be also utilized for a complete elucidation on the ECM integrity.

The sole evaluation of the decellularization effectiveness protocols does not allow the concluding that no adverse host responses will be verified *in vivo*. Although decellularization methodologies have been much investigated in the last decades, the concept of decellularized matrix by quantitatively metrics has not yet been well established. Clear knowledge on the relation between cellular remnants and a hostile response is not yet available. However, in order to prevent adverse cell and host responses, minimal criteria were suggested by Crapo and colleagues as follows:

- < 50ng dsDNA per mg ECM dry weight;
- < 200 basepairs DNA fragment length;
- lack of visible nuclear material in tissue sections stained with DAPI or H&E stain [109].

Other criteria will be necessary to clarify, and to prevent, the extent to which cytoplasmic and membrane-associated constituents are also involved in adverse host responses. Moreover, the criteria already defined are fundamental to allow investigators and ECM product manufacturers to criteriously conclude on the effectiveness among protocols, to compare between decellularized tissue products and to eliminate variations in cell and host responses to acellular tissues that may be caused by dissimilarities in residual DNA. The latter efforts simplify the interpretation and comparison of *in vitro* and *in vivo* results and contribute for standardization in the field.

Aim

Recent data revealed that the mammalian heart appears to retain a degree of regenerative capacity on the fetal to neonatal transition which is ceased at the end of the first week after birth [36, 37]. This capacity showed to be similar to that verified in regenerative low vertebrates, such as in zebrafish [38]. The mechanisms underlying the switch-off of regenerative ability are not completely understood. The complex "triggering" and "silencing" of molecular pathways (*e.g.* cell-cycle machinery, tumor suppressor factors) associated to the establishment and stabilization of the mature cardiomyocytes' phenotype and to those of adult fibroblasts and CSC homeostasis demonstrated in some studies may in part explain the phenomenon.

The developmental-stage specific expression of distinct ECM elements and thus the respective dynamic network (re)-arrangement of several protein epitopes, structure and catalytic domains are likely associated to the display of regenerative ability along ontogeny [40]. The high ECM secretion of collagen, fibronectin and heparin-binding growth factors characteristic during heart development, is illustrative of the creation of a niche propitious for cardiomyocytes proliferation and consequently to a regenerative response if damage is to happen at this stage [39]. Moreover, alteration in the laminin organization overlapping with cardiomyocytes cell cycle arrest has been reported [40, 74].

We hypothesize that the ECM plays a key role on determining whether the wound healing process develops along a regenerative response (fetus/early neonate) or leads instead to scar formation (adult). Hence, with the ultimate purpose of identifying ECM component(s) that may be used to drive a regenerative response, we set out to investigate specific changes in the ECM throughout ontogeny that may correlate with cardiogenesis *vs.* adult cardiac repair. To meet this, an *in vitro* model system of decellularized ECM from fetal and adult hearts will be developed to study the ECM network and its elements. It is anticipated that the latter will ease the analysis of cardiogenic potential and thus, in the future, constitute a biocompatible and cardioinductive patch that, after proper pre-clinical testing in MI surgically-induced

animal-models, could be translated to the human clinical context. Furthermore, regarding the present limitations to the *in vitro* culture of cardiac cells, we intend to explore the cardioinductive potential of the acellular-heart matrices and thus establish bioscaffolds-systems that replicate fundamental cues of the *in vivo* microenvironment.

The herein thesis work was specifically designed to accomplish the development, characterization and analysis of biocompatibility of decellularized fetal (E18)- and adult-derived cardiac ECM. To this end, we focused on the following tasks:

- Production of decellularized cardiac matrices from fetal and adult tissues;
- Biochemical characterization of acellular cardiac tissue from fetal and adult heart;
- *In vitro* evaluation of the biocompatibility and cardiogenic potential of the decellularized matrices obtained.

Materials and Methods

Animals

All animal experiments were performed in accordance with the IBMC.INEB Animal Ethics Committee and to the DGVet. Humane endpoints were performed in accordance to the OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000).

Adult pregnant C57BL/6 with eighteen days of gestation (E18) and their fetus were used for this study.

Decellularization of cardiac tissue

Pregnant mice C57BL/6 (E18) were euthanized by carbon dioxide asphyxiation and a Caesarean section was performed. E18 fetuses were deeply anesthetized with Isoflurane (MARK) prior to decapitation. A median sternotomy was performed in a sterile fashion and the hearts excised and cleaned in phosphate buffer saline (PBS). The left ventricle (LV)of adult heart was divided in small fragments with (Ø=2mm) a tissue biopsy puncher (PUN200, Zivic Instruments). Intact fetal hearts and LV tissue fragments from adult hearts were embedded in OCT (Cryomatrix 6769006, THERMO Scientific) and cryopreserved in liquid nitrogen-cooled isopentane (2-methyl butane, SIGMA) and stored at -80ºC until further use.

Cardiac tissue decellularization was achieved through a sequence of incubation steps and washes (Figure 4). Following thawing and washing in PBS to remove the OCT, an overnight incubation (~18 hours) in Hypotonic Buffer (Table 3) and three washes in PBS (1 hour per wash) were performed. Samples were then emerged in Detergent Solution (Table 3) and incubated for 24 hours. Different detergents, previously used for cardiac tissue decellularization [103, 105, 108, 111, 113, 114], were selected and used at different formulations: 0,1% Sodium Dodecyl Sulfate (SDS), 0,2% SDS, 0,5% SDS combined with 1% Triton X-100, 1% SDS combined with 1% Saponin. As a result of

protocol optimization, 0,2%SDS was selected as the most efficient condition, and used in further experiments.

After incubation with the Detergent Solution samples were washed three times (20 minutes per wash) with a Hypotonic Wash Buffer and incubated 3 hours, at 37ºC, in DNAse Solution. Three final washes with PBS were performed (20 minutes per wash) (Table 3, Figure 4) to remove residual detergent and DNAse.

Table 3. Composition of the solutions required for the decellularization protocol.

Solutions	Composition
Hypotonic Buffer	10mM Trisma BASE (T6066, Sigma Aldrich), 0.1% EDTA (ED2SS, Sigma Aldrich), 10µg/mL gentamicin (15710,Gibco), in deionized water at pH 7,8.
Detergent Solution	*x*% SDS (71729, Sigma Aldrich) with or without 1% TritonX-100 (X100-500mL, Sigma Aldrich) or 1%Saponin (47036-50G-F, Sigma Aldrich), 10mM Tris BASE, 10µg/mL gentamicin, in deionized water, at pH 7,8.
Hypotonic Wash Buffer	10mM Tris BASE, 10µg/mL gentamicin in deionized water, at pH 7,8.
DNAse Solution	10mM Tris BASE, 50 U/mL DNAse I (AplliChem), 10µg/mL gentamicin in deionized water, at pH 7,8.

All the decellularization steps were performed at room temperature (RT), unless stated otherwise, and under mechanical agitation (165 rotations per minute (rpm)). An experimental control, hereinafter designated PBS control, was subjected to the same experimental conditions with the exception that it was emerged in PBS with 10µg/mL gentamicin throughout the procedure.

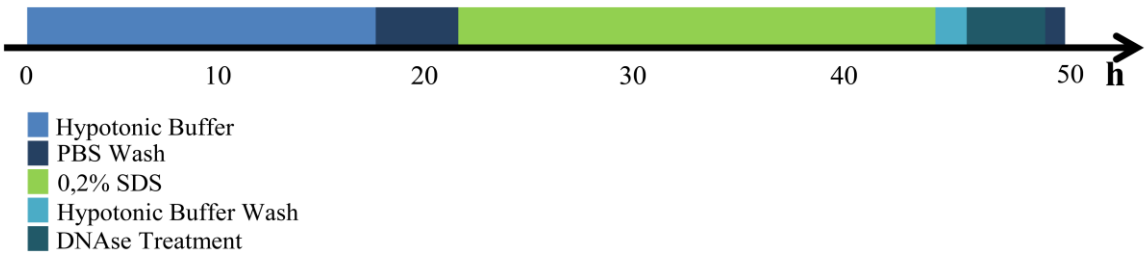

Figure 4. Timeline of the decellularization protocol. h-hours

iCPC^{Sca-1} cell maintenance

iCPC^{Sca-1} is a cell-line model system for adult Sca-1 cardiac-resident progenitor that was recently characterized at our laboratory. Cells were expanded *in vitro* in complete medium (αMEM (Minimum Essential Medium α, 11900-073, Gibco) supplemented with 10% FBS (Lonza), 1% P/S (100U/mL Penicillin and 100µg/mL Streptomycin, PAA), and 10mM HEPES). Cells were kept under an atmosphere of 5% CO_2 at 37ºC. When the cell culture reached 80% of confluence, cells were subcultured by washing in PBS twice, to remove culture medium and were then incubated with a trypsin/EDTA solution for 3minutes at 37ºC. Following inactivation of the detaching agent by the addition of complete medium cells was centrifuged at 300xg for 5 minutes at 4ºC and the supernatant was discarded. The cells were resuspended in culture medium, and an aliquot was stained with trypan blue (1:1) to discriminate viable cells. Cells were counted in a Neubauer's chamber and the cell density was adjusted for seeding onto the cardiac decellularized ECM (5.000cells/µl).

Passive seeding of decellularized cardiac matrices

Decellularized matrices were incubated overnight at 4ºC on αMEM with 1% P/S, transferred to a 96-well plate and seeded with iCPC^{Sca-1} as represented in Figure 5. Briefly, the cell suspension (50.000 cells in 10µl per decellularized matrix) was directly pipetted to the surface of the scaffold and complete medium was carefully added to cover the entire scaffold. The plate was incubated 24 hours under 37ºC and 5% CO_2 and then the matrices were transferred to a 24-well plate for the following culture period. The medium was replaced every 2 days.

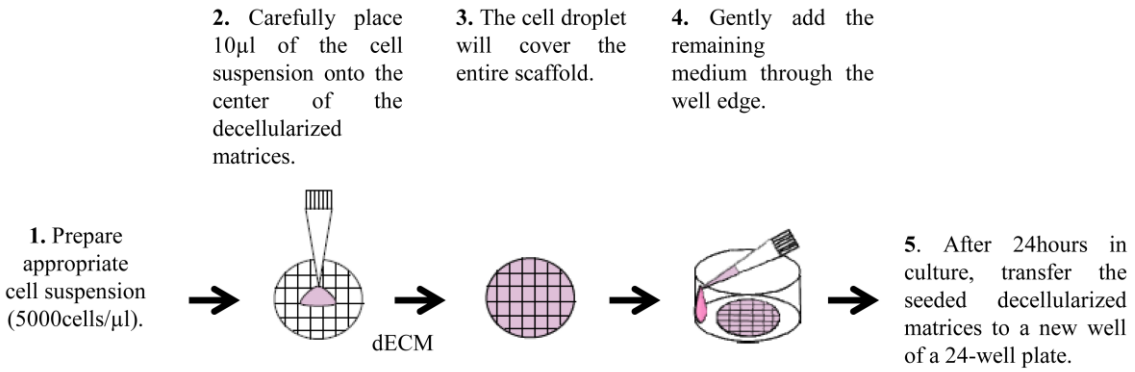

Figure 5. Schematic representation of cell seeding onto the decellularized matrices.

Histological assessment

Decellularized and recellularized matrices were fixed in 10% Formalin neutral buffer (VWR BDH & Prolabo) with 0,02% aqueous Eosin (01592E, SURGIPATH) either during 2 hours at RT or overnight at 4ºC.

For paraffin embedding the samples were processed for 12 hours in an automated system through of successive PBS washes, crescent series of alcohols (Aga), Clear Rite 3® (Richard-Allan Scientific) and Shandon Histoplast (Thermo Scientific) at 56ºC. Paraffin blocks were the cut into 3 µm sections, until the center of samples was reached. The sections were then stained with and stained with Hematoxylin and Eosin staining (HE) to highlight tissue changes following decellularization. Briefly, sections were dewaxed and rehydrated by three changes of xylene, followed by sequential alcohol gradients and rinsing in deionized water. An incubation of 5 minutes with Gill's Hematoxylin (GHS232, Sigma-Aldrich) was performed, followed by bluing in 0,5% ammonia for 4 seconds. Finally, sections were incubated with alcoholic eosin during 2 minutes, diafanized in xylene and mounted in DPX Mountant for histology (06522, Sigma Aldrich).

Sections were observed on an inverted microscope (Axiovert 200M, Carl Zeiss) and the images captured with a heterochromatic camera (AxioCam HRC, Carl Zeiss) using the MosaiX software (AxioVision modules, Carl Zeiss).

Immunostaining

Paraffin sections were dewaxed and rehydrated by three changes of xylene, followed by sequential alcohol gradients and rinsing in deionized water. If antigen retrieval was required heat-induced epitope retrieval (HIER) or enzymatic epitope retrieval (PrER) were applied. For HIER, sections were incubated 30 minutes at 98ºC on a water bath, in 10mM sodium citrate, pH 6,0 or in 10mM Tris 1mM EDTA (Tris-EDTA), pH 9,0 and let to cooled down at RT during 20 minutes. When targeting epitopes that require cell membrane permeabilization (cytoplasmic and intra-membrane epitopes) sections were treated with 0,2% Triton X-100. Tissue sections were blocked for 1hour with 4%FBS and 1% bovine serum albumin (BSA). If the primary antibody was produced in mice, the M.O.M.™ Immunodetection Kit (Vector Lab) was applied to enable blocking of endogenous Fc receptors that can be recognized by the secondary antibody [115].

Incubation with primary antibody was performed in a humidity chamber 2 hours at RT or overnight at 4ºC. The primary antibodies used were: Cardiac Troponin T (cTroponin T) (Mouse IgG, ab33589, Abcam), at 1:200 dilution; Collagen IV (Goat, AB769, Millipore), at 1:10 dilution; Ki67 (Rabbit IgG, ab15580, Abcam), at 1:100 dilution; Fibronectin (Rabbit, F-3648, Sigma Aldrich), at 1:400 dilution; Laminin (Rabbit IgG, L9393, Sigma Aldrich) at 1:25 dilution; Sca-1 (Rat IgG2a,k, 553333, BD Pharmingen), at 1:100 dilution; and Vimentin (Mouse IgG1/K, MS-129-P, Thermo) at 1:50 dilution. The incubation with secondary antibodies was carried out for 1 hour at RT. The secondary antibodies used were: Alexa Fluor 594 donkey anti-mouse IgG (A-21203, Invitrogen) at 1:1000 dilution; Alexa Fluor 647 donkey anti-rabbit IgG (A042, Invitrogen) at 1:1000 dilution; Biotinylated anti-rabbit IgG (BA-1000, Vector Lab) at 1:200 dilution; Biotinylated anti-rat IgG (BA-9400, Vector Lab) at 1:200 dilution; Biotinylated MOM IgG (MKB-2225, Vector Lab) at 1:250 dilution. Whenever using a biotinylated secondary antibody, sections were incubated 20 minutes with Streptavidin conjugated with Alexa 555 (S32355, Invitrogen) at 1:500 dilution. Sections were mounted using Fluoroshield™ with DAPI (Sigma Aldrich), observed on an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss) and images captured with a monochrome camera (AxioCam MNC, Carl Zeiss) and MosaiX software's (AxioVision modules, Carl Zeiss).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The samples were fixed by 2,5% Glutaraldehyde (R1011, AGAR Scientific) and 4% PFA (19208, EMS) in 0,1M Na Cacodylate (20840, Fluka), overnight at 4ºC. After washing in 0,1M Na Cacodylate, a post fixation of 1 hour was performed with 2% OsO4 (19152, EMS) in 0,1M Na Cacodylate followed by a 30minutes fixation with 2% uranil acetate (22400, EMS) in deionized water, at RT. The fixed tissues were dehydrated using a crescent series of alcohols (70%, 90% and 100%) (Aga) prior to inclusion in Epon 812 (14120, EMS). Samples were oriented and positioned in labeled molds, and placed on an incubator for curing.

Semi thin sections of 1,0 mm of thickness were obtained using a glass knife. To analyze the entire cross section by SEM the secondary electrons and back-scattered electrons (BSE) detector was used.

Ultrathin sections of 50 nm of thickness were obtained using a diamond knife and collected in copper grids. Ultrathin cuts were double contrasted with uranyl acetate (aqueous saturated solution) during 1 minute and lead citrate [103] during 3 minutes. Digital images were acquired using a Jeol JEM 1400 electron microscope coupled by a Orius 1000w.

DNA extraction and quantification

DNA was extracted using the PureLink Genomic DNA Mini Kit (K1820-00, Invitrogen). Briefly, complete tissue lysis was achieved using the PureLink Genomic Digestion Buffer and Proteinase K, at 55ºC on a thermomixer (Eppendorf). Following RNA removal with RNAse, the DNA was collected on a PureLink Collection Tube.

DNA quantification was performed using the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen) and according to manufacturer's instructions. The fluorescence was measured using a Fluorimeter (Excitation: 480 nm, Emission: 520 nm) and the concentration of DNA present on each sample was calculated based on the high and low-range standard curves obtained. All samples were run in triplicate.

Sulfated Glycosaminoglycans (sGAG) quantification

The sGAG were extracted using the Blyscan Sulfated Glycosaminoglycan Assay (B1000, Biocolor). Samples were minced into small fragments (<1 mm) and incubated during 3 hours at 65ºC in 1ml of a Papain-based extraction solution. Digested tissues were centrifuged 10 minutes at 10.000xg and the supernatant was collected.

For quantification the supernatant was incubated with Blyscan Dye Reagent for 30 minutes with gentle agitation. This reagent contains 1,9-Dimethyl-Methylene Blue zinc chloride double salt, a cationic dye that binds to sulfated glycosaminoglycans. Samples were centrifuged for 10 minutes at 13500xg, the supernatant was decanted and the centrifuge tube was carefully dried, to avoid any loss of the deposit formed. The deposit was dissolved in the dissociation reagent for 10 minutes prior to sample measurement. The absorbance was measured at 656nm on a Biotek Plat Reader. sGAG were quantified based on a standard curve and expressed as µg/mg of wet tissue. All samples were run in triplicate.

Soluble Collagen quantification

Collagen was extracted using the Sircol Soluble Collagen Assay (S1000, Biocolor). Briefly, tissue digested with 0,5M Acetic Acid (457040, FLUKA) and 0,1 mg/mL pepsin (P7000-100G, Sigma Aldrich) overnight at 4ºC. Extracted collagen was concentrated overnight at 4ºC by addition of Acid Neutralizing Reagent and cold Isolation and Concentration Reagent. Samples were centrifuge at 13500xg for 10 minutes, the supernatant was discarded and the pellet of hydrated collagen was used for the quantification. The hydrated collagen was incubated with 1mL of Sircol Dye Reagent for 30 minutes under gentle agitation. Samples were subjected to a centrifugation at 13500xg for 10 minutes, the tubes were drained and the pellet was washed with ice-cold Acid-Salt Wash Reagent to remove unbound dye. Samples were again centrifuged and the hydrated collagen was drained and incubated for 5 minutes with Alkali Reagent to dissolve the dye. The absorbance was measured at 555nm on a Biotek Plat Reader, the collagen was quantified based on the standard curve and expressed as µg/mg of wet tissue. All samples were run in triplicate.

Assessment of apoptotic cells

To assess the extent of apoptosis on recellularized matrices, paraffin sections were treated following the standardized protocol of ApopTag (ApopTag Fluorescein *In Situ* Apoptosis Detection Kit, S7110, Chemicon), a commercial apoptosis-detection kit based in the terminal deoxynucleotidyl transferase dUTP nick en labeling (TUNEL) assay. Briefly, paraffin tissue sections were dewaxed and rehydrated with changes of xylene, followed by a sequential alcohol gradient and rinsing in deionized water. The sections were briefly washed in PBS and incubated for 15minutes in Proteinase K (20µg/ml). Following a PBS wash samples were incubated for 10 seconds in Equilibration Buffer (15µl/cm^2). The liquid in excess was carefully removed and a new incubation with Working Strenght TdT Enzyme (11µl/cm^2) was performed for 1hour in a humidified chamber.

After enzyme incubation, the Working Strength Stop/Wash Buffer was applied to the samples for 10 minutes at RT and a new wash with PBS was performed. Warmed working strength Anti-Digoxigenin Conjugate (13µl/cm^2) was added to the tissue sections and incubated in the dark. Sections were washed in PBS and mounted with Fluoroshield™ with DAPI, results were observed on an inverted fluorescence

microscope (Axiovert 200M, Carl Zeiss) and the images captured with a monochrome camera (AxioCam MNC, Carl Zeiss) and MosaiX software's (AxioVision modules, Carl Zeiss).

Statistical analysis

Results of matrix cell colonization, proliferation and apoptosis were quantified using the Fiji software.

All the results were obtained in five independent experiments, unless stated on the *Results* section, and shown as mean ± standard error of the mean. The statistical analysis of the data was performed using the IBM SPSS Statistics 20 software. $p<0,05$ was considered for statistical significance. Whenever normality was verified the t-Student test was applied and the Independent- Samples Mann-Whitney U test was applied for remaining analysis.

Results

1. Optimization of decellularization protocol for fetal (E18) hearts and adult heart fragments

Aiming at dissecting the cardiogenic potential of fetal (E18) and adult ECM we designed a strategy based on the decellularization of both tissues. Thus, in order to avoid the variation produced by different decellularization methods we engaged on the optimization of a common protocol that efficiently removed cellular components and provided for ECM integrity of both the fetal (E18) and the adult cardiac tissue. To ease this task, prior to decellularization, the adult left ventricle tissue was cut in fragments of a size similar to the E18 fetal hearts (~2 mm). Fragments were obtained in a standardized fashion using a tissue puncher with 2 mm of diameter.

The decellularization protocol was structured on three phased incubations in different solutions: (I) hypotonic buffer to induce cell lysis by osmotic shock and thus produce low impact on ECM composition and architecture; (II) detergent solution which is the 'decellularization agent' *per se* and solubilizes the cellular membranes and dissociates the DNA from proteins; and (III) a DNAse containing solution that hydrolyzes the DNA, promoting the clearance of the nucleic acids anchored to the ECM.

During experimental tests different detergents previously applied for cardiac decellularization, *i.e.* Triton X-100 and Saponin [108, 111, 113], were tested in combination with SDS, which is the gold-standard detergent for cardiac application [103, 105, 114, 116] (Figure 6). Following decellularization, samples were subjected to paraffin embedding, sectioning, and HE stain for qualitative assessment of the protocol efficiency (Figure 7). Of notice, in accordance with the *3Rs* guidelines [117] and to minimize the use of experimental animal, all variations to the starting protocol were tested using adult fragments and the most efficient conditions were further applied on fetal hearts. In regard to the adult cardiac fragments, the treatments using only SDS as decellularization agent resulted on a porous structure without remnants of cellular components and defined nuclei when compared to the non-manipulated control (Figure 7). In addition, the adult fragments subjected to higher SDS concentrations of 0,5% and

1% resulted on more friable acellular matrices that appeared less preserved in the HE stain

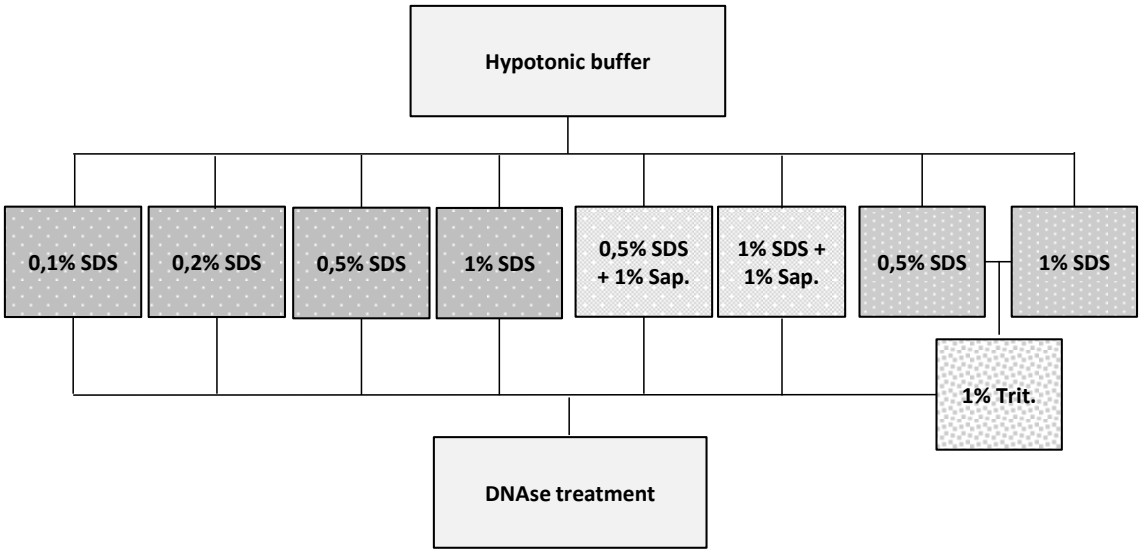

Figure 6. Schematic representation of the decellularization protocol optimization. Sap.:Saponin; Trit.:TritonX-100.

The treatments involving the combination of SDS with either Saponin or TritonX-100 resulted on samples with cytoplasm residues highlighted by an eosin based intense pink stain (Figure 7). Considering the results obtained for the adult heart fragments, only protocols using different SDS concentrations were further tested for the decellularization of the fetal (E18) hearts (Figure 7). Besides the protocol that used 0,1% SDS in which clear nuclei were observed, the other SDS concentrations tested reproduced the results obtained for the adult tissue. Thus, the treatment of 0,2% SDS was elected as the less invasive protocol that allowed for efficient removal of cell constituents from both fetal (E18) and adult cardiac tissue and was further used on the subsequent experiments.

On a second round of optimizations we focused on the removal of DNA that remained attached to the ECM. Although, the HE stain showed no defined nuclei on the acelular matrices, DAPI staining was also performed on those sections (Figure 8). DAPI staining showed that the nuclear membrane was disrupted and defined nuclei were not present although a diffuse staining could be observed in the background regardless of the SDS concentration used (Figure 8). This result indicates poor sheared-DNA clearance upon nuclear disruption and thus DNA quantification using the Pico Green assay was performed.

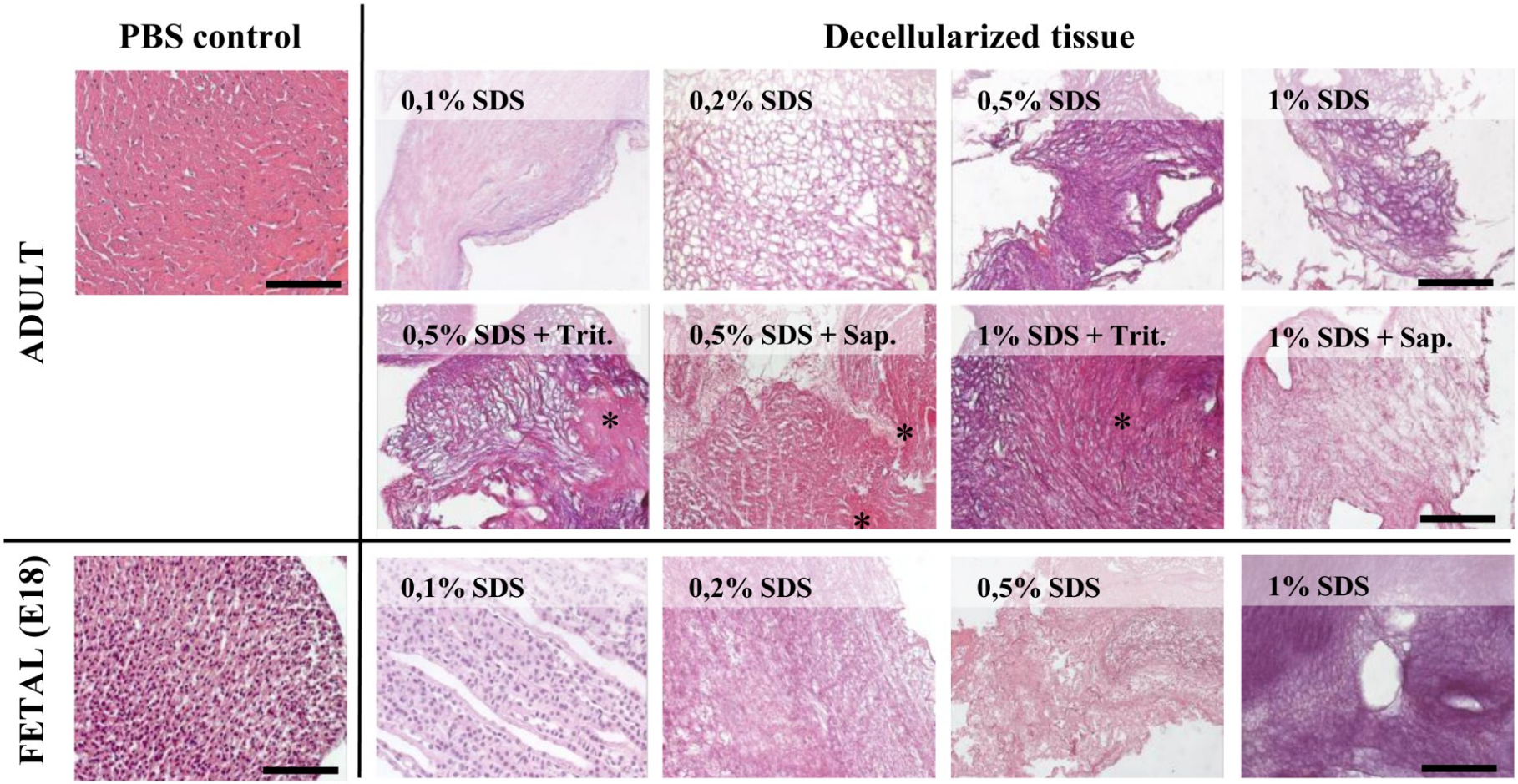

Figure 7. HE stain of representative sections of adult cardiac fragments and fetal (E18) hearts following decellularization using distinct detergent combinations and concentrations. Scale bars, 100µm. Sap.:Saponin; Trit.:Triton X-100. (*cytoplasmatic remnants)

DNA quantification clearly demonstrated that DNA persisted on the biological scaffolds after SDS treatment (data not shown). To improve the DNA clearance, an incubation with Hypotonic Wash Buffer was included in the protocol prior to the DNAse treatment to enhance the disruption of DNA-protein interactions and to reduce the remnants of SDS that may inhibit the enzymatic activity. In addition, the buffer containing DNAse was supplemented with magnesium chloride ($MgCl_2$), a DNAse co-factor that potentiates the DNAse hydrolytic activity. The decellularized protocol was repeated with the new improvements and the DNA was quantified. A 99,9%- and 99,8% fold reduction of the DNA was obtained when compared to the samples decellularized with the previous protocol and to the native tissue, respectively (data not shown). Thus, the new modifications on the protocol increased the efficiency of DNA removal. Once again protocol adjustments were tested preferentially in adult samples; further characterization of the matrices regarding nuclei acid contents is fully detailed on the section *2.Validation of the optimized decellularization protocol*.

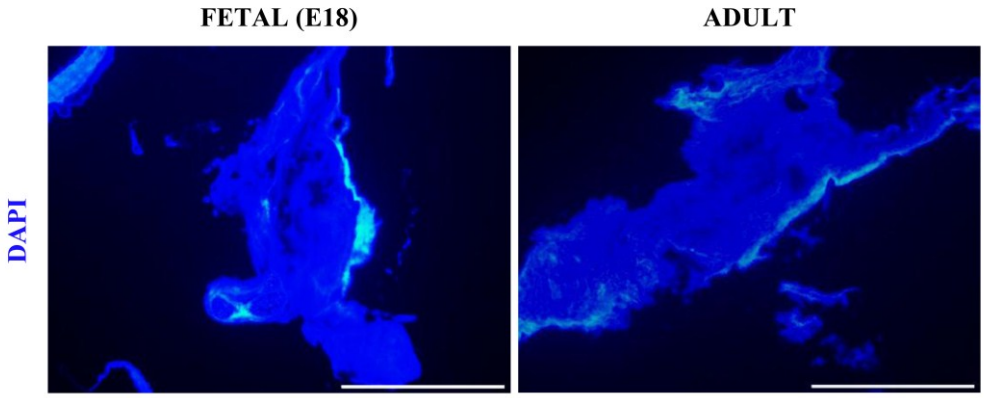

Figure 8. DAPI staining of fetal (E18) and adult cardiac fragments following decellularization. Scale bar, 100µm.

Although not included in this thesis, other small adjustments to the decellularization protocol were also performed, *i.e.* agitation, decellularization container, volume of solutions. The final decellularization protocol is fully detailed on the *Methods* section.

2. Validation of the optimized decellularization protocol

The adjustments on the initial decellularization protocol described in the previous section were further validated (*n=5*) for the decellularization of the fetal (E18) and adult heart biopsies.

Stereoscopic observation of the samples demonstrated that following the incubation with the detergent a translucent scaffold emerged when compared to the PBS control group. Of notice, the PBS control was subject to the same mechanical conditions but was emerged in PBS throughout the procedure, therefore escaping the detergent action. Maintenance of the anatomic structure was evident in the case of fetal hearts, where the preservation of atria, ventricles and vascular system architecture was confirmed.

Likewise, the adult-derived decellularized fragments conserved the shape and approximately the same size of the intact tissue (Figure 9). All results were shown to be reproducible across independent experiments.

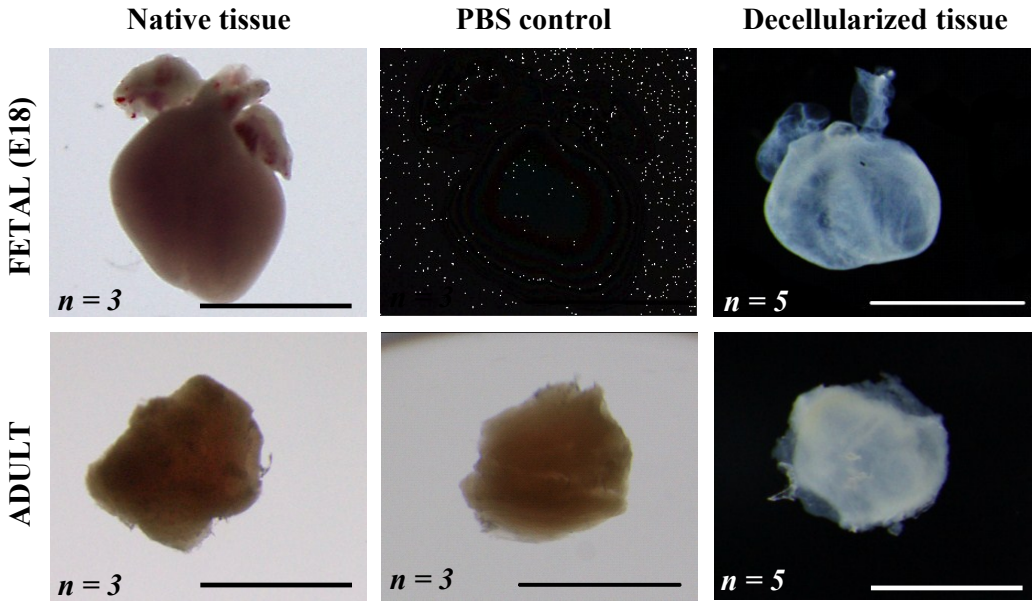

Figure 9. Macroscopic analysis of the decellularized fetal (E18) heart and adult heart fragments. Scale bars, 2mm.

To further validate the decellularization protocol fetal- and adult-derived ECM were paraffin-embedded, sectioned and HE stained to evaluate the bioscaffold architecture and the presence of cytoplasmic and nuclei remnants. Absence of nuclei and cytoplasmic vestiges was confirmed in all samples (Figure 10).

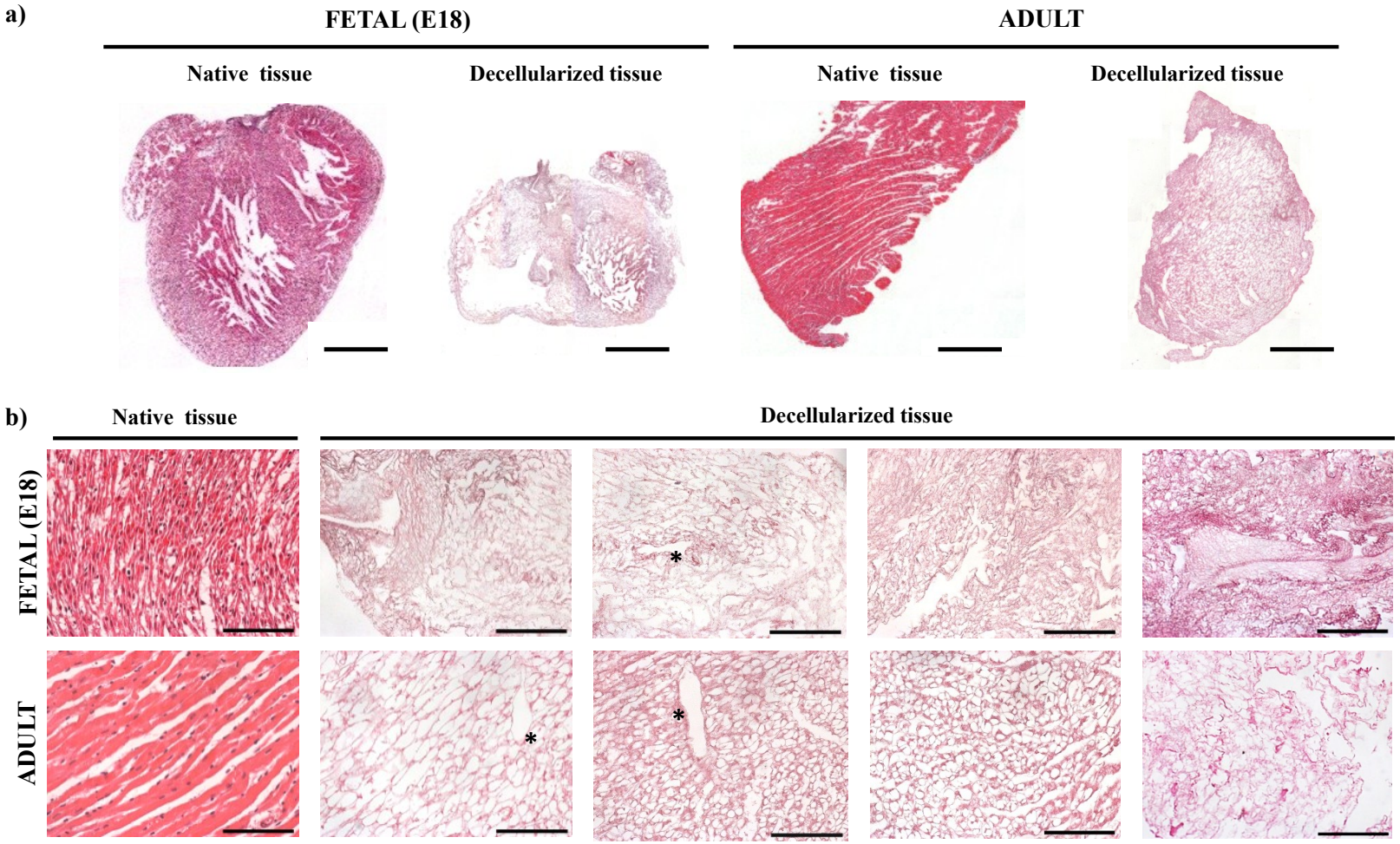

Figure 10. HE stain of representative sections of adult cardiac fragments and fetal (E18) hearts following decellularization. All cross sections (first row) were evaluated (a), with further assessment of porosity and cell removal (b). The matrices revealed to be efficiently decellularized, without cytoplasm remnants or defined nuclei. Scale bars, 500µm (a) and 100µm (b). (* vasculature conduits)

However it was observed that in 3 out of 5 analyzed adult-fragment samples the outer surface of the bioscaffold was labeled by an intense eosin stain typical of cytoplasmatic remnants (data not shown). The latter occupied a very small fragment area and thus appeared as irrelevant when considering the proportion to the fragment size. It is well known that the fetal and the adult cardiac tissue are histologically very different. The fetal heart is composed of rounded or oval cells with lower ratio cytoplasm/nuclei content and with high nuclear density. On other hand, the adult cardiac tissue, due to the maturation of cardiomyocytes is a compact tissue containing highly organized long rod shaped cardiomyocytic cells with more cytoplasm and lower nuclei density [118] (Figure 10). Overall, ECM-derived scaffolds presented a porous structure in accordance with the respective native tissue anatomy, *i.e.* the fetal ECM displayed a less organized porous structure with small porous when compared to the adult scaffolds. Moreover, the frequent presence of vascular conduits was verified in both fetal and adult acellular scaffolds demonstrating the preservation of the native vasculature following decellularization (Figure 10, *).

To further investigate the efficient clearance of abundant cytoplasmatic proteins on the cardiac tissue immunofluorescence was employed. Immunostaining against cTroponin T and vimentin allowed the discrimiantion between cardiomyocytes and fibroblasts/ endothelial cells, respectively (Figure 11). Overall, it was clearly demonstrated by the absence of labelled structures that the cellular remnants from such cells were well removed from the scaffolds. Assessment of nuclear integrity and nucleic acid removal were verified by DAPI stain and PicoGreen dsDNA quantification, respectively (Figure 12).

Absence of defined nuclei and of any nuclear material was clearly demonstrated in acellular matrices derived from both fetal (E18) and adult hearts. Moreover, a significant reduction of 99,5% of DNA content was shown in both conditions (Figure 12).

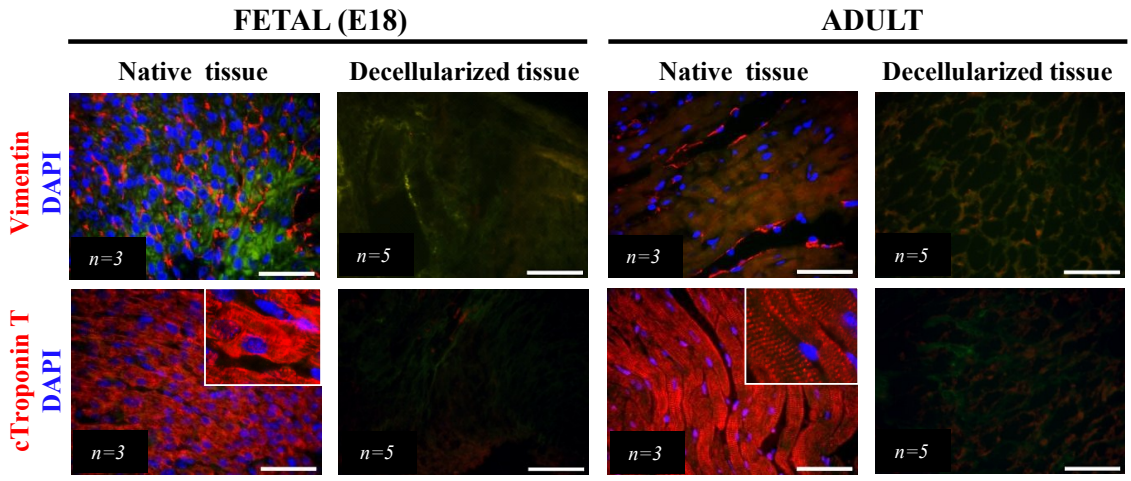

Figure 11. Representative immunostainings of Vimentin (upper panel) and cTroponin T (lower panel) in sections of adult cardiac fragments and fetal (E18) hearts before and after decellularization. Clear absence of fibroblasts and endothelial cells as well as cardiomyocytes was demonstrated by the lack of Vimentin and cTroponin T, respectively, in the decellularized scaffolds. Green: tissue autofluorescence. Scale bars, 50µm.

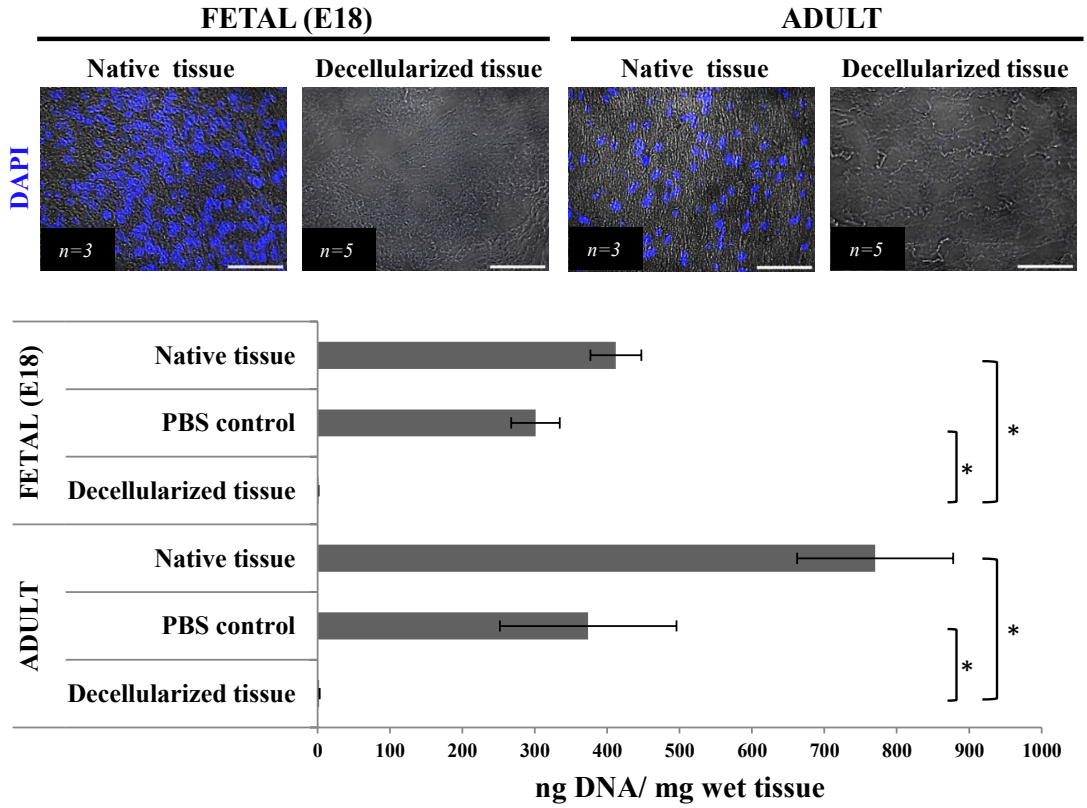

Figure 12. DNA assessment. DAPI staining images revealed the absence of defined nuclei or disperse DNA. PicoGreen DNA quantification confirmed significant reductions of 99,5% of DNA in the fetal and adult acellular ECM. Scale bars, 50µm. *$p<0,05$.

3. Characterization and preservation of decellularized cardiac ECM

Following the decellularization protocol validation based on the assessment of efficient cell removal, ECM preservation analyses were performed. Distinct histological approaches were used to access the presence, distribution and pattern of ECM molecules (*e.g.* collagen, laminin) and quantitative assays, to quantify sGAG and collagen content in the fetal- and adult- derived ECM as compared to the native tissues.

Components of the basal lamina namely, laminin, fibronectin and collagen IV, were first studied due to the critical role on modulating the cells behavior (*e.g.* adhesion, proliferation and differentiation). A strong and homogeneous presence of laminin, fibronectin and collagen IV within the thinned decellularized heart matrices was confirmed by immunofluorescence (Figure 13). No changes were verified between the decellularized and the respective native tissues, confirming the preservation of the basal lamina network after decellularization; as previously shown in the HE stain, a typical organization identified on the native cardiac tissues, was also be seen (Figure 10).

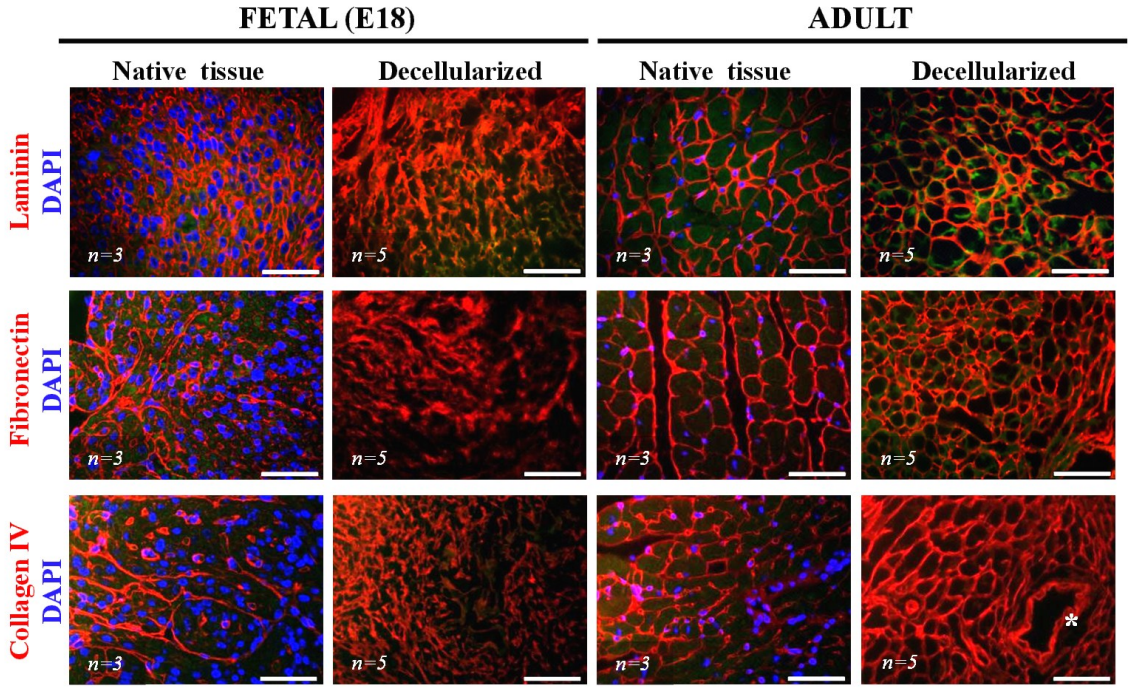

Figure 13. Basal lamina ECM molecules preservation after decellularization. The matrices were evaluated by immunofluorescence (IF) showing the presence and preservation of major ECM molecules of basal lamina, such as: laminin (first row), fibronectin (second row) and collagen IV (Third row). Green: tissue autofluorescence. Scale bars, 50 µm. (* vasculature conduits)

Aiming at evaluating the preservation of other ECM components, GAGs and collagen were extracted and quantified from acellular scaffolds and compared to the native tissue (Table 4).

GAGs are important players in cell modulation, allowing the anchorage of growth factors and cytokines to the ECM network. The content in sGAG were evaluated by colorimetric quantification methods, *i.e.* Blyscan Sulfated Glycosaminoglycan assay that allows quantification of sulfated PGs and GAGs, therefore not detecting the HA. Because the kit requires a minimal start weight (20-50mg) for efficient and reliable quantification, a pool of scaffolds was performed. Of notice, in the timeframe of this Master dissertation was not possible to generate enough acellular matrices to repeat the quantification using several pools and thus ensure statistical analysis.

Overall, a reduction of sGAG content was verified in fetal- and adult- derived acellular ECM when compared to the native tissue (Table 4). In fetal and adult decellularized samples the content of sGAG dropped 98% and 88% in relation to the native tissue, respectively. Although the native fetal tissue had a sGAG content (4,10µg/mg) four times higher when compared to the adult (0,62µg/mg), the final sGAG values in both type matrices were comparable (~0,10µg/mg). A decrease in the amount of sGAG was also observed in the PBS control.

Table 4. Sulfated glycosaminoglycans (sGAG) and collagens quantification by Blyscan and Sircol assay, respectively.

	FETAL(E18)			ADULT		
µg/mg wet weight	**Native**	**PBS control**	**Decellularized**	**Native**	**PBS control**	**Decellularized**
sGAG	4,10	2,22	0,14	0,62	0,60	0,10
Collagen	9,90	5,24	2,10	12,68	8,61	3,28

The collagens are the main constituents of cardiac ECM and are of key importance for the maintenance of the tissue architecture and mechanical properties. In the herein work, the collagen content was quantified by Sircol Soluble Collagen Assay and the fiber distribution and structural preservation were evaluated by Masson's Trichrome stain and TEM, respectively. As it was the case for the sGAG quantification, a pool of samples was required for collagen quantification. Adult native hearts showed higher collagen levels (12,68 µg/mg) in comparison with fetal hearts (9,90 µg/mg). Collagen

losses of 78% and 74% were obtained for the fetal and adult tissue following decellularization, respectively (Table 4). A reduction of collagen content was also observed in the PBS control when compared to the native tissue. The Trichrome Masson stain revealed to be consistent (data no shown); on the other hand, TEM images allowed to visualize long and preserved collagen fibers in both decellularized matrices (Figure 14).

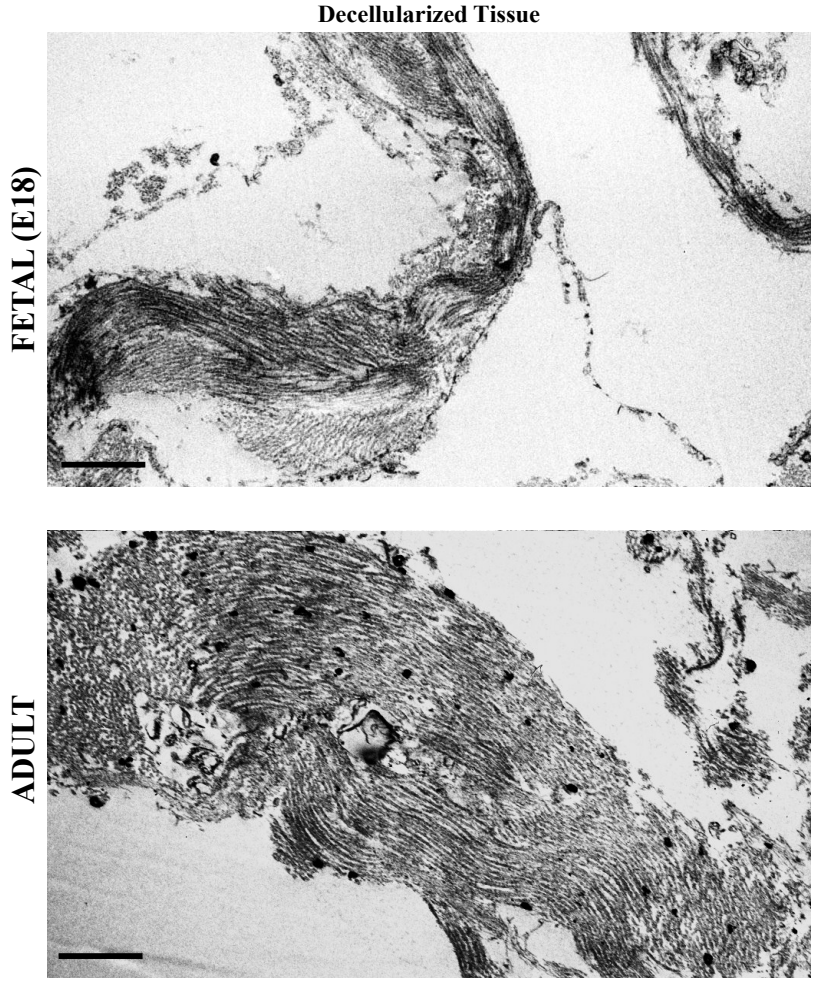

Figure 14. Representative images of collagen fibers of fetal (E18)- and adult-derived ECM obtained by TEM. Scale bars, 1µm.

Decellularized sample porosity and architecture were aspects that we aimed to characterize, hence, therefore, due to the high hydration degree of samples cryoSEM was selected as the method of choice. However, due to the small size of the samples it was technically over challenging the observation of samples using this technique. Therefore, in order to assess the porosity of the ECM-derived scaffolds, the samples were fixed and processed for TEM and semithin (1µm) sections were visualized using SEM (Figure 15). Acellular scaffolds displayed a porous structure with well-preserved cell lacunae, easily observed in the adult-derived ECM, characterized by

interconnecting open pores in contrast with the dense native tissue (Figure 15). The porous patterns presented by both types of decellularized matrices were distinct: fetal decellularized matrix displayed a thin and fibrillar-like network which was opposed to a more defined and homogeneous structure showed by the adult ECM (Figure 15).

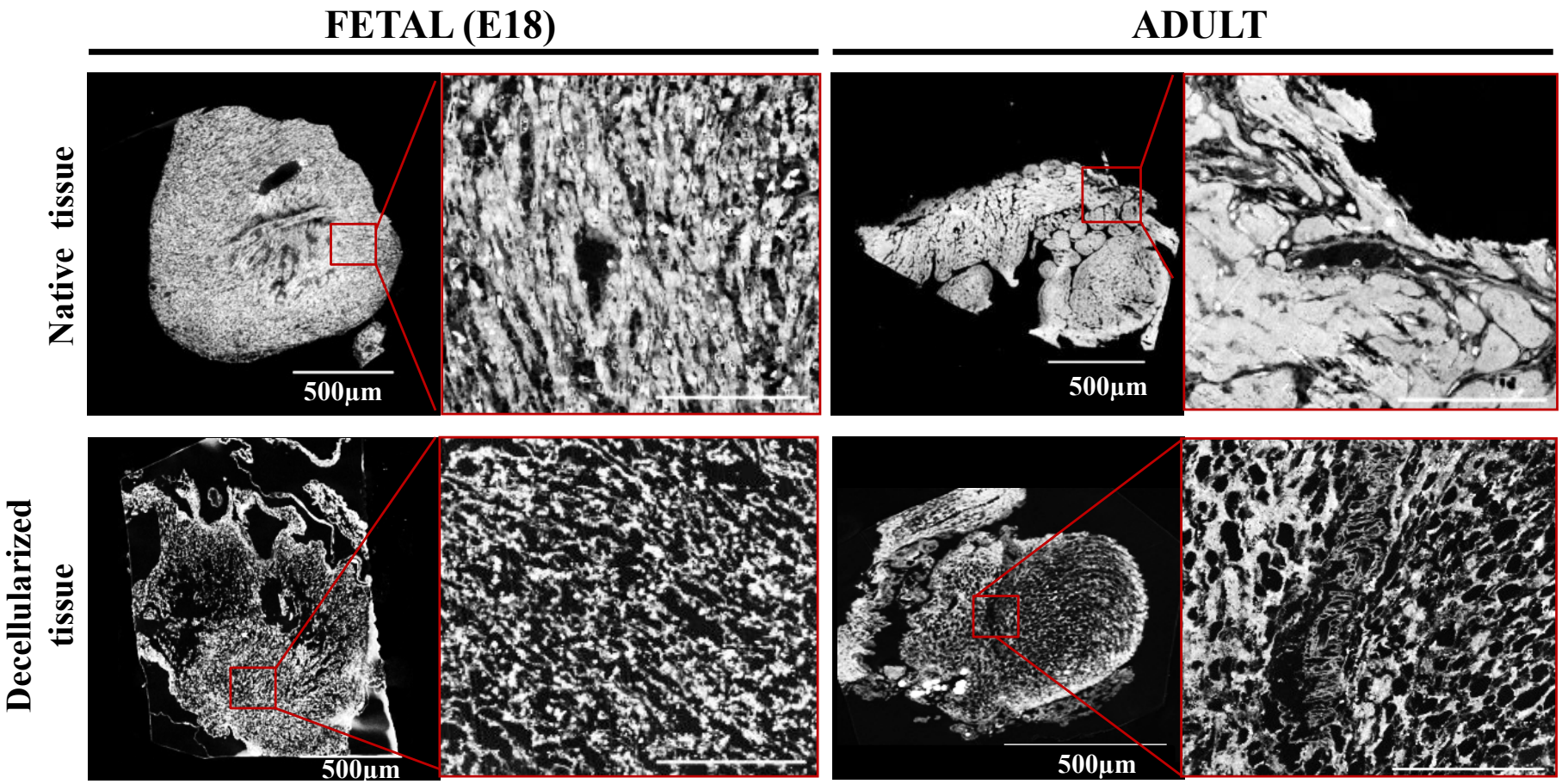

Figure 15. Porosity and architecture analysis of fetal- and adult –derived acellular ECM. Scale bars, 500µm (1st and 3rd column) and 100 nm (2nd and 4th column).

4. Biocompatibility and repopulation of acellular cardiac ECM

The biocompatibility of decellularized scaffolds was analyzed *in vitro* using iCPC^{Sca-1}, a cell line model for adult-derived cardiac progenitor cells (Freire AG, Nascimento DS, Forte G et al., manuscript). Cells were seeded on the matrices by passive seeding and analyzed after 8 and 15 days in culture to evaluate cell colonization (Figure 16), proliferation and apoptosis (Figure17).

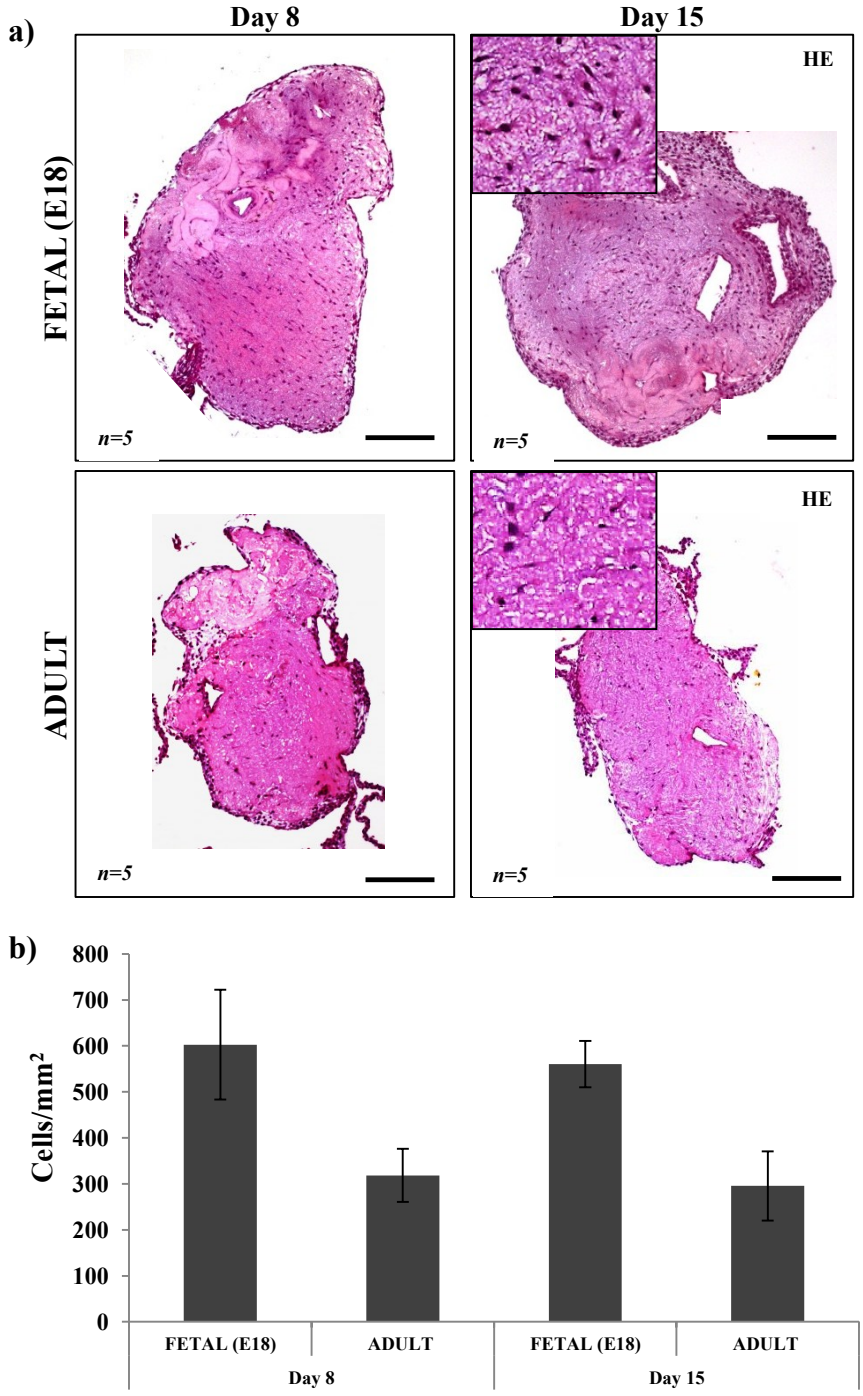

Figure 16. iCPC^{Sca-1} colonization of the ECM scaffolds derived from fetal hearts and adult heart fragments. Matrices were seeded with the cells at day 0 and colonization was accessed after 8 and 15 days on static culture conditions. The matrices were analyzed by HE stain (a) and the index of colonization calculated (b). Scale bars, 200 µm.

The indexes of colonization of matrices were calculated as a fraction of the number of nuclei labeled with DAPI inside the matrices per matrix area (mm^2). Of notice, previous preliminary results demonstrated that the cell line proliferated on the surface of the matrix and invaded the adjacent tissue culture plastic and formed a continuous monolayer. Although prior to histologic processing the recellularized matrix was dissected and the matrices' surrounding cells removed, the scaffolds size and its loose structure compromised the task. Therefore, in order to obtain quantifiable and reproducible results, only cells that were found embedded in the matrix were included in the analysis. HE stain of recellularized matrices showed that fetal matrices, when compared to the adult, displayed a higher number of cells in the interior of the matrix (Figure 16). The mean colonization index corroborated this observation since it was 47% higher in the fetal-derived ECM than that obtained for the adult at both time points. A 7% decrease in the colonization index was obtained from day 8 to day 15 in both fetal and adult decellularized matrices (Figure 16). The assessment of proliferating cells within the matrices was performed by immunostaining against Ki67, a typical proliferation marker. The number of proliferating cells was expressed in relation to the total number of cells that colonized the scaffold and presented as a percentage. Overall, the number of cells in division was low when considering the mean percentage of proliferating cells under the cell-line maintenance-conditions culture (~30%) at the same cell passage number (data not shown). In addition a statistically significant reduction of 88% and 87% was observed in the fetal and adult acellular scaffolds, respectively, from day 8 to day 15 in culture. A tendency to higher ratio of proliferating cells was found on the adult when compared to the fetal scaffold, however no statistical differences were verified. The presence of apoptotic cells embedded in the matrices was evaluated using the ApopTag Fluorescein *In Situ* Apoptosis Detection kit, a commercial kit that labels the nuclei of cells undergoing apoptosis. Overall a small percentage of apoptotic cells was found at day 8 (<2%) but an increase of approximately 86% was observed at day 15, although not enough to reach statistical significance. In addition, fetal-derived scaffolds demonstrated a propensity for higher percentage of apoptotic cells when compared to the adult (Figure 17). Of importance is that, due to restrains related to the timeframe of the present dissertation, only one histological section was used for assessing proliferation and cell death within the scaffolds. However, all samples were analyzed using selected sections of the same scaffold region. This work

has already been extended to include more sections in order to analyze samples representative of the entire scaffold and therefore reduce the standard deviation (work underway).

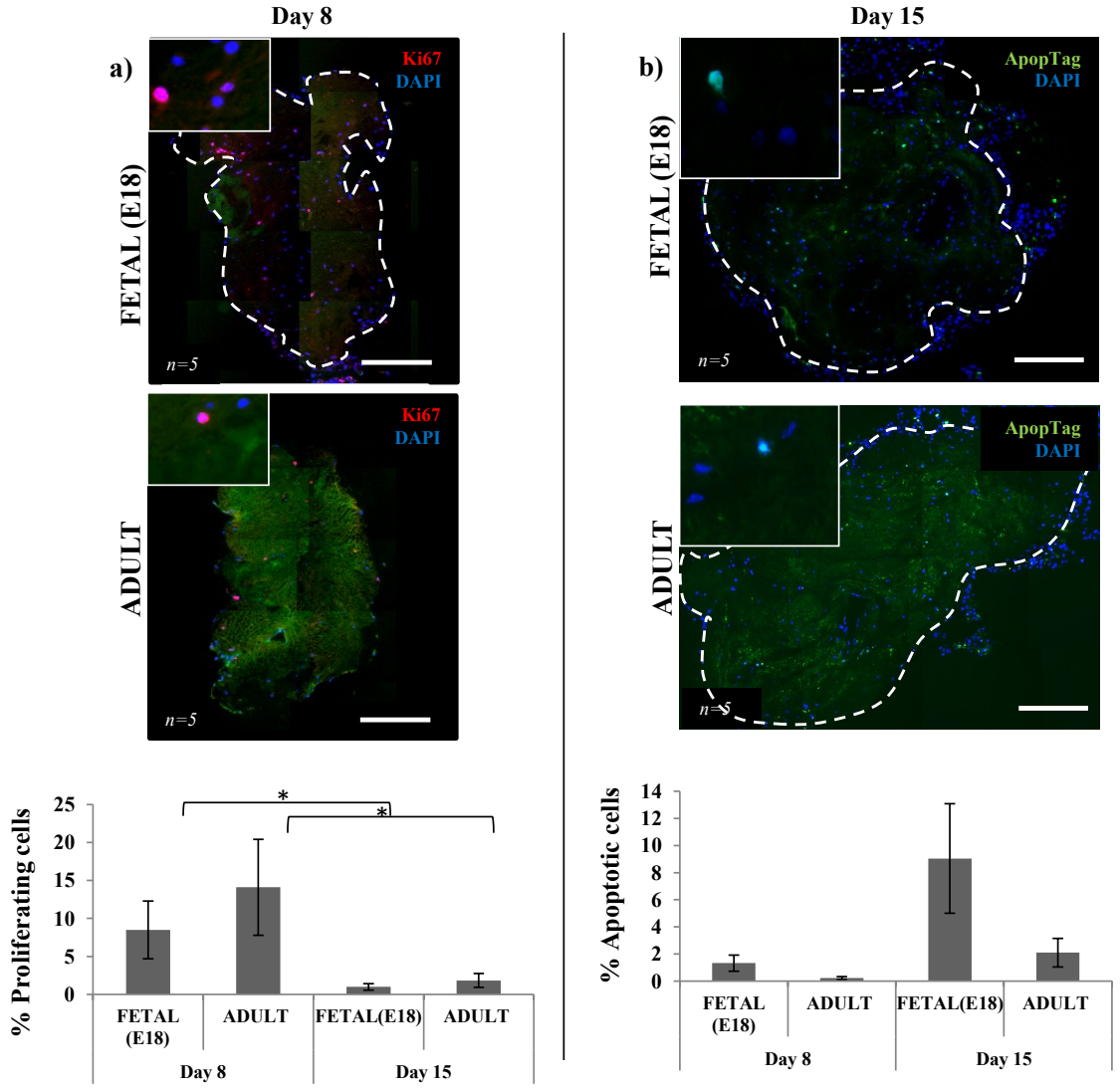

Figure 17. Assessment of proliferation and apoptosis of iCPC$^{Sca\text{-}1}$ embedded in the ECM-derived matrices at day 8 and day 15 of *in vitro* culture. ECM-derived scaffolds were analyzed by immunofluorescence to access the percentage of: (a) proliferating cells (Ki67 positive; red) and (b) apoptotic cell death (Apoptosis Detection kit; light green). Scale bars, 200 µm. (- - - matrix boundary).

Discussion

The use of decellularization to produce biological scaffolds, *i.e.* bioscaffolds, for tissue regeneration or organ replacement has been explored in a high range of tissues [73, 100, 102, 103, 119, 120]. The novelty of the herein study was to use decellularization as a tool for addressing the paradigm that the ECM itself contains critical information for driving cardiogenesis and cardiac tissue regeneration, phenomena that occur naturally only at earlier stages in the individuals' ontogeny. Thus, the decellularization was used to assess the ECM of fetal (E18) and adult heart with the purpose of dissecting its cardioinductive potential *in vitro* and *in vivo* Decellularized matrices will be continuously produced and further characterized in order to create *in vitro* model-systems for maintaining cells of the cardiac lineage, *i.e.* neonatal cardiomyocytes, cardiac progenitors cells isolated from the adult heart and cardiomyocyte precursors isolated from the developing embryoid body (embryonic stem cell derived). In parallel, ECM-based cardiac patches will be generated and their cardiogenic potential will be tested *in vivo* following their application on the hearts of mice with surgically induced MI. The work developed in the time frame of this *Master* dissertation was determinant for the implementation of the decellularization technique, the characterization of the acellular matrices and constitutes indeed the first report on the parallel decellularization of fetal and adult myocardial tissue. Hence, it constitutes the start-off of a new investigation line in our laboratory and a pioneer concept in the field.

The decelularization protocol that was herein used as starting baseline was developed at INEB (M. A. Barbosa and M. J. Oliveira) for histological sections of human intestine. Different conditions such as the detergent composition and concentration, the enzymatic treatments and the agitation conditions were tested on the pursuit for the least invasive and efficient protocol for fetal and adult cardiac tissue decellularization. The experimental sequence using 0,2% SDS during 24 hours, improved DNAse treatment and using 165 rpm of agitation throughout the procedure was shown to be the most reproducible and efficient protocol. Therefore an effective removal of the three main heart residing cells types, *i.e.* cardiomyocytes, fibroblasts and endothelial cells, was

confirmed by immunofluorescence. HE stain of the paraffin embedded bioscaffolds revealed the porous structure largely free of cytoplasmatic remnants and with preserved vascular conduits. The appearance of adult scaffolds is similar with other reports of cardiac tissue decellularization *i.e.* a network of lacunae that previously housed cardiac cells. However, in some adult-derived ECM was possible to identify occasional scaffold boundaries with cellular remnants, although irrelevant when considering the proportion to the fragment size. Indeed it has been proposed that it is extremely unlikely to remove 100% of the cellular material and thus methods which efficiently remove most or all of the visible cellular remnants are suitable for implantation [109].

Different methods for cardiac tissue decellularization have been described and the majority of reports propose the use of SDS at 1% for efficient cell removal [103, 108, 121]. Lower SDS concentrations have however been applied for the decellularization of non-muscular tissues, such as the lung [106] and liver [122]. A similar approach was used for the decellularization of porcine myocardium fragments (2mm), in which an agitation for several weeks in a solution of 0,1% SDS with 0,01% trypsin was required [114]. Our results demonstrate that by using 0,2% SDS we were able to reduce the SDS exposure to 24 hours and dispense the use of trypsin which can, in case of prolonged exposure, remove laminin, fibronectin, elastin and GAGs [110].

In addition, the absence of nuclei and nucleic acids was further demonstrated using different methodologies. Pico Green DNA quantification showed a reduction of ~99,7% in the content of DNA and thus decellularized matrices contained less than 2 ng/mg. The amount of DNA considered acceptable following decellularization is not well defined. However, a DNA concentration below 50 ng/mg and DNA fragments with less than 200 base pars length were previously suggested as requirements of a successful decellularization [109]. Indeed, our results outreached the latter value and hence we consider that the DNAse treatment herein applied resulted on efficient DNA hydrolysis and clearance from the acellular matrices.

Fetal and adult cardiac scaffolds were subjected to biochemical characterization by immunofluorescence to dissect the ECM components that resisted to the decellularization procedure. The elements that compose the basal lamina enable cells to sense the microenvironment and influence cellular behavior by the triggering of intracellular signaling pathways [46]. Fetal and adult ECM scaffolds showed the

maintenance of key basal lamina components, such as laminin, fibronectin and collagen IV. Immunolabelling produced a reticular pattern compatible with that of a tissue skeleton to which cells have been removed, similarly to what has been obtained in other decellularization studies [103]. Moreover, the labeling was homogeneous throughout the sample meaning that the basal lamina was not largely affected with the decellularization process.

The GAGs, important members of basal lamina and stromal matrix were also analyzed. The GAGs have critical functions in the tissue, *e.g.* modulate cell fate, act as elements that mediate the anchorage of cytokines and growth factor to the matrix and confer a high hydration of the tissue, affecting their properties.

GAGs are polysaccharide chains comprising repeating amino sugar and uronic acid disaccharide units. There are four classes of GAGs: the hyaluronic acid (HA), which is the most frequent GAG in the developing heart and the other three types of GAGs are chondroitin sulfate/dermatan sulfate, heparan sulfate/heparin and keratin sulfate that are found in the ECM as proteoglycans, *i.e.* associated with core proteins [47, 77, 89, 123]. The colorimetric Blyscan quantification assay that was used in the present work for the assessment of GAG content only quantifies sulfated GAGs and thus excluding HA. A reduction of sGAG content was verified in the PBS control but a more prominent decrease was observed in decellularized matrices. This absence of GAGs contrasts with frequent reports of GAGs maintenance following cardiac tissue decellularization [73, 102, 103]. The use of SDS as decellularization agent leads to GAGs removal in a time dependent manner, nevertheless the 0,2 % SDS for 24 hours is less aggressive than most treatments proposed for heart decellularization which report not to affect the content of GAGs [103, 108, 111]. However, other important aspects to consider in this equation are also the temperature, and the delivery method of the decellularization agent used, *i.e.* immersion and agitation vs. perfusion. We cannot also exclude that because no protease inhibitor was used throughout the procedure, and sGAG are secreted as proteoglycans, that proteolysis may have occurred thus reducing the amount of GAGs. The latter can explain the reduction of GAGs also observed in the PBS control. Yet, other studies have also reported a decrease on the content of GAGs following decellularization without compromising scaffold performance *in vitro* as well as *in vivo*, in an experimental injury setting [124]. Overall the methodology used for GAGs quantification is highly variable amongst laboratories and so distinct content in sGAGs

is frequently described for the same tissue [103, 108]. The presence of GAGs can alternatively be evaluated qualitatively using histological stains such as Alcian Blue or Safranin O; those techniques are now under implementation in our laboratory but no consistent results have yet been obtained.

The collagen superfamily comprises important molecules responsible for the support of tissue architecture and mechanical properties as well as molecules with a key role on the modulation of cell behavior [40, 43, 50, 54]. Multiple approaches can be used to evaluate the amount of collagen present in acellular scaffolds such as histological stains (Masson Trichrome stain or Movat stain), immunofluorescence, western blotting, Transmission electron microscopy (TEM) and also quantification methods as the Sircol assay. In the herein work we have assessed collagen by different methodologies. The collagen quantification obtained using the Sircol assay showed a decrease on the content of collagens. Nevertheless, only one pool of matrices was measured and therefore these results need to be further confirmed using experimental replicates. Conversely, immunofluorescence against collagen IV showed maintenance of this molecule upon decellularization. In addition, TEM clearly revealed structural preservation of collagen fibers in decellularized matrices of both fetal and adult cardiac tissue. Immunofluorescence analysis for collagen I and III, the most common collagen of the cardiac ECM, are now underway to clarify whether collagens are being cleared out with the process of decellularization.

It is well known that the ECM can influence cell behavior, *i.e*. cell migration [48-50, 54, 89], proliferation [48, 49, 125] and differentiation [40, 48, 49, 89], but can also promote tissue remodeling [48, 50, 54, 62]. Moreover, ECM geometry also regulates intracellular architecture and cell polarization [126]. To access the ECM architecture in the interior of the acellular matrices SEM was applied in 1µm sections previously fixed and processed as if they would be observed by TEM; this ensure optimal ultrastructure preservation. Overall, fetal- and adult-derived ECM matrices showed preservation of cardiomyocyte lacunae and homogeneity. This is, to the best of our knowledge, the first report of such technical modification which was simultaneously successful for the evaluation of the architecture and porosity of the entire cross section of samples.

Recent findings demonstrated that mammalian heart retains a transient regenerative capacity during fetal/neonatal life [36, 37] and that this capacity appeared to be associated with the time-specific high expression of particular ECM molecules, such as

fibronectin, collagen and HBEGF [39] and ECM organization [40, 48]. In regard to that, we hypothesized that fetal (E18) ECM, contrarily to the adult ECM, contains crucial key ECM elements for cardiac regeneration. To assess the native cardiogenic potential between fetal (E18) and adult cardiac ECM, the respective decellularized matrices produced were cultured *in vitro* with a cardiac progenitor/stem cell line.

Fetal- and adult-derived ECM were seeded by passive seeding with a cardiac progenitor/stem cell line and cultured during several days. Even though previous reports have shown that passive seeding provides low efficiency seeding of approximately 10%–25% [127], the decellularized cardiac matrices were easily colonized by cardiac progenitors. Fetal-derived ECM showed a tendency for higher colonization when compared to the adult bioscaffold at both 8 and 15 days in culture. Cells migrated into the acellular matrices and colonized the scaffold from the periphery to the center. Cell counting is presently extended to more sections in order to obtain representative sampling of the entire scaffold and thus reach statistical significance (work underway). Overall, and despite the apparent loss of sGAG after decellularization, fetal and to a less extent adult matrices appear to preserve essential key elements to (re)create a microenvironment favorable for cell migration and adhesion. These results corroborate our premise that there are differences in the ECM composition throughout development that may translate in different potential to house cells and modulate cell behavior. Hence, adult matrices demonstrated to represent a niche less favorable for the maintenance of cardiac progenitors when compared to the fetal scaffold. Whether these differences have an impact on cell differentiation and can be extended to other cardiac cell types will be further investigated. In order to dissect the cellular dynamics during the *in vitro* culture, proliferation and apoptotic death were evaluated and quantified. Overall it was shown that while proliferation decreased from 8 to 15 days in culture, apoptosis was increased. This event maybe explained by the static culture conditions that were used in the present work that do not provide enough oxygenation and nutrients to the interior of the bioscaffolds. Thus we are planning to develop a bioreactor to enhance culture conditions and improve diffusion of nutrients to the interior of the matrix.

Conclusions

Besides the advances on cardiovascular research along the years the regeneration of the damaged adult heart is still to be accomplished. With this work we intended to analyze the differences of cardiogenic potential between fetal (E18) and adult ECM by the generation of decellularized matrices. The ultimate goal was to dissect how different ontogenic-specific ECM components could improve adult heart regeneration after MI.

In the present work we developed a decellularization protocol to efficiently remove cytoplasmatic and nuclear contents from fetal and adult cardiac fragments with preserved structure and minimal loss of ECM contents. Homogeneous porous scaffolds preserving the cardiac cells lacunae were produced using 0,2% SDS as the main decellularization agent. Important ECM elements of basal lamina, such as laminin, fibronectin and type IV collagen were preserved. Preliminary results on sGAG quantification show that decellularized matrices were largely depleted of this ECM component. Inconsistent results were obtained regarding the collagen(s) content and thus further confirmation of these data is now underway Biocompatibility studies demonstrated that fetal (E18)-derived ECM induced more cell migration and adhesion when compared to the adult counterpart. Cells were observed throughout the entire matrix with homogeneous distribution. However, proliferation and apoptosis assessment demonstrated that static culture conditions were not providing enough oxygenation and nutrient diffusion to the interior of the matrices. This barrier will be surpassed by the use of an appropriate bioreactor (underway project).

Worthy of remark, this is the first report for the parallel decellularization of fetal and adult myocardial tissue. Preliminary characterization demonstrated that decellularized matrices preserved the ECM structure and composition. *In vitro* culture of decellularized scaffolds with a cardiac progenitor cell line demonstrated the biocompatibility of the acellular scaffolds. The characterization of recellularized matrices biochemical composition and cell phenotype is underway.

The herein work constitutes the foundation for the generation of (i) *in vitro* model-systems for dissecting molecular mechanisms of cardiac-cells expansion and differentiation and of (ii) ECM-derived cardiac patches for therapeutic application.

Future Work

In order to share the data compiled in this thesis with the scientific community a manuscript will be submitted to an international peer reviewed journal. To that end the issues to be addressed next are as follow:

- Make small adjustments to the decellularization protocol such as adding a cocktail of protease inhibitors throughout the procedure in order to improve the preservation of sGAG on the decellularized matrices;
- Optimize the extraction of collagen from the native and decellularized tissue and use immunofluorescence to identify the mainly collagen types present in the cardiac ECM, *i.e.* collagen I and collagen III;
- Evaluate the viscoelastic properties of the acellular scaffolds by dynamic mechanical analysis (DMA);
- Test other techniques for improving the cell seeding, as *vacuum* or centrifugation approaches [127, 128];
- Continue the analysis of cell seeding into the decellularized matrices;

In parallel, other key aspects for the accomplishment of the proposed goal will be also addressed:

- Development of a bioreactor to enhance cell culture conditions onto 3-D acellular scaffolds;
- Evaluate the behavior of other cell types following seeding in the fetal and adult decellularized matrices, such as ESC and neonatal cardiomyocytes;
- Test the lyophilized decellularized matrices as 2-D culture-systems.

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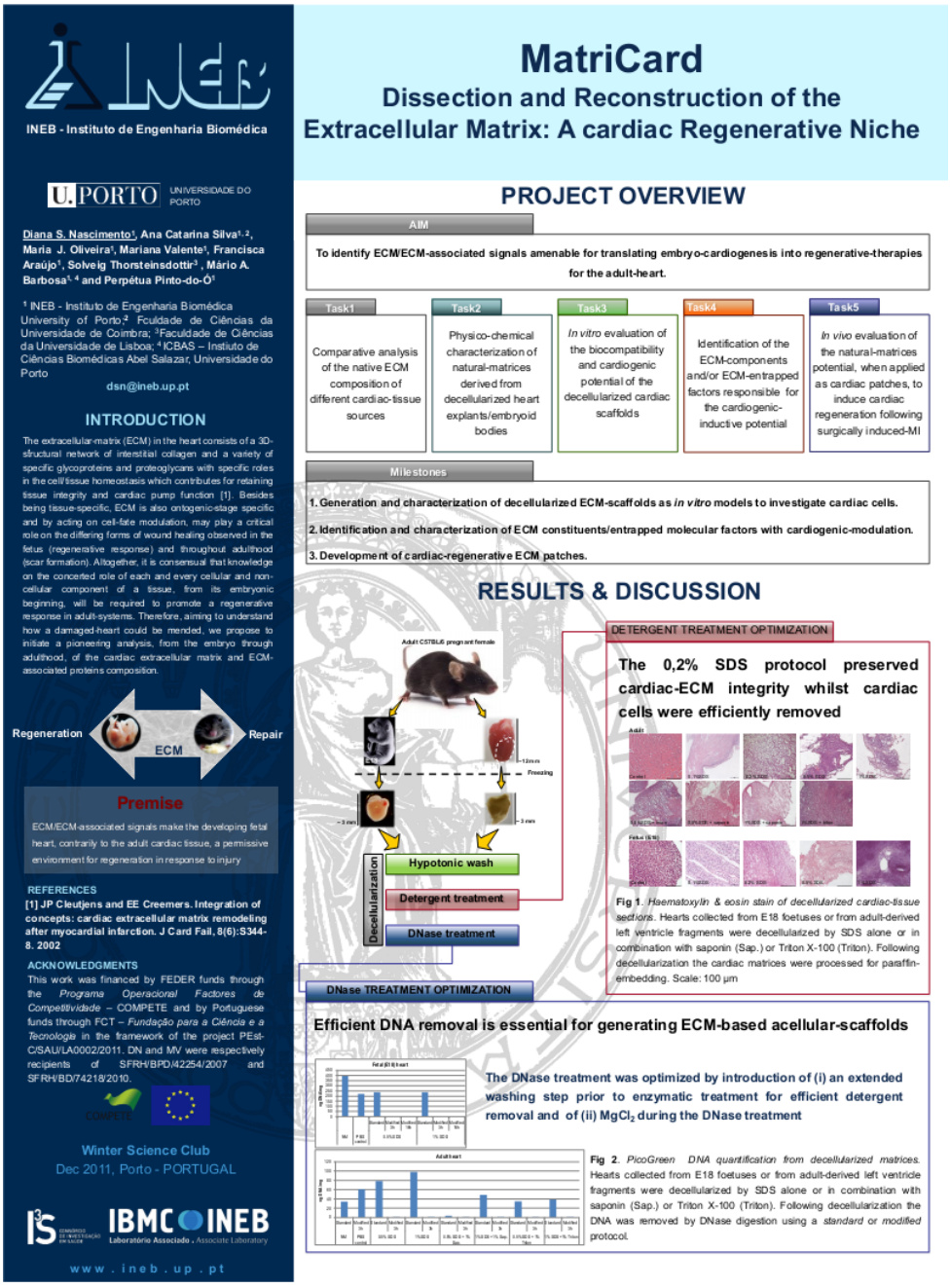
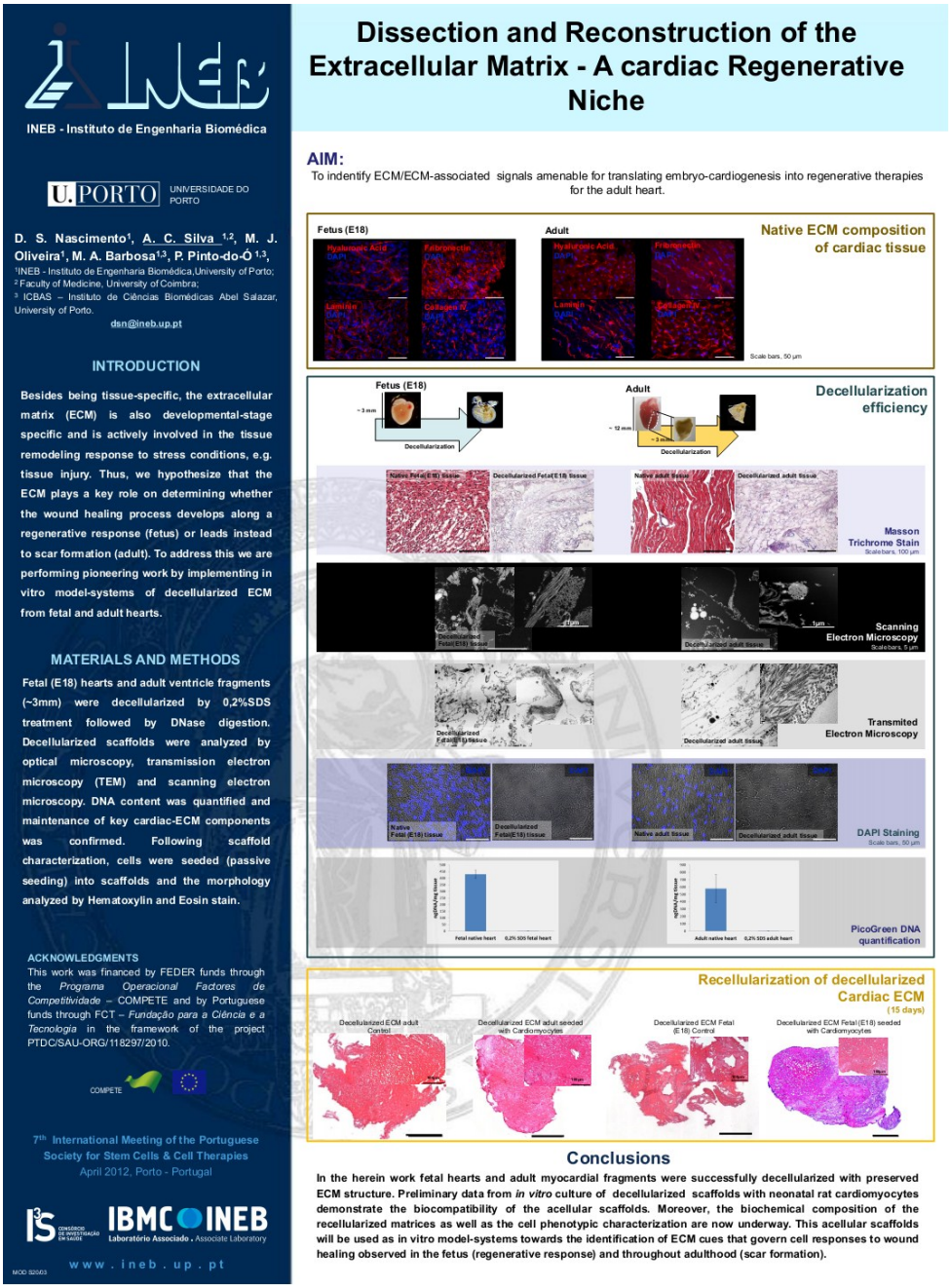
Attachments

1. Poster presentations

Winter Science Club, December 2011



7th International Meeting of the Portuguese Society for Stem Cells & Cell Therapies, April 2012



Dissection and Reconstruction of the Extracellular Matrix - A cardiac Regenerative Niche

INEB - Instituto de Engenharia Biomédica

U.PORTO UNIVERSIDADE DO PORTO

D. S. Nascimento[1], A. C. Silva [1,2], M. J. Oliveira[1], M. A. Barbosa[1,3], P. Pinto-do-Ó [1,3],
[1]INEB - Instituto de Engenharia Biomédica,University of Porto;
[2] Faculty of Medicine, University of Coimbra;
[3] ICBAS – Instituto de Ciências Biomédicas Abel Salazar, University of Porto.
dsn@ineb.up.pt

INTRODUCTION

Besides being tissue-specific, the extracellular matrix (ECM) is also developmental-stage specific and is actively involved in the tissue remodeling response to stress conditions, e.g. tissue injury. Thus, we hypothesize that the ECM plays a key role on determining whether the wound healing process develops along a regenerative response (fetus) or leads instead to scar formation (adult). To address this we are performing pioneering work by implementing in vitro model-systems of decellularized ECM from fetal and adult hearts.

MATERIALS AND METHODS

Fetal (E18) hearts and adult ventricle fragments (~3mm) were decellularized by 0,2%SDS treatment followed by DNase digestion. Decellularized scaffolds were analyzed by optical microscopy, transmission electron microscopy (TEM) and scanning electron microscopy. DNA content was quantified and maintenance of key cardiac-ECM components was confirmed. Following scaffold characterization, cells were seeded (passive seeding) into scaffolds and the morphology analyzed by Hematoxylin and Eosin stain.

ACKNOWLEDGMENTS
This work was financed by FEDER funds through the *Programa Operacional Factores de Competitividade* – COMPETE and by Portuguese funds through FCT – *Fundação para a Ciência e a Tecnologia* in the framework of the project PTDC/SAU-ORG/118297/2010.

COMPETE

7th International Meeting of the Portuguese Society for Stem Cells & Cell Therapies
April 2012, Porto - Portugal

IBMC INEB Laboratório Associado . Associate Laboratory

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AIM:

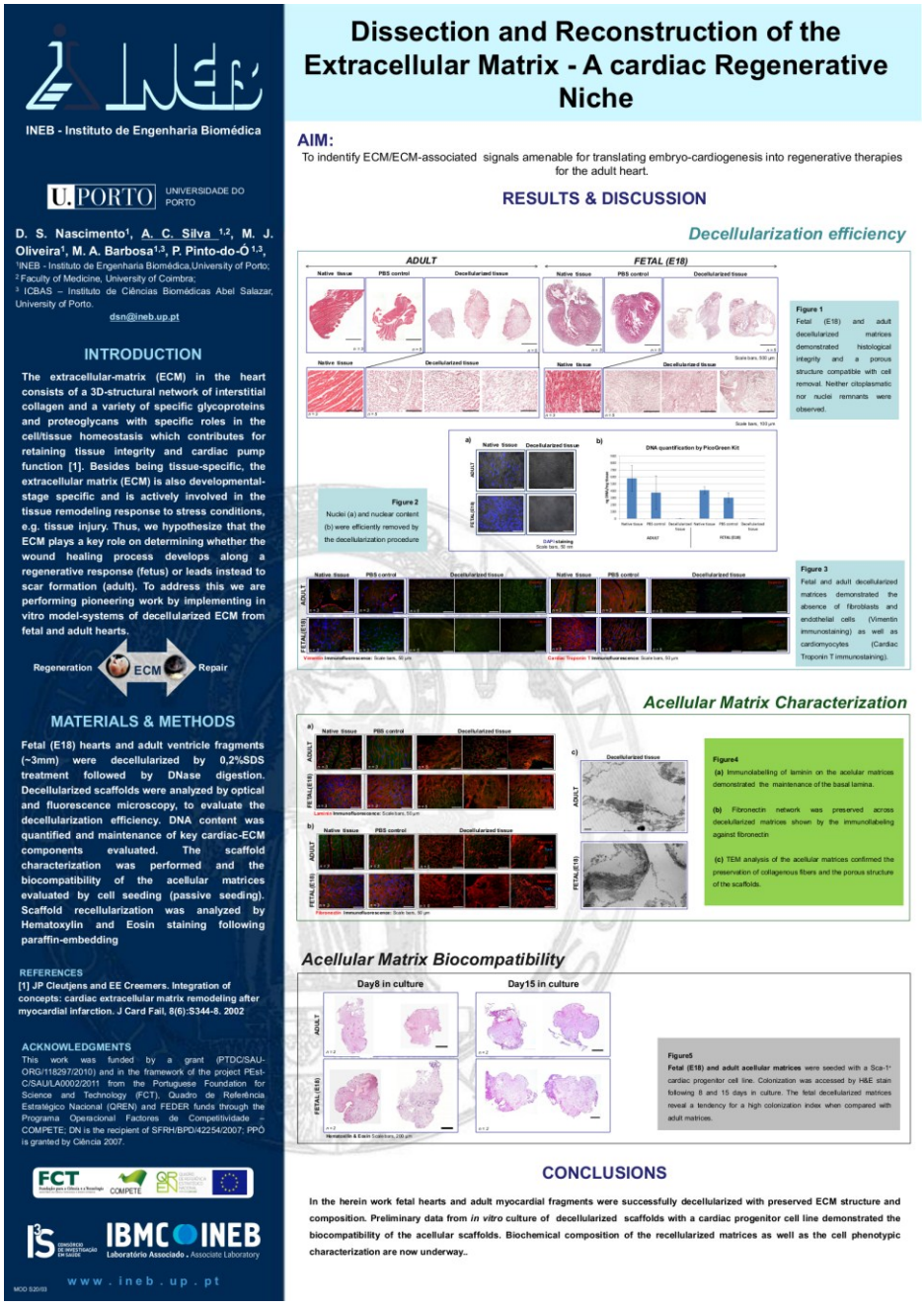
To indentify ECM/ECM-associated signals amenable for translating embryo-cardiogenesis into regenerative therapies for the adult heart.

Conclusions

In the herein work fetal hearts and adult myocardial fragments were successfully decellularized with preserved ECM structure. Preliminary data from *in vitro* culture of decellularized scaffolds with neonatal rat cardiomyocytes demonstrate the biocompatibility of the acellular scaffolds. Moreover, the biochemical composition of the recellularized matrices as well as the cell phenotypic characterization are now underway. This acellular scaffolds will be used as in vitro model-systems towards the identification of ECM cues that govern cell responses to wound healing observed in the fetus (regenerative response) and throughout adulthood (scar formation).

3rd I3S Scientific Retreat, May 2012

2nd Interrogations at the Biointerface Advanced Summer School, June 2012



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INTRODUCTION

The extracellular-matrix (ECM) in the heart consists of a 3D-structural network of interstitial collagen and a variety of specific glycoproteins and proteoglycans with specific roles in the cell/tissue homeostasis which contributes for retaining tissue integrity and cardiac pump function [1]. Besides being tissue-specific, the extracellular matrix (ECM) is also developmental-stage specific and is actively involved in the tissue remodeling response to stress conditions, e.g. tissue injury. Thus, we hypothesize that the ECM plays a key role on determining whether the wound healing process develops along a regenerative response (fetus) or leads instead to scar formation (adult). To address this we are performing pioneering work by implementing in vitro model-systems of decellularized ECM from fetal and adult hearts.

Regeneration ECM Repair

MATERIALS & METHODS

Fetal (E18) hearts and adult ventricle fragments (~3mm) were decellularized by 0,2%SDS treatment followed by DNase digestion. Decellularized scaffolds were analyzed by optical and fluorescence microscopy, to evaluate the decellularization efficiency. DNA content was quantified and maintenance of key cardiac-ECM components evaluated. The scaffold characterization was performed and the biocompatibility of the acellular matrices evaluated by cell seeding (passive seeding). Scaffold recellularization was analyzed by Hematoxylin and Eosin staining following paraffin-embedding

REFERENCES

[1] JP Cleutjens and EE Creemers. Integration of concepts: cardiac extracellular matrix remodeling after myocardial infarction. J Card Fail, 8(6):S344-8. 2002

ACKNOWLEDGMENTS

This work was funded by a grant (PTDC/SAU-ORG/118297/2010) and in the framework of the project PEst-C/SAU/LA0002/2011 from the Portuguese Foundation for Science and Technology (FCT), Quadro de Referência Estratégico Nacional (QREN) and FEDER funds through the Programa Operacional Factores de Competitividade – COMPETE; DN is the recipient of SFRH/BPD/42254/2007; PPO is granted by Ciência 2007.

IBMC INEB Laboratório Associado . Associate Laboratory

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AIM:

To indentify ECM/ECM-associated signals amenable for translating embryo-cardiogenesis into regenerative therapies for the adult heart.

RESULTS & DISCUSSION

Decellularization efficiency

Figure 1
Fetal (E18) and adult decellularized matrices demonstrated histological integrity and a porous structure compatible with cell removal. Neither citoplasmatic nor nuclei remnants were observed.

Figure 2
Nuclei (a) and nuclear content (b) were efficiently removed by the decellularization procedure

Figure 3
Fetal and adult decellularized matrices demonstrated the absence of fibroblasts and endothelial cells (Vimentin immunostaining) as well as cardiomyocytes (Cardiac Troponin T immunostaining).

Acellular Matrix Characterization

Figure4
(a) Immunolabelling of laminin on the acellular matrices demonstrated the maintenance of the basal lamina.

(b) Fibronectin network was preserved across decellularized matrices shown by the immunolabeling against fibronectin

(c) TEM analysis of the acellular matrices confirmed the preservation of collagenous fibers and the porous structure of the scaffolds.

Acellular Matrix Biocompatibility

Figure5
Fetal (E18) and adult acellular matrices were seeded with a Sca-1+ cardiac progenitor cell line. Colonization was accessed by H&E stain following 8 and 15 days in culture. The fetal decellularized matrices reveal a tendency for a high colonization index when compared with adult matrices.

CONCLUSIONS

In the herein work fetal hearts and adult myocardial fragments were successfully decellularized with preserved ECM structure and composition. Preliminary data from *in vitro* culture of decellularized scaffolds with a cardiac progenitor cell line demonstrated the biocompatibility of the acellular scaffolds. Biochemical composition of the recellularized matrices as well as the cell phenotypic characterization are now underway..

3rd TERMIS World Congress, September 2012

Dissection and Reconstruction of the Extracellular Matrix - A cardiac Regenerative Niche

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[3] ICBAS – Instituto de Ciências Biomédicas Abel Salazar, University of Porto.

perpetua@ineb.up.pt

INTRODUCTION

In the normal heart the extracellular-matrix (ECM) consists of a 3D structural network of interstitial collagen, which helps retaining tissue integrity and cardiac pump function, and a variety of specific glycoproteins and proteoglycans with specific roles in cell and tissue homeostasis [1]. Besides being tissue-specific, the extracellular matrix (ECM) is also developmental-stage specific and is actively involved in the tissue remodeling response to stress conditions, e.g. tissue injury. Thus, we hypothesize that the ECM plays a key role on determining whether the wound healing process develops along a regenerative response (fetus/neonate) or leads instead to scar formation (adult). To address this we are performing pioneering work by implementing *in vitro* model-systems of decellularized ECM from fetal and adult hearts.

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3rd TERMIS World Congress 2012
"Tissue Engineering and Regenerative Medicine"
September 2012, Vienna - Austria

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RESULTS & DISCUSSION

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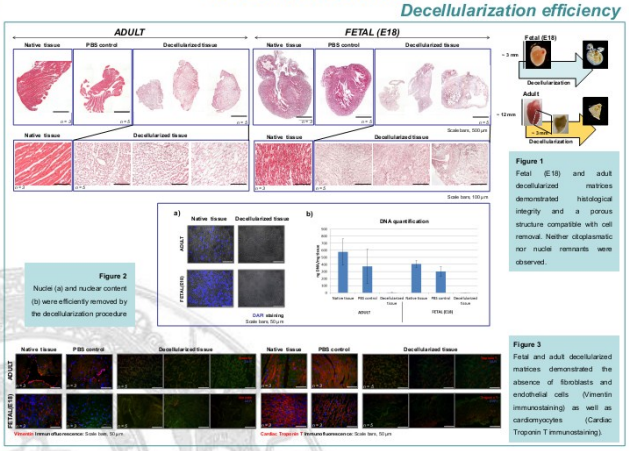

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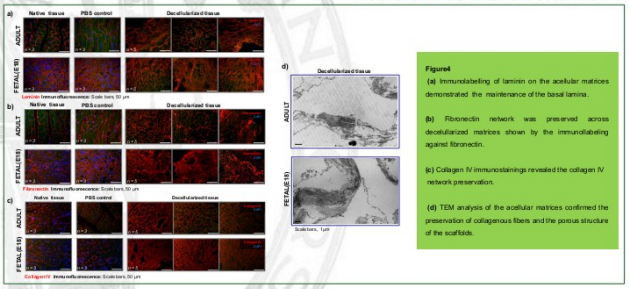

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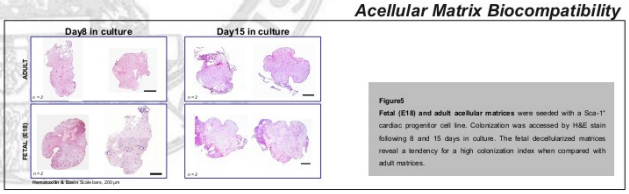
(a) Immunolabeling of laminin on the acellular matrices demonstrated the maintenance of the basal lamina.
(b) Fibronectin network was preserved across decellularized matrices shown by the immunolabeling against fibronectin.
(c) Collagen IV immunostainings revealed the collagen IV network preservation.
(d) TEM analysis of the acellular matrices confirmed the preservation of collagenous fibers and the porous structure of the scaffolds.

Acellular Matrix Biocompatibility



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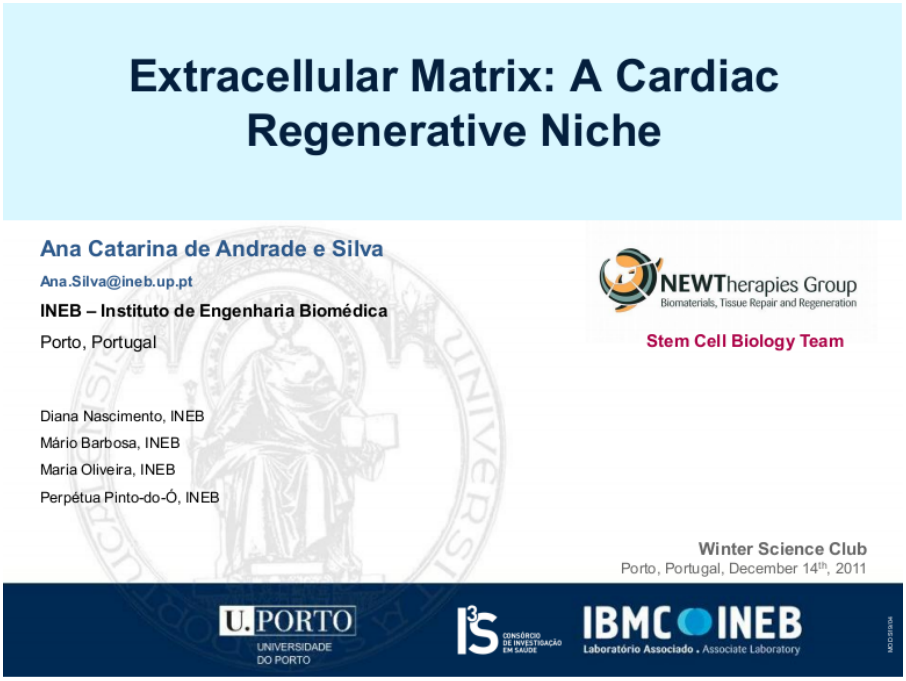
CONCLUSIONS AND FUTURE PERSPECTIVES

This is the first report on parallel decellularization of fetal and adult myocardial tissue. Preliminary characterization demonstrated that decellularized matrices preserved the ECM structure and composition. *In vitro* culture of decellularized scaffolds with a cardiac progenitor cell line demonstrated the biocompatibility of the acellular scaffolds. Characterization of recellularized matrices biochemical composition and cell phenotype is underway.

The herein work constitutes foundation for the generation of (i) *in vitro* model-systems for dissecting molecular mechanisms of cardiac-cells expansion and differentiation and of (ii) ECM-derived cardiac patches for therapeutic application. The role of ECM intrinsic and anchored clues for heart regeneration will also be investigated in comparative studies along ontogenesis and across species.

2. Oral Presentations

Winter Science Club, December 2011



Science Club, June 2012