Guide to the heart: Differentiation of human pluripotent stem cells towards multiple cardiac subtypes

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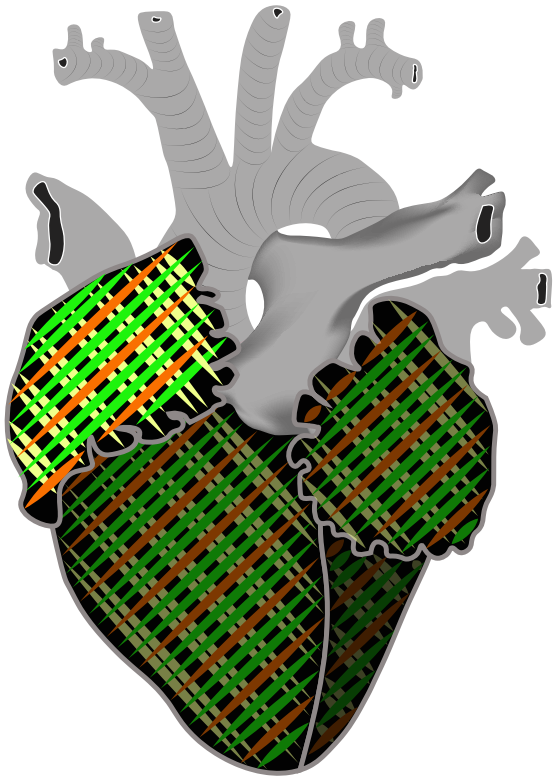

Chapter 7:

Native cardiac environment and its impact on engineering cardiac tissue

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Abstract

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) generally have an immature fetal-like phenotype when directly compared to isolated CMs from human hearts, despite significant advance in differentiation of human pluripotent stem cells (hPSCs) to multiple cardiac lineages. Therefore, hPSC-CMs may not accurately mimic all facets of healthy and diseased human adult CMs. During embryonic development, the cardiac extracellular matrix (ECM) experiences a gradual assembly of matrix proteins that transits along the maturation of CMs. Mimicking these dynamic stages may contribute to hPSC-CMs maturation *in vitro*. Thus, in this review, we describe the progressive build-up of the cardiac ECM during embryonic development, the ECM of the adult human heart and the application of natural and synthetic biomaterials for cardiac tissue engineering with hPSC-CMs.

Introduction

Since the first isolation of human pluripotent stem cells (hPSCs), their remarkable capacity to self-replicate and differentiate into every cell type of the human body aroused great interest and has advanced the fields of human disease modeling and pre-clinical pharmacology. Particularly because of the limited regenerative capacity of the human heart, as well as the difficulty to propagate primary cardiomyocytes (CMs) *in vitro*, hPSC-derived CMs (hPSC-CMs) are increasingly acknowledged as an alternative and accessible cell source for disease modeling, as well as drug and safety pharmacology or regenerative cardiac repair (Braam et al., 2010; Caspi et al., 2009; Chong et al., 2014; Doherty et al., 2015; Himmel, 2013; van Laake et al., 2007; Laflamme et al., 2007; Liang et al., 2010, 2013; Navarrete et al., 2013; Sharma et al., 2014; Shiba et al., 2016; Shinozawa et al., 2012; Stancescu et al., 2015; Talbert et al., 2015). Protocols to generate distinct cellular subtypes of the human heart, which include ventricular (Burridge et al., 2014; Mummery et al., 2012), atrial (Devalla et al., 2015; Laksman et al., 2017; Pei et al., 2017; Schwach et al., 2017) and pacemaker CMs (Birket et al., 2015; Protze et al., 2016), as well as epicardial cells and epicardial-derived smooth muscle cells and fibroblasts (Guadix et al., 2017; Iyer et al., 2015; Witty et al., 2014) and endothelial cells (Giacomelli et al., 2017; Orlova et al., 2014) have been developed and are continuously optimized. Despite these significant advances in directing differentiation to multiple cardiac lineages, hPSC-CMs are generally characterized by an immature fetal-like phenotype and may therefore not accurately mimic all facets of healthy and diseased human adult CMs. In a direct comparison to human hearts, gene expression patterns and morphological and functional characteristics of hPSC-CMs resemble that of fetal hearts (Table 7.1) (van den Berg et al., 2015; Braam et al., 2010; Nunes et al., 2013; Ribeiro et al., 2015). For a detailed overview about the immaturity of hPSC-CMs, we refer to other comprehensive reviews on this topic (Denning et al., 2016; Robertson et al., 2013; Veerman et al., 2015; Yang et al., 2014).

Table 7.1: Properties of fetal and adult CMs (Adapted from (Denning et al., 2016; Yang et al., 2014).

		Immature fetal CMs	**Mature adult CMs**
Morphology	Cell morphology	Circular	Rod-shaped
	Sarcomere morphology	Disorganized	Highly organized
	Sarcomere length	~ 1.6 µm	~ 2.2 µm
	T-tubules	-	Yes
	Nucleation	Mostly mono-nucleated	25 – 30% bi – or polynucleated
Mechanical parameters	Contractile force	~ nN for single cells	~ µN for single cells
0.08 - 4 mN/mm2 3D tissues	~ µN for single cells	0.08 - 4 mN/mm2 3D tissues	
40 - 80 mN/mm2 3D tissues			
	Conduction velocity	~ 0.1 - 0.2 m/s	0.3 - 1 m/s
Expression	Isoform expression	MYH6 (α-MHC) > MYH7 (β-MHC)	
MYL2 (MLC2v) : MYL7 (MLC2a)			
TNNI (fetal ssTnI) > TNNI3 (cTnI)	MYH7 (β-MHC) > MYH6 (α-MHC)		
MYL2 (MLC2v) > MYL7 (MLC2a)			
TNNI3 (cTnI) > TNNI (fetal ssTnI)			
	Gap junctions	Circumferential	Polarized to intercalated discs
Electrophysiological parameters	Upstroke velocity	10 - 50 V/s	150 - 350 V/s
	Resting membrane potential	-20 - 60 mV	-80 - 90 mV

Various strategies have been applied to enhance maturity of hPSC-CMs *in vitro*, including prolonged time in culture, electrical or mechanical stimulation, addition of chemical or biological factors, co-culture with non-CMs, three-dimensional (3D) assembly into tissues and the use of specific scaffold materials (Figure 7.1) (Sun and Nunes, 2017; Veerman et al., 2015; Yang et al., 2014). Despite distinct improvements - those strategies often only induce some aspects attributed to cardiac maturity - hPSC-CMs

never acquired an adult cardiac phenotype. Therefore, further progress necessitates the development of more advanced models, as for example engineering 3D cardiac tissue surrogates closely resembling the native cardiac microenvironment.

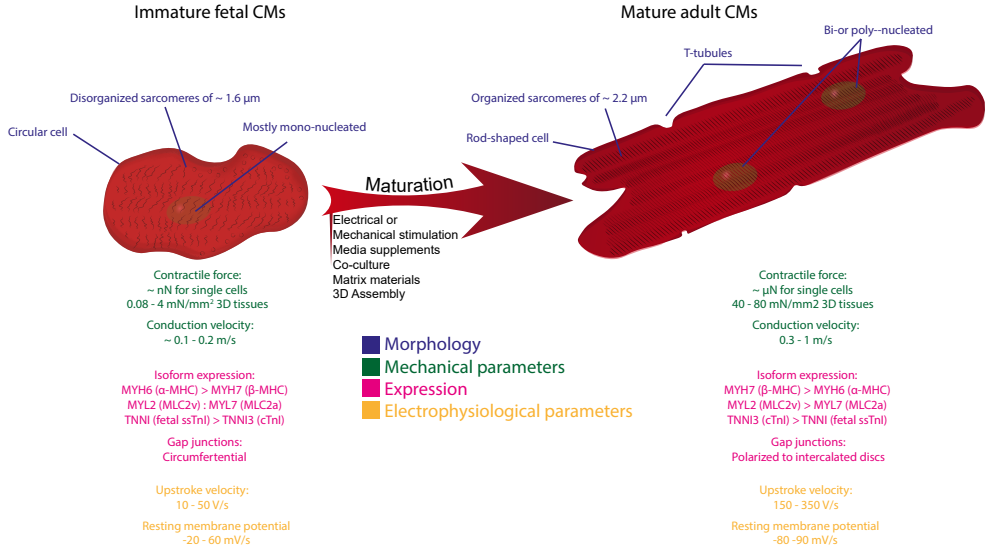

Figure 7.1: Strategies to enhance the maturity level of hPSC-CMs.

In this review, we describe the expression of extracellular matrix (ECM) proteins in the native microenvironment of human CMs with focus on developmental changes and respect to different heart compartments, such as atria and ventricles and also the right and left side of the heart. Moreover, we discuss the application of natural and synthetic biomaterials in the context of *in vitro* cardiac tissue engineering with hPSC-CMs and their effect on hPSC-CM maturity.

The native environment of CMs

Cells in their native environment are embedded within a network of ECM components, which are crucial for proper development and function of tissues and organs. The ECM delivers vital cues for various cellular responses, which include fate determination, migration, proliferation, morphology, cell

arrangement, survival and maturation. The cardiac ECM additionally needs to provide a strong support and elastic anchorage for precisely aligned CMs and creates a specialized environment to permit electrical coupling and cardiac impulse propagation between adjacent cells and transmission of contractile forces of CMs to the surrounding matrix for the repetitive blood pumping function of the heart (Chen et al., 2014; Heras-Bautista et al., 2014; Kléber and Rudy, 2004; Marijianowski et al., 1994; McCain et al., 2014). The basic structural unit of the cardiac ECM is primarily composed of collagen, but also includes laminin, fibronectin, fibrillin and elastin, which are continuously secreted by different cell types and assembled into a hierarchically organized scaffold (Table 7.2 and Figure 7.2) (Bishop and Laurent, 1995).

Table 7.2: Selection of proteins of the ECM in the heart

	Expression	Structure	Function	Secreted by:
Collagen I	Extra- and intracellularly in all tissues, but not in basement membrane	Triple-helical fibrils from fibrillary collagen	Structural support and strength	Fibroblasts
Collagen III	Extra- and intracellularly in all tissues, but not in basement membrane	Triple-helical fibrils from fibrillary collagen	Structural support and elasticity	Fibroblasts and smooth muscle cells
Collagen IV	Predominant protein in basement membrane	Rope-like, very long fibrils and sheet-like network	Cell-ECM interaction, connection to other ECM proteins	Fibroblasts, smooth muscle cells and CMs
Collagen VI	Extracellularly in all tissues, but not in basement membrane or intracellularly	Fine filaments in 90° orientation to other collagens	Adhesion and migration of cells, connection to other ECM proteins	Fibroblasts and smooth muscle cells
Fibronectin	Extra- and intracellularly in all tissues and plasma	Two chains connected by disulfide bridges. Dimers connect into fibrils only via integrin receptors on cell surfaces	Cell-ECM interaction via cell surface receptors, such as integrins	Fibroblasts and smooth muscle cells
Laminin	Most prominent glycoprotein in basement membrane	Self-assemble into asymmetric planar sheets	Cell-ECM interaction via cell surface receptors, such as integrins	Fibroblasts, smooth muscle cells and CMs
Elastin and fibrillin	Extracellularly	Long, very hydrophobic protein. No stretch: Circular compact Stretch: elongated interconnected strains	Elasticity	Fibroblasts and smooth muscle cells

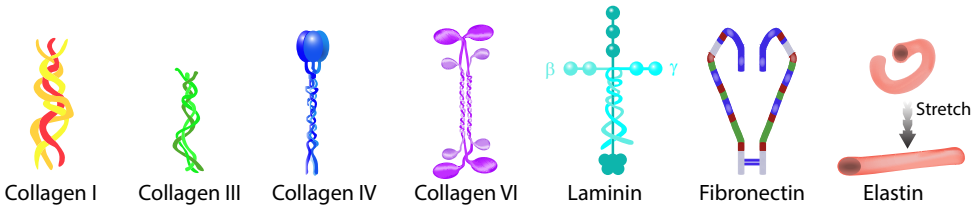

Figure 7.2: Most common ECM proteins in the heart.

Fibrillary collagens type I and type III form a complex fiber scaffold composed of thicker collagen I and thinner collagen III triple helical fibrils that have interconnected into larger fibers (fibril diameter depends on the tissue: in adult healthy cardiac tissue collagen I fibril diameter varies around 75 nm and collagen III fibrils around 45 nm (de Souza, 2002)). Thicker collagen I fibers are important to convey the necessary rigidity and stability, while collagen III fibers support the elasticity of the cardiac ECM (Marijianowski et al., 1994). Additional elastic flexibility of the cardiac wall is regulated by distribution and content of interspersed elastic fibers. Elastic fibers are primarily made from microfibrillary fibrillin with amorphous elastin as core protein (Votteler et al., 2013). Together with collagen type IV, laminin has a key role in linking CMs to their surrounding ECM and serves as cell adhesive protein. (Kim et al., 1999; Speiser et al., 1991, 1992). Similarly, fibronectin anchors cells to the ECM and transduces extracellular signaling to mediate cellular response (Farhadian et al., 1996). In mice, knockout of fibronectin has been shown to result in differentiation of cardiac progenitors towards CMs while inhibiting proliferation of cardiac progenitor cells in the pre-cardiac mesoderm during cardiac morphogenesis (Mittal et al., 2013). Another key component of the ECM are glycosaminoglycans, which interact with several signaling molecules to regulate differentiation, protection against oxidative stress and also cardiac remodeling after injury (Rienks et al., 2014). In human left and right atrial and ventricular walls, five different glycosaminoglycans have been identified: hyaluronic acid, heparan sulfate, dermatan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate (Masuda and Smcmjo, 1981; Shichijo and Masuda, 1980).

Most ECM proteins interact with cells via transmembrane receptors, so-called integrins, which do not only function as principal anchor for the cells to their surrounding ECM, but also mediate bi-directional communication

across the membrane to regulate cellular behavior via ligand binding on the outside (outside-in), or receptor activity by intracellular signaling (inside-out signaling). In the heart, integrins are also linked to the cytoskeletal protein alpha-actinin via talin and vinculin, thereby transmitting the mechanical forces from the inside of CMs to the ECM (Legate et al., 2009; Shen et al., 2012).

Native cardiac ECM during human heart development

Composition of the ECM is dynamic throughout embryonic development and adulthood, thereby providing stage-dependent clues for maturation of CMs (Figure 7.3).

Early during heart development, around the end of the first trimester of human development, laminin, fibronectin and most collagen fibers predominantly localize along the endocardial and epicardial layers (Jackson et al., 1993; Kim et al., 1999) CMs within the myocardium are supported by a premature collagen network primarily build from thin, loose fibers of collagen type III only (Jackson et al., 1993).

At the beginning of the second trimester with the onset of increased fetal growth and higher energy demands in the embryo, fibronectin and notably laminin become more widely distributed and strongly expressed within the myocardium (Kim et al., 1999; Speiser et al., 1992). Laminin is particularly expressed in the basal membrane of CMs, but also smooth muscle and capillary endothelial cells (Speiser et al., 1991). The basal membrane is a dense, highly organized layer that connects each cell with their surrounding ECM. Laminins are heteromerically formed by cell subtype-specific structural subchains, e.g. the A1-chain is primarily present in the basal membrane of vascular cells and not CMs, whereas the A2-chain is only present around CMs (Kim et al., 1999; Oliviéro et al., 2000; Saetersdal et al., 1992; Speiser et al., 1992). Fibronectin is predominantly localized around blood vessels close to smooth muscle cells and the basal membrane of fibroblasts and along endo- and epicardial layers (Kim et al., 1999; Oliviéro et al., 2000; Saetersdal et al., 1992; Speiser et al., 1992). Along with progressive build-up of the ECM, collagen fiber thickness increases and the premature collagen network quickly develops into a highly aligned, interconnected and

intercoiled network with similar ratios of collagen type I and III, or at the most a small shift in favor of collagen I (Jackson et al., 1993; Marijianowski et al., 1994; Speiser et al., 1991).

Postnatally, laminin strands have interconnected and built a fine, complex fibrillary network throughout the myocardium (Kim et al., 1999; Oliviéro et al., 2000; Saetersdal et al., 1992; Speiser et al., 1992). Despite no differences in total laminin, cardiac A2-laminin, fibronectin and total collagen proportions decrease immediately after birth, leading to a reduction of approximately 50% in the adult heart (Kim et al., 1999; Marijianowski et al., 1994; Oliviéro et al., 2000).

Less attention has been paid to the expression and distribution of less prominent collagens, such as collagen type IV and VI, as well as fibrillin and elastin in human myocardium during development. Moreover, little is known about expression of fibronectin isoforms in human fetal or adult cardiac tissues. However, in the mouse heart, three different isoforms of highly conserved fibronectin are generated by alternative splicing. Domains A and B, but not C of fibronectin are specifically expressed during embryonic development of the heart, but not in adult stages (Lu et al., 2015).

Native ECM in adult heart

In adulthood, collagen type III is twice more abundant than collagen type I (Marijianowski et al., 1994; Speiser et al., 1991). It is important to note that collagen is not evenly distributed throughout the adult heart, most of the collagen resides in the endo- and epicardium with a dense, thick subendocardial network, which gradually changes to a thinner subepicardial network. Similarly a dense, thick network of collagen fibrils can be observed at the base of the heart, close to the atrioventricular valves below the papillary muscles, which gradually declines in thickness in the direction of the apex of the heart (Jackson et al., 1993). In conjunction with lower atrial pressures and less compliant atrial compared to ventricular muscle, collagen I and III are twice more abundant in the myocardium of both atria than ventricles (Oken and Boucek, 1957; Smorodinova et al., 2015). Similarly, collagen I and III content is higher in the right heart compared to the left (Oken and Boucek, 1957; Smorodinova et al., 2015). In contrast

to intraventricular differences which are already obvious during the first trimester of human development (Jackson et al., 1993), differences in collagen expression between heart chambers only become apparent after birth, coinciding with the first respiration of the newborn and transition to independent systemic circulation resulting in pressure differences between the four cardiac chambers (Oken and Boucek, 1957).

In adult hearts, collagen VI has an overlapping expression pattern with collagen I and III, although fibers are much finer. To assemble into a complex fibrillary network, collagen VI fibers orientate in perpendicular orientation to collagen I and III (Speiser et al., 1991). Collagen IV is the only collagen secreted by CMs and the majority of collagen IV associates with laminin and fibronectin in the basal membrane of single atrial or ventricular CMs (Bashey et al., 1992; Borg et al., 1982; Jiang et al., 2016; Speiser et al., 1991). In human adult ventricular myocardium, collagen IV, laminin and fibronectin span the inner lining of the t-tubular network, thereby preventing collapse of t-tubules during cardiac contraction (Kim et al., 1999; Speiser et al., 1991, 1992). In the adult heart, elastin fibers assemble into an irregular network with fibers parallel to the long axis of muscle fibers. Importantly, not only the elastin content, but also fiber length is at least twice as long in the ECM of the right atria and ventricle compared to that of the left heart compartments (de Carvalho Filho et al., 1996; Smorodinova et al., 2015). Moreover, as opposed to irregular, less interconnected and more circular elastin in the left atrium, straight, parallel elastic fiber strands interconnect into a highly organized fibrillary scaffold in the right atrium (Smorodinova et al., 2015). Differences between right and left atrium might be due to diverse biomechanical stimulation of cardiac fibroblasts or different cell origins, but need further investigation for clarification (Smorodinova et al., 2015).

With increasing age the ratio of thick, densely packed collagen and elastin fibers increase in all chambers of the heart, but predominantly in the left ventricle (Alings et al., 1995; de Carvalho Filho et al., 1996; Gazoti Debessa et al., 2001).

The sinoatrial node is a specialized region in the posterior wall of the right atrium responsible for the initiation of the electrical impulse during each cardiac contraction cycle. The uniformly distributed bundles of collagen fibers are only sparsely dispersed within the human sinoatrial node during embryonic development, but comprise about 40% of the entire nodal

volume after birth and up to ~70% in adult hearts to form a dense regular framework. In rats, fibroblasts in the sinoatrial node predominantly secrete collagen I and III (Yanni et al., 2010), but it is not confirmed which collagens predominate in the human sinoatrial node. The collagen network of the sinoatrial node, is key to the mechanical support of nodal CMs, which rely on extra support because of their underdeveloped contractile machinery when compared to CMs of the working myocardium. The dense collagen network, which isolates groups of sinus node cells plays a negative role in maturation by limiting contact between groups of sinoatrial CMs, as well as sinoatrial CMs and their adjacent atrial CMs (Alings et al., 1995; James, 1970; Nabipour, 2012). In combination with fatty tissues, collagen may also be important in insulating pacemaker automaticity from surrounding atrial CMs (Csepe et al., 2015).

Matrix changes have not only been associated with cardiac development, but also various cardiac diseases. Post myocardial infarction (MI) around the infarct zone or in fibrotic scar tissue of hearts with cardiomyopathy, not only enhanced deposition of collagen type I, but also III, IV and VI, as well as fibronectin and laminin has been observed (Bishop et al., 1990; Kapelko, 2001; Kitamura et al., 2001; Lombardi et al., 2003; Ma et al., 2014; Oken and Boucek, 1957; Pauschinger et al., 1999). Interestingly, also permanent atrial fibrillation leads to an increase in collagen I, III and IV in both atria (Boldt et al., 2004; Jiang et al., 2016). No gender differences in collagen content or distribution have been detected during development or later (Alings et al., 1995; Marijianowski et al., 1994; Oken and Boucek, 1957).

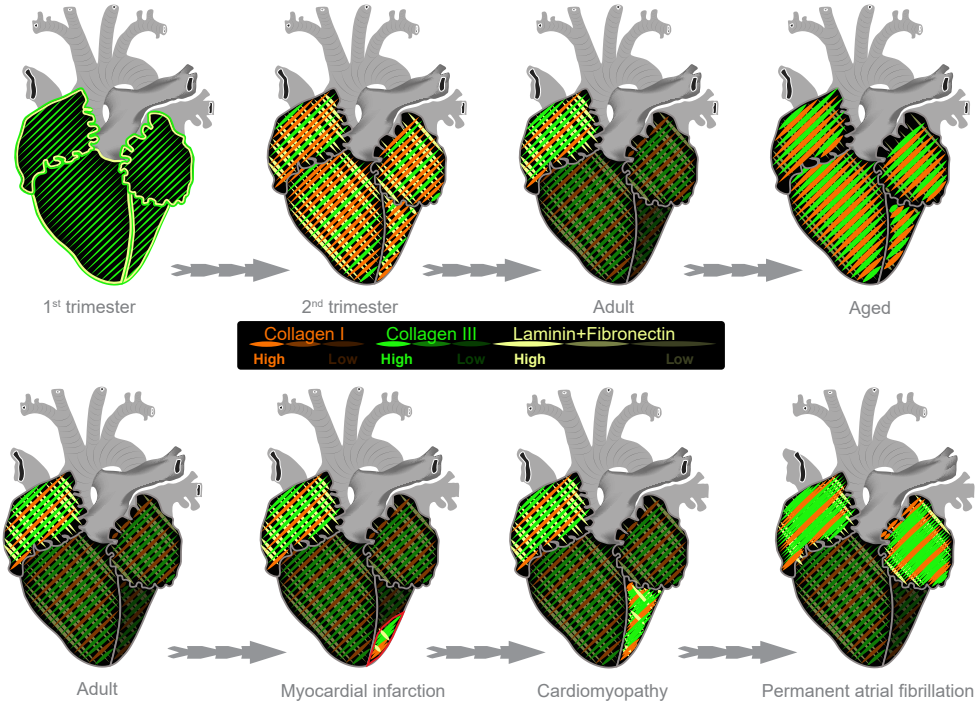

Figure 7.3: Upper panel: Schematic representation of the dynamic changes of the expression of ECM proteins in the heart. During the first trimester, the ECM network is primarily composed of collagen III. Laminin and fibronectin localize to endo- and epicardial layers. In the second trimester, collagen I, as well as laminin and fibronectin become more widely expressed and collagen fiber thickness increases. The premature collagen network develops into a highly organized network. In postnatal and adult hearts, laminin strands have interconnected to form a complex fibrillary network. Collagen III is now twice more abundant than collagen type I. Additionally, collagen I and III are twice more abundant in the myocardium of both atria than ventricles and collagen I and III amounts are higher in the right heart compared to the left. With increasing age the ratio of thick, densely packed collagen, as well as the fiber thickness increase. Lower panel: Schematic representation of the ECM changes in the diseased heart. Post myocardial infarction around the infarct zone (marked in red in left ventricle) or in fibrotic scar tissue in cardiomyopathy (see left ventricle), enhanced deposition of collagen type I, III, IV and VI, as well as fibronectin and laminin has been observed. Similarly, permanent atrial fibrillation leads to an increase in collagen I, III and IV in both atria.

Natural and synthetic biomaterials

Although a variety of biomaterials have been used for tissue-engineering replacements for different tissues, including bone, cartilage, tendon or skin, lungs and bladder (Shapira-Schweitzer et al., 2009), the ability to utilize natural or synthetic biomaterials for cardiac repair has proven more challenging than originally anticipated.

The use of natural biomaterials for engineering of tissue constructs shows many obvious benefits for mimicking endogenous tissues and organs. Nonetheless, important drawbacks of natural materials are their limited availability and shelf life, as well as batch-to-batch variation and high costs. Even more importantly, reproducible scaffold design from pure natural materials often can be challenging. Alternatively, synthetic polymers have adjustable mechanical and degradation properties and unlimited availability. Nevertheless, in contrast to natural biomaterials, synthetic materials have reduced biocompatibility and may provoke adverse rejection responses. Especially for the *in vitro* generation of soft tissue, such as mechanically challenging cardiac tissue, only few synthetic materials with appropriate combination of biodegradability, biocompatibility and mechanical properties without compromising cellular function of CMs have been applied so far (O'Brien, 2011; Patel et al., 2015; Shapira-Schweitzer et al., 2009). Selection of natural and synthetic biomaterials for cardiac tissue engineering are presented in Table 7.3 and Table 7.4, respectively.

Table 7.3: Selection of natural polymers

	Structure	Origin	Properties
Gelatin	Fine fibrillary scaffold	Hydrolysis of collagen	Deforms rapidly, low tensile strength, rapidly bioresorbable,
Matrigel	Hydrogel	Secretion by mouse sarcoma cells: Laminin, collagen IV, heparin sulfate proteoglycans, entactin/nidogen	Resistance against remodeling
Fibrin	Fibrillary scaffold	Plasma-glycoprotein, for blood clotting Fibrinogen as precursor for blood clotting Fibrinogen as precursor	Tunable properties and quickly remodeled

Table 7.4: Selection of synthetic polymers

		Approval for clinical application	Application
Poly-esters	Poly-glycolic acid (PGA)	Approved by the FDA	Re-absorbable suture material
	Poly-lactic acid (PLA)	Approved by the FDA	Rigid thermoplastic materials
	Co-polymers poly-lactic-glycolic acid (PLGA)	Approved by the FDA	Scaffold for controlled drug release
Poly-lactones	Poly-caprolactone (PCL)	Approved by the FDA	Implantable drug delivery
	Carboxylated-PCL (cPCL)		
Elastomers	Poly-dimethyl-siloxane (PDMS)	Approved by the FDA	Medical devices
	Poly-glycerol sebacate (PGS)	Approved by the FDA	Nerve and vascular tissue engineering
PEG	Poly-ethylene glycol	Approved by the FDA	PEGylated drugs

Desired biomaterial properties for cardiac tissue engineering and *in vitro* modeling of hPSC-CMs

CMs display a high sensitivity to environmental factors, such as elasticity, geometry and topography (Farouz et al., 2015; Nakane et al., 2017) and extracellular substrates have already been shown to induce some aspects of cardiac maturity (Sun and Nunes, 2017; Veerman et al., 2015). In order to emulate the native cardiac environment, the preferred biomaterial needs to meet the following essential criteria:

1) Long-term elasticity and mechanical strength (suitable stiffness) to sustain the high repetitive mechanical stress and non-linear elasticity of contracting muscle

2) Adaptable biodegradability long enough to facilitate cell attachment and bear the proteolytic activity of diseased myocardium, while avoiding a persisting response against a foreign structure

3) Biocompatibility that is not toxic for the cells and supports cell survival

4) Structural facilitation of neovascularization and remodeling

5) Controllable electrical properties that do not interfere with the electrical conductance of the action potential

6) Outside-in-signaling enabling optimal CM attachment, survival, growth, maturation and function *in vitro*, and support of functional cardiac contraction and integration into the host tissue after implantation *in vivo*

Scaffold-mediated engineered cardiac tissues

Natural materials as ECM and multicellular approaches in human cardiac tissues engineered from hPSC-CMs

To generate cardiac tissues which include the natural ECM components in a developmentally and physiologically relevant organization, approaches have been undertaken to decellularize cardiac tissue, resulting in a 3D cell-free cardiac ECM meshwork, followed by repopulation with cardiac cells or their progenitors in order to study processes like survival, migration, proliferation, maturation and communication. Repopulation of a decellularized mouse heart with hPSC-derived KDR_{low}/C-KIT_{neg} cardiovascular progenitor cells (CPCs) with the capacity to differentiate into CMs, smooth muscle cells and endothelial cells allowed formation of a bioartificial heart suitable for analyzing ECG traces, contraction and drug response (Lu et al., 2013). Here, stable interaction between the ECM of the decellularized heart allowed about 10-15% of the CPSs to be retained in the natural cardiac ECM meshwork. These CPCs formed a revascularized spontaneously beating tissue within 20 days after repopulation. Interestingly, endothelial cells formed a single layer along the endocardial surface, as well as the inner surface of small coronary vessels suggesting that the natural ECM effectively supported correct arrangement of *in situ* differentiated cardiac cells. Although these studies indicate the importance of a natural scaffold of ECM components for the formation of 3D cardiac tissue, alternative strategies are required for controlled production of cardiac tissues, since material source is limited and morphology is difficult to adjust to the desired shape of the tissue. Instead of using a complex ECM network, many approaches for generation of cardiac tissues are based on only a few components or a mixture thereof.

Several features attributed to maturation of hPSC-CMs have been achieved in engineered 3D human cardiac tissues utilizing fibrin in combination with matrigel, a mixture of laminin together with collagen IV and proteoglycan derived from mouse sarcoma cells, as ECM material (Hirt et al., 2014; Mannhardt et al., 2015; Schaaf et al., 2011; Stoehr et al., 2014; Zhang et al., 2013, 2017). Fibrin is an insoluble fibrous protein, involved in blood clotting and wound healing, with little presence in the healthy fetal or adult heart. Instead, increased plasma levels of fibrin have been acknowledged as important cardiovascular risk factor (Stec et al., 2000). However, practical reasons, such as tunable physical and mechanical properties, as well as biodegradability, made fibrin the protein of choice for tissue engineering. Consistent with enhanced expression of genes important for cardiac contractile function and calcium handling, sarcomeres in cardiac tissues with fibrin as scaffold material were highly organized and evenly distributed with a longitudinal orientation along force lines and enhanced sarcomere length of approximately 1.6 μm (Mannhardt et al., 2015) and 2.1 μm (Zhang et al., 2013). Structural maturation led to a sustained contraction force of ~0.15 mN (Mannhardt et al., 2015) or ~3 mN (Zhang et al., 2013) and higher conduction velocities (10 cm/s up to 25 cm/s with increased hPSC-CM purity)(Zhang et al., 2013). Interestingly, hPSC-CMs in heterogeneous cardiac tissues, typically consisting of hPSC-CMs mixed with fibroblasts and other non-CMs, developed immature t-tubular-like structures (Mannhardt et al., 2015). T-tubules are a manifestation of CM maturation typically absent in hPSC-CMs. However, as evidenced by metabolic phenotype, sarcomeric organization, intercellular connection, electrical function and low sensitivity to calcium, 3D cardiac tissues failed to induce full maturation. Continuous electrical pacing (Hirt et al., 2014) or mechanical load (Zhang et al., 2017) by stretching had additive positive effect on CM morphology, as proven by sarcomeric organization, transcriptional profile and contraction force. Recently, it was shown that physical conditioning by electromechanical stimulation of early-stage hPSC-derived CMs in fibrin hydrogel cardiac tissues with supporting dermal fibroblasts induced maturation to an adult-like stage regarding gene expression, ultrastructural organization, such as physiological sarcomere length of 2.2 μm, presence of mitochondria and t-tubules, as well as oxidative metabolism, positive force–frequency relationship and functional calcium handling. However, electromechanics did not reach maturity seen in adult human myocardium (Ronaldson et al., 2018).

Since fibrin only has a minor role in the cardiac ECM and therefore may be less optimal as ECM component for CMs in engineered cardiac tissues, it is well possible that collagens may be more appropriate for cardiac tissue engineering based on cardiac expression of ECM proteins. However, in contrast to cardiac tissues with fibrin as scaffold, cardiac tissues with collagen require at least 25% of non-CMs (Mannhardt et al., 2015). The majority of non-CMs in the heart are cardiac fibroblasts which play a key role in the production and remodeling of cardiac ECM proteins, including collagen I and III, fibronectin and laminin (Castaldo et al., 2013). Importantly, in scaffold-free cardiac tissues, only co-culture with adult cardiac fibroblasts, but not fibroblasts of dermal origin, successfully improved CM maturity, as evidenced by enhanced calcium signaling and amended contractile response to inotropic agents (Ravenscroft et al., 2016). This indicates that the origin of cells plays an important role for proper CM function and suggests that co-culture with cardiac cells such as endothelial cells, smooth muscle cells and fibroblasts are required to closely mimic the native cardiac environment and induce CMs maturity. While the co-culture of hPSC-CMs with irradiated human foreskin fibroblasts or hPSC-derived fibroblasts yielded synchronously contracting engineered cardiac tissues based on collagen I in combination with matrigel (Kensah et al., 2013; Thavandiran et al., 2013), only treatment of hPSC-CMs with ascorbic acid in combination with mechanical stimulation yielded some degree of structural maturation with a contraction force of 4.4 mN/mm^2 and a conduction velocity of up to 4.9 cm/s (Kensah et al., 2013); similarly alignment of collagen I and electrical stimulation yielded some aspects of maturation, such as increased transcriptional activity of genes encoding for structural proteins and high conduction velocity (25 cm/s) (Thavandiran et al., 2013).

Not only the presence of fibroblasts, but also endothelial or smooth muscle cells has been demonstrated to advance structural or electrical maturity of hPSC-CMs (Giacomelli et al., 2017; Pasquier et al., 2017; Vuorenpää et al., 2017). However, based on gene expression patterns and morphological characteristics, such as increased cell surface area with well-defined Z discs and nascent intercalated discs, the maturation of hPSC-CMs was only partially accomplished. hPSC-CMs maturity was further enhanced by electrical stimulation as indicated by improved calcium handling. Nonetheless, hPSC-CMs did not develop crosslinking of myosin thick filaments, so-called M-bands, nor t-tubules (Nunes et al., 2013).

Similarly, the inclusion of hPSC-derived vascular endothelial and mural cells (smooth muscle cells and pericytes) into collagen I-matrigel scaffold-based 3D cardiac tissues especially led to structural maturation in hPSC-CMs, which was indicated by organized mitochondria localized between myofibers and enhanced expression of structural proteins, such as β-MHC. Together this led to more organized myofibrillar structure with highly aligned, parallel sarcomeres that demonstrated distinct Z-lines between adjacent sarcomeres, thin filaments (I-bands) and thick filaments (A-bands), but no thick filament crosslinking (M-bands). This resulted in medium force generation (average force 0.62 mN/mm^2). Despite immature calcium signaling, structural maturation allowed multicellular engineered 3D cardiac tissues to sustain high frequency pacing by maintaining higher active force (Masumoto et al., 2016).

Under defined culture conditions (without matrigel), using a selected cocktail of growth factors, FGF-2, IGF-1, TGF-ß, and extracellular calcium led to improved CM maturity in collagen I-based 3D cardiac tissues with hPSC-CMs and fibroblasts, as evidenced by increased sarcomeric organization with defined M-bands and a sarcomere size of 1.9 µm, yielding a positive force-frequency behavior with a contraction force of 6 mN/mm^2 and N-cadherin-positive intercalated disc-like structures. However, despite some improvement, the transcriptional profile was comparable to fetal heart at 13 weeks of development. Unexpectedly, laminin and fibronectin failed to improve function of 3D cardiac tissues (Tiburcy et al., 2017). Together this indicates that collagen I or fibrin may not be sufficient for mimicking the ECM in order to engineer cardiac tissues *in vitro*.

Synthetic materials as supportive scaffold in human engineered cardiac tissues

A small range of synthetic materials have been tested as alternative for natural scaffold materials in engineered cardiac tissues from hPSC-CMs. Porous scaffolds engineered from poly(2-hydroxyethyl methacrylate-co-methacrylic acid) (pHEMA-co-MAA) enhanced selected survival and increased proliferation of hPSC-CMs in mixed cardiac tissues, but failed to induce maturity (Madden et al., 2010). Particularly in combination with natural materials, such as collagen, gelatin, matrigel or vitronectin, synthetic

polymers like PEG, PCL, c-PCL, PLGA, polyethylene and PGS successfully supported viability and permitted cell adhesion, spontaneous contraction and also migration of hPSC-CMs *in vitro* or after transplantation *in vivo* (Chen et al., 2010, 2015; Chun et al., 2015; Wang et al., 2013, 2015; Xu et al., 2013). Precisely aligned hPSC-CMs on micropatterned PLGA, coated with gelatin, and matrigel-coated poly-ethylene, exhibited accelerated conduction velocity and reduced predisposition to arrhythmia in optical mappings *in vitro*. Analogous results on PLGA (Chen et al., 2015) and poly-ethylene (Wang et al., 2013) suggest that the decreased sensitivity to arrhythmia can be attributed to the physical alignment of the CMs on micromolded grooves and not the substrate matrix. Interestingly, a co-polymer of 4% PEG and 96% PCL, was able to induce expression of genes attributed to cardiac maturity and improved some functional aspects of cardiac maturation, such as increased expression of structural proteins, isoform switch, mitochondrial function and contractility during *in vitro* culture (Chun et al., 2015).

Synthetic biodegradable, porous PLLA/PLGA scaffolds in combination with matrigel allowed survival and synchronous contraction of hPSC-CMs in engineered 3D human cardiac tissues (Caspi et al., 2007). Importantly, inclusion of endothelial cells into the tissue had an advantageous effect on CM proliferation. However, prominent vascularization occurred only in multicellular tissues with hPSC-CMs, endothelial cells and embryonic fibroblasts. Interestingly, hPSC-CMs within vascularized tissues exhibited features of maturation, such as increased sarcomeric organization and increased functional coupling (Caspi et al., 2007).

PEGylated fibrinogen is a biosynthetic and rapidly biodegradable hydrogel that photo-polymerizes in response to low intensity, long wave UV-light (Almany and Seliktar, 2005; Habib et al., 2011; Shapira-Schweitzer and Seliktar, 2007; Shapira-Schweitzer et al., 2009). Importantly, physical and mechanical properties, such as matrix stiffness, can accurately be regulated by the ratio of synthetic PEG to biological active fibrinogen or the amount of cross linker (Almany and Seliktar, 2005; Habib et al., 2011; Shapira-Schweitzer and Seliktar, 2007; Shapira-Schweitzer et al., 2009). PEGylated fibrinogen hydrogels were the first biosynthetic materials to promote survival and reorganization of isolated neonatal rat CMs, as well as hPSC-CMs into functional contracting tissues without negatively affecting their contractile phenotype even after several weeks (Habib et al., 2011; Shapira-Schweitzer and Seliktar, 2007; Shapira-Schweitzer et al., 2009). Typical

response to chronotropic agents, carbamylcholine and the beta-adrenergic agonist isoproterenol, validated responsiveness to cardiac drugs (Shapira-Schweitzer et al., 2009). Engineered cardiac patches with PEGylated fibrinogen as scaffold for hPSC-CMs abridged adverse cardiac remodeling and proved beneficial for ventricular performance post myocardial infarction due to improved fractional shortening (Habib et al., 2011). By preserving contractile function, promoting some features of maturation in hPSC-CMs, PEGylated fibrinogen may serve as promising biosynthetic material for engineering 3D *in vitro* cardiac tissues.

Recent advances in the design of hydrated polymeric hydrogels renders them very useful for mimicking the native ECM environment and controlled tissue engineering either for *in vitro* applications or for *in vivo* tissue regeneration. In general, hydrogels have a high water content, a high biocompatibility and elasticity and consist of either natural materials such as hyaluronic acid, collagen and alginate, or synthetic polymers such as PEG, PVA or PAM. The chemical, mechanical and degradable properties of hydrogels can be modified and biological processes, such as proliferation, differentiation, migration and maturation can be influenced by incorporation and crosslinking of bioactive molecules, indicating the versatile nature of hydrogels. It goes beyond the scope of this review to discuss all the latest developments in hydrogel technology and their possible applications for regenerative medicine and *in vitro* assays. Excellent reviews on this topic have been published elsewhere (Flégeau et al., 2017).

As mentioned before, it has become clear that it is important to mimic the 3D microenvironment of the native cardiac tissue, ensuring dynamic interplay between cardiac cells, ECM components, and bioactive molecules in an optimal structural architecture and topographic organization with appropriate biomechanical (elasticity and stiffness) and electrical conductive properties Several innovative approaches have been followed in order to generate functional 3D cardiac tissues using engineered 3D fiber-based scaffolds or hydrogel-based. Recently, melting electrowriting, an electrohydrodynamic printing technology, was used to fabricate stretchable microfiber scaffolds with hexagonal microstructures using medical grade polycaprolactone as a polymer. Interestingly, these printed scaffolds supported and enhanced maturation of hPSC-derived CMs. Moreover, these scaffolds were also successfully applied on a beating porcine heart while maintaining their structure (Castilho et al, 2018). Although these hexagonal

structures clearly demonstrated advantages in comparison to rectangular scaffolds, the pores are much larger (approximately 20-fold) than the honeycomb structure of the heart. It remains to be seen whether these scaffolds will support correctly organized multicellular cardiac constructs for optimal cardiac function and intercellular communication.

Another important aspect of cardiac function and maturation is electrical activity. To increase electrical conductivity of biomaterials various polymers have been used, such as polyaniline (PANI), polypyrrole (PYY) and polythiophene as reviewed by Guo and Ma in 2018 (Guo and Ma, 2018). In addition, mixing polymers with graphene or other conductive materials, such as carbon or gold nanoparticles have been shown to increase the electrical conductivity. In addition to their properties related to electrical activation of cardiac tissue, conducting polymers may also have favorable effects on various biological processes. Blending conducting polymers with other polymers like PLA, PLGA and PCL can change biomaterial properties such as degradability, elasticity and stiffness and can also be used in combination with different techniques including electrospinning for the production of ECM-like nanofibers or generation of hydrogels. PPY/PCL and PLA/PANI conductive films or nanofibrous sheets positively affected cardiomyocyte survival, morphology or function *in vitro* (Spearman et al., 2015; Wang et al., 2017) whereas injection of conductive hydrogels encompassing eNOS-expressing nanocomplexes, improved heart function in an experimental model of heart failure (Wang et al., 2018). It will be of interest to study the role of conducting polymers on cardiac function in highly advanced *in vitro* models and ***in vivo***.

Advanced human cardiac models: towards a pumping mini-heart

To generate more advanced models more closely resembling cardiac geometry and to allow measurement of hemodynamic cardiac output, Li et al. created for the first time a human ventricle-like cardiac organoid chamber (hvCOC) (Li et al., 2018). In order to achieve this, dissociated hPSC-derived CMs and dermal fibroblasts (for compaction) were reaggregated in the presence of a mixture of collagen I and Matrigel and were transferred to an agarose mold with a centrally placed inflatable balloon, which allowed to form a 3D

human engineered cardiac organoid. HvCOCs were able to pump fluid and ejection fraction and generation of pressure volume loops could be measured in these heart models. Although these results are very promising, cardiac maturation and function may be improved if cardiac fibroblasts will be used instead of dermal fibroblasts (vide supra). Macqueen and colleagues followed another approach to generate a ventricle-like tube (MacQueen et al., 2018). In this study scaffolds were made by pull spinning a mixture of PCL and gelatin onto a mandrel in the shape of an ellipsoidal ventricle. Following sterilization by UV, scaffolds were coated with fibronectin for the attachment of hPSC-derived CMs. Following suturing of the ventricular engineered tissue on a tubing system ejection fraction and pressure volume loops could be measured and were responsive to pharmaceutical compounds. Although it is clear that further improvements are required, it is promising that clinically relevant parameters could be measured in these ventricle-like tubes.

Conclusions and future directions

Although differences in experimental approaches, including timing, origin of cells, use of biomaterials, culture conditions, engineering technologies and endpoint evaluation, make it difficult to directly compare results from different studies, it is evident that the level of cardiomyocyte maturation can be further increased in more advanced 3D tissues when compared to standard monolayer or aggregate differentiation cultures. Nevertheless, even in multicellular 3D cardiac tissues, hPSC-CMs fail to acquire an adult phenotype. So far, little attention has been paid to mimic the native cardiac microenvironment. Instead the majority of cardiac tissues are based on fibrin or collagen I, a matrix which is far from comparable to native cardiac environment, consisting of a complex scaffold of collagen I, III, IV and VI and also laminin, fibronectin, fibrillin and elastin, as well as other components. Temporal and spatial cues from cardiac cells and their microenvironment during human cardiac development (from fetal to adult stages), exemplified by localized and progressive build-up of cardiac ECM components, are essential for a proper understanding of cardiomyocyte maturation. Mimicking the same dynamic stages with gradual assembly of matrix proteins recapitulating cardiac native environment may contribute to maturation of hPSC-CMs *in vitro*, which may be dependent on the self-organization of the engineered cardiac tissue in combination with physical

factors such as mechanical load and electrical stimuli. In this context, it is intriguing to speculate that hPSC-derived cardiac cells, either progenitor cells or functional subtypes, and biomaterials may be positioned in the appropriate architecture by 3D-bioprinting technology. Although many hurdles need to be overcome before vascularized cardiac tissues can be printed (including survival or cells and printing resolution), recent advances have shown the feasibility to generate viable organized tissues (Duan, 2016; Murphy and Atala, 2014). Advances in differentiation, characterization and purification of hPSC-derived cardiac cells along with those in biomaterials, polymers and tissue engineering will facilitate controlled and defined construction of heart tissue, using fetal and adult stages of the human heart as a molecular, morphological and functional blueprint, which undoubtedly has a major impact on regenerative medicine. In addition, with this in prospect, we expect to gain insight to what level of organization and maturation of engineered heart tissues can be achieved *in vitro* and to what extent this will be sufficient to reveal key aspects of cardiac disease using patient-derived hPSCs. With the development of innovative smart hydrogels and other materials new opportunities have arisen for dynamic interaction with cells and tissues in a temporal and spatial manner. In combination with other recent advances in other fields such as microfabrication, nanotechnology, electrical engineering, biosensors, membranes, chemistry, membrane sciences, 3D printing, etc., new opportunities have been created to mimic tissue or organ function in small (normally micrometre- or millimetre-sized) chambers or chip-like devices. In these so-called “organs-on-chips” the microenvironment can be controlled and biological and functional readouts can be implemented in a flexible and multiplex manner. Increased understanding of how ECM components and synthetic polymers affect biological responses and function under physiological and pathophysiological conditions, will facilitate development of predictable human *in vitro* models for safety pharmacology and drug discovery and generation of multicellular tissue constructs for clinical use.

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