Myocardium in a dish : the influence of cellular and extracellular perturbations on cardiomyocyte contractility

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Myocardium in a dish: The influence of cellular and extracellular perturbations on cardiomyocyte contractility

PROEFSCHRIFT

ter verkrijging van de graad van doctor
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voor een commissie aangewezen door het College voor Promoties,
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door

Ariane Catharina Cornelia van Spreeuwel

geboren te Eindhoven

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Het onderzoek dat in dit proefschrift wordt beschreven is uitgevoerd in overeenstemming met de TU/e Gedragscode Wetenschapsbeoefening.

Contents

Summary

Myocardium in a dish: The influence of cellular and extracellular perturbations on cardiomyocyte contractility

Cardiac tissue models have been developed to study cardiac physiology *in vitro*. With a high degree of experimental control, different tissue properties can be mimicked to unravel their contribution to the complex pathological conditions observed *in vivo*. A small perturbation in the cardiac microenvironment may trigger a cascade of adverse remodeling events, leading to the onset of cardiac disease. Nowadays treatments for cardiac disease mainly focus on relief of the symptoms rather than addressing the cause of the disease itself. To develop new treatments, more knowledge is needed about the effect that these small perturbations in cells or matrix have on cardiomyocyte function.

The aim of this thesis was to investigate the effect of changing microenvironments on cardiac contractility. A microtissue model was developed, consisting of both cardiomyocytes and cardiac fibroblasts, that allows for manipulation of cell number, cell ratio, and matrix organization and content, as well as quantification of tissue generated contractile forces.

Literature review revealed that in current tissue models limited attention is paid to the cellular microenvironment and the response of cardiomyocytes to differences in matrix organization and composition. Detailed information of the native healthy and diseased cardiac microenvironment can be used as guidelines to develop or improve in vitro models that mimic specific disease hallmarks. Therefore, changes in extracellular matrix properties were studied in genetic and acquired mouse models of heart disease, which all showed (local) fibrosis. In the fibrotic areas of the myocardium, alignment of cells and matrix was deteriorated. To investigate the influence of matrix (an)isotropy on cardiomyocyte contraction, collagen orientation of the cardiac microtissues was manipulated. Uniaxial constraints were used to obtain an aligned organization, while biaxial constraints resulted in disorganization of the matrix, representing a “healthy” and “diseased” tissue organization respectively. Disorganization of the matrix did not decrease the generated tissue force and beating frequency, although it did disturb homogeneity of force distribution.

Cardiac fibrosis is often accompanied by cardiac fibroblast proliferation and/or increased matrix production. To quantify the effect of these factors on contractility, both the number of cardiac fibroblasts and collagen content of the cardiac microtissues were manipulated. Interestingly, a threshold of 50% cardiac fibroblasts was found, after which synchronized beating of the microtissues was severely hampered. Next to beating frequency, contraction force also decreased with increasing fibroblast content. Increasing collagen content only affected the generated contraction force, but not the beating frequency of the microtissues.

Next to collagen, elastin is an important extracellular matrix protein in the heart. Impaired elastic fiber formation is the underlying cause of cardiomyopathy in mice with a reduced fibulin-4 expression. However, it remains unclear if this mutation also directly affects cardiomyocyte functionality. Therefore, microtissues were seeded with neonatal cardiac

cells from wild-type mice and from mice with reduced fibulin-4 expression. For the first time, our results revealed a decreased contractility in fibulin-4 deficient microtissues compared to wild-type cardiac microtissues, suggesting that this mutation also directly affects the cardiac cells and not only their matrix production.

When comparing all data of the different chapters, it is clear that increased fibroblast density had the most detrimental effect on tissue contractility. From a clinical perspective, this means that increasing the number of cardiomyocytes, for example via stem cell injection, is the most promising option to regain cardiac function. Of course, to completely recover the pump function of the heart, it is also important to reduce collagen accumulation and restore the alignment of both cells and matrix.

In conclusion, the results of this thesis demonstrate that not only the cardiomyocyte itself but also its microenvironment is essential for efficient contractility of cardiac tissue. Furthermore, we showed that the developed cardiac microtissues are suitable to systematically study pathophysiological properties that have been observed during cardiac disease *in vivo.* As such, these tissue models may facilitate in the optimization of new regenerative therapies and have the potential to be used as a screening tool for drugs or therapies.

1

General introduction

Part of this chapter is based on:

M.H. van Marion
N.A.M. Bax
A.C.C. van Spreeuwel
D.W.J. van der Schaft
C.V.C. Bouten

Material-based engineering strategies for cardiac regeneration, Current Pharmaceutical Design (2014), 20: 2057-2068.

General introduction

The adult human heart contracts more than 100,000 times per day to pump the blood through the body. This is realized by the rhythmic contraction of cardiomyocytes in the myocardial wall. To act as a syncytium, a coordinated interaction between the cardiac cells and the surrounding extracellular matrix (ECM) is required. The ECM provides structural support and guides cellular alignment. Even small perturbations in this specialized and delicate interplay may trigger a cascade of adverse remodeling events in both cells and matrix, leading to the onset of cardiac disease. Even though there are many different causes for cardiac disease, both acquired and genetic in nature, generally the end stage of the disease is chronic heart failure. Nowadays treatments for cardiac disease mainly focus on relief of the symptoms rather than on curing the disease itself, simply because the heart has only limited regenerative capacity. To develop treatments to cure or even prevent specific diseases, it is important to know how perturbations in cells or matrix affect cardiomyocyte function and thus lead to the onset of cardiac disease.

Therefore, the aim of this thesis was to elucidate the impact of cellular and extracellular disturbances, such as cell and matrix composition or (an)isotropy, on cardiomyocyte function. Since the most important function of the cardiomyocyte is to generate contractile forces, this will be the main functional readout in this thesis. As it is extremely difficult to quantitatively investigate the effects of cellular and extracellular perturbations of cardiomyocyte contractility in the complex *in vivo* environment, we developed an in vitro cardiac tissue model. This model allows us to mimic essential elements of the native cardiac tissue and at the same time manipulate the different parameters of the cardiac microenvironment, such as cell types and numbers, or matrix structure and composition. This approach will help to systematically study the different parameters that influence cardiomyocyte contraction. The current chapter provides a basal introduction to the cardiac microenvironment and its perturbations in cardiac disease, focusing on cardiac contractility.

Cell types in the myocardial microenvironment

The myocardial wall consist of multiple cell types and all of them contribute to the structural, biochemical, mechanical, and electrical properties of the functional heart under physiological and pathophysiological conditions. In this thesis, we focus on the cells that are most relevant for contractility of the myocardium: the cardiomyocytes and fibroblasts. At the tissue level, these cells are embedded in a fibrous extracellular matrix (ECM). Cells and matrix together are assembled in an anisotropic and layered structure which is essential for efficient pump function (1).

Cardiomyocytes are the cardiac muscle cells and represent around 75% of the heart's volume. Yet, they account for only one third of the total cell number in the human heart. However, these numbers vary between different species. A study by Vliegen et al. reported 28% cardiomyocytes in adult human hearts (2), while Banerjee et al. found 56% of all cells to be cardiomyocytes in the adult mouse heart and even higher numbers in neonatal mouse hearts (3). Cardiomyocytes generally have a diameter of 10-15 µm and a length of up to 100 µm (4). The length/width ratio which is optimal for contraction was found to be 7:1 in

healthy cardiomyocytes. Cardiac hypertrophy or dilation will lead to an increase or decrease of this ratio and hence less optimal contraction at the cellular level (5). Also at the cellular level, the myocardium is well structured: Cardiomyocytes contain many myofibrils consisting of actin and myosin, which are organized into sarcomeres (figure 1D). For contraction, the actin and myosin filaments slide along each other, thereby shortening the sarcomeres. In rest, the length of a single sarcomere in adult mice is ~1.8 µm (6).

Cardiomyocytes connect to each other at the long ends via intercalated discs, which contain three types of cell-cell contacts: Adherens junctions, desmosomes and gap junctions (figure 1C). Adherens junctions connect bundles of actin filaments from cell to cell, desmosomes connect the intermediate filaments between cells and gap junctions allow small molecules to pass from cell to cell (7). The intercalated disc has two main functions: mechanical coupling via the adherens junctions and desmosomes and electrical coupling via the gap junctions. Contact of the cell with the laterally surrounding ECM occurs via so-called costameres. Costameres align with the Z-disk of the sarcomere (8). There are two main protein complexes in the costamere: The dystrophin-glycoprotein complex and the integrin complex (figure 1B). Forces generated by contraction of the cardiomyocytes and externally applied forces on the cardiomyocytes are transmitted via these cell-matrix interactions (9).

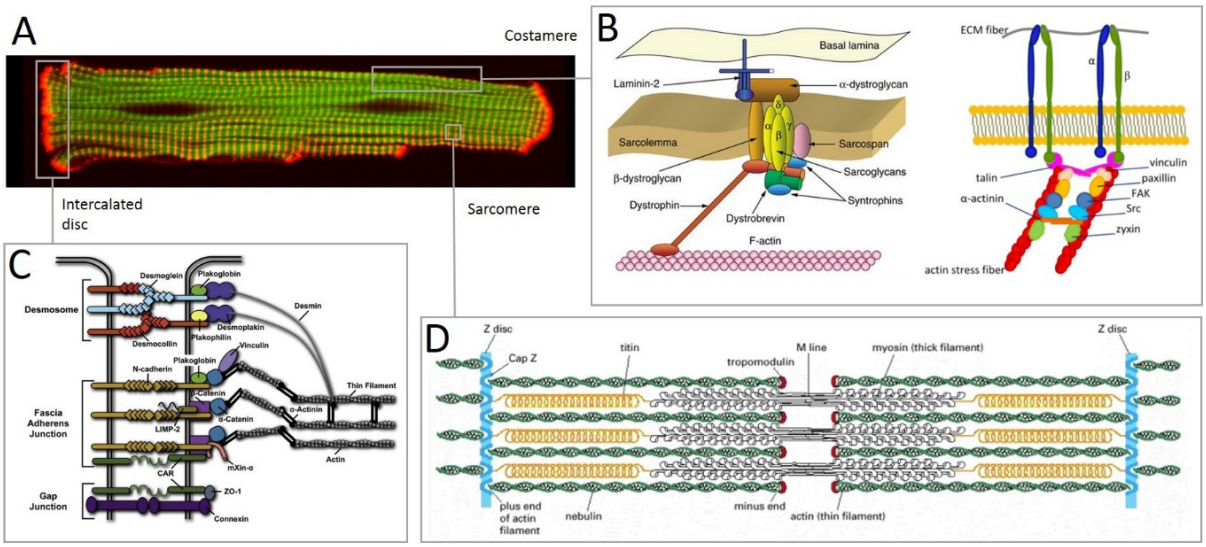

Figure 1 *Individual cardiomyocyte (A) with its cell-matrix (B) and cell-cell (C) interactions and sarcomere structure (D). Adapted from (7, 10-13).*

Cardiac fibroblasts maintain the ECM of the myocardium. They have a spindle shaped and elongated morphology and reside in the collagen network that is surrounding the cardiomyocytes. Specific fibroblast markers are lacking, although vimentin, an intermediate filament protein of the fibroblasts, is a well-accepted and frequently used marker to distinguish them from cardiomyocytes (14-16). The primary function of cardiac fibroblasts is the synthesis and degradation of the ECM (17). Furthermore, they distribute mechanical forces and electrically separate the cardiomyocyte bundles to allow proper contraction. Via cell-cell interaction, fibroblasts can communicate directly with cardiomyocytes (18, 19). Experiments have shown that cardiomyocytes and fibroblasts connect via connexin (cx40, cx43 and cx45) coupling (20). Through these connections, they can pass electrical signals

from the cardiomyocytes but the fibroblasts cannot generate these signals themselves (14). Next to ECM protein production, cardiac fibroblasts also produce matrix metalloproteinases (MMPs) and their inhibitors, TIMPs. MMPs can degrade the ECM to facilitate cell migration and release of bioactive molecules. In healthy myocardium, MMP activity is low, and ECM synthesis and degradation are in balance to maintain a stable collagen content (21).

Extracellular matrix of the myocardium

The myocardial ECM supports the cells to maintain the structural integrity of the heart and guide alignment and coordinated contraction. The cardiac cells are surrounded by a basement membrane, mainly consisting of laminin and collagen IV. The basement membrane is essential for sarcomeric development and interaction with the surrounding ECM. Via other proteins, such as fibronectin, the basement membrane is connected to the collagen network of the heart (22). The main components of this network, collagen I and III, are both fibrillar types of collagen, which form a scaffold around the cardiac cells (figure 2). In total, collagen accounts for less than five percent of the myocardial volume. Yet, it is is essential for transmitting forces generated by the cardiomyocytes (23). Collagen type I represents around 80% and type III around 20% of total collagen content in the myocardium (24). Collagen I gives the heart its tensile strength and guides cellular alignment, while collagen III, together with elastin, maintains the elasticity of the myocardium (25). Furthermore, negatively charged proteins, called proteoglycans, attract water to preserve hydration of the myocardial tissue and provide resistance to compressive forces. They assist in maintaining the integrity of the tissue by interacting with other ECM proteins (26). Altogether, the matrix connects cells, prevents overextension of the myocardium and deformation of blood vessels, and facilitates intracellular signaling (21). The myocardial ECM even preserves the alignment of myofibrils within the myocytes (27).

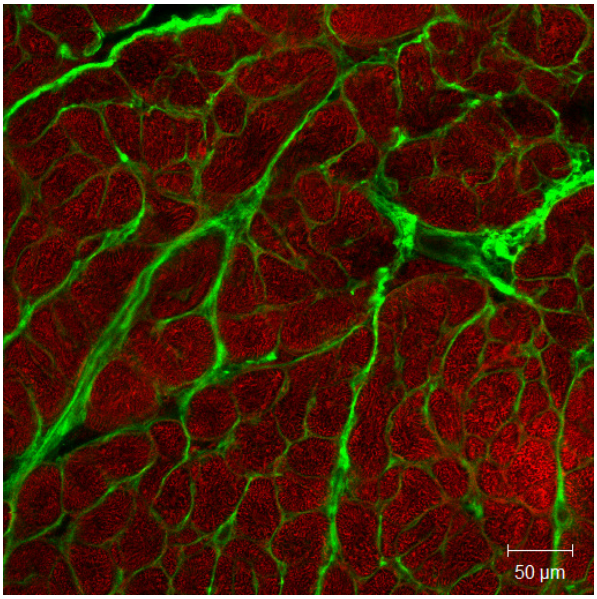

Figure 2 *Cells (red) embedded in a fibrillar collagen network (green) in a transverse section of porcine myocardium stained with Cell Tracker Orange (Invitrogen Molecular Probes) and CNA35-OG (28).*

Perturbations in the myocardium during disease

There are many different causes of heart disease, both acquired and genetic in origin. Since we intend to investigate the effect of matrix perturbations on cardiomyocyte contractility, we focus on diseases that affect the ECM or the cell-matrix interactions between the cardiomyocyte and its environment. Because the heart has only limited regenerative capacity, cardiac disease will always be associated with remodeling. In the majority of acquired cardiac pathologies, the development of fibrosis, or collagen accumulation, severely affects the extracellular environment of the cardiomyocyte. Replacement or scarring fibrosis, formed after a myocardial infarction, results in localized fibrosis, usually in combination with cardiomyocyte death. Myocardial stress or inflammation, usually caused by pressure or volume overload, results in reactive fibrosis with a diffuse distribution (29). As a result, the microenvironment of the cardiomyocyte will change, both in structure and composition. These changes in structure and composition may lead to altered mechanical cues acting of the cardiac cells. For the fibroblasts, pathological changes may induce activation and differentiation into myofibroblasts. These cells can be distinguished from fibroblasts by the presence of smooth muscle actin stress fibers (14). Differentiation of fibroblasts into myofibroblasts leads to increased contractile, proliferative and migratory properties (30). Their increased contractiliy activates mechanosensitive channels in cardiomyocytes and thereby affects conduction (31). On the other hand, myofibroblast proliferation leads to changes in the cardiomyocyte-fibroblast and cell-matrix ratios. For example, within and surrounding scar tissue formed after a myocardial infarction, increased fibroblasts numbers have been found (32). The unbalanced matrix degradation and production contribute to changes in collagen content, collagen subtypes and crosslinking enzymes, thereby affecting the ECM composition (33, 34). In dilated cardiomyopathy, it has been shown that altered ECM production and degradation led to an increased collagen I to III ratio (35). On the other hand, ischemic cardiomyopathy has been associated with a decreased collagen I to III ratio (36), which emphasizes the variability in ECM composition under pathological conditions. The formation of fibrosis also disturbs the perfect alignment of cardiomyocytes and surrounding ECM, leading to a chaotic organization of the myocardium (1). Upregulation of MMPs also leads to degradation of the structural integrity of the ECM in cardiac disease.

Next to acquired pathologies, cardiac disease can also be the consequence of mutations in genes that affect the cytoskeleton, cell signaling, cell-matrix interactions or calcium handling of the cardiomyocytes (37). To act as a syncytium, cardiomyocytes require the coordinated interaction between the cardiac cells and their ECM. A mutation in one of the proteins involved in cell-matrix interactions will therefore lead to contractility problems. A clinical example is a mutation in dystrophin, a protein of the dystrophin-glycoprotein complex found in the costameres, which is the cause of Duchenne muscular dystrophy. Truncation of this protein causes mechanical instability of the cardiac muscle during contraction, leading to loss of muscle tissue and finally resulting in dilated cardiomyopathy (38, 39). Mutations in genes coding for matrix proteins also disturb the cell-matrix interactions and may therefore affect cardiac contractility. Indeed, recently it was discovered that a mutation in Fibulin-4,

which is involved in elastic fiber assembly and known for causing aortic aneurysms, leads to cardiac remodeling and dysfunction (40).

In vitro mimics of the myocardium

For a long time, the golden standard for research on cardiac cells has been the 2D monolayer culture. Even though this has provided a lot of basic knowledge of cardiac cell function, translating these results to clinically relevant insights is difficult. By culturing the cells on plastic or glass substrates, they encounter a highly artificial environment which is stiff and isotropic, compared to the soft fibrous ECM found *in vivo*. Moreover, cell-matrix interactions which have proven to be important for cell behavior (41), are limited to the 2D cell-substrate interface. All these limiting factors have driven the development and advance of 3D *in vitro* cardiac tissue models. *In vitro* engineered cardiac tissues provide excellent models for studying normal and diseased cardiac development and physiology. Their use enables researchers to focus on cardiac contractility in real time and at the molecular, cellular and tissue level. The models can be cultured under highly controlled conditions and can give important insights into the responses of cells and ECM on isolated biochemical and biophysical stimuli. Over the past years, several groups have optimized cardiac tissue engineering methods with the goal to regenerate damaged tissue (42) or to develop models of the myocardium "in a dish" (43-45). Different approaches are used to create cardiac tissues *in vitro.* For instance, spontaneous self-assembly of cardiomyocytes and non-myocytes is used to generate scaffold free cardiac tissues (46), which may represent the most natural mimics of myocardium. Other approaches to generate engineered cardiac tissues include the seeding of cardiac cells in hydrogels (47, 48) or scaffolds (49). Developments are advancing progressively and tissues now show *in vivo*-like cell composition and electrophysiological properties, although they generally lack the right organization (50). Topological cues and mechanical loading protocols are used to improve and control the architecture of cardiac tissues (51, 52), while electrical stimulation is applied to enhance the maturation and contractile behavior of the cells (53). Depending on the model system design, functional properties such as action potentials, calcium fluxes, twitch forces, and frequencies can be measured *in vitro.* Moreover, scaling down the size of the tissues, i.e. to create microtissues, and combining these with microfabrication technology, provides a way to study multiple parameters in a high throughput fashion (54). Together, these advancements have led to the development of "heart on chips" to be used as research tool or even as drug screening platforms to approach the *in vitro* clinical trial (44, 55, 56).

However, the structure and composition of both healthy and diseased cardiac ECM are only limitedly incorporated in these tissue models. Since this is the focus of this thesis, the relevance of ECM structure and composition for engineering the cardiac microenvironment will be discussed in more detail in chapter 2.

Contractility measurement

The physiologically most relevant readout of engineered cardiac tissues is contractility. Methods to quantify cell generated forces include traction force microscopy (57) and flexible micropost arrays (58). Both methods have also been successfully employed to

measure contractility of cardiomyocytes and found forces of ~75 nN per cell on substrates with a stiffness of 15 kPa (59, 60). Force measurement on cardiac monolayers instead of single cardiomyocytes has been developed by the Parker group (43, 45, 61). Thin polydimethylsiloxane films serve as culture substrates, and contractility is measured by bending of the material due to contraction. Compared to single cell studies, this systems allows for studies on cell-cell interactions and limited manipulation of the substrate properties, such as introducing anisotropy, although it is still limited to 2D (43, 45, 62). Traction force microscopy has advanced towards 3D applications, but is only possible in a mechanically homogeneous materials such as a PEG hydrogel (63), which limits the possibilities for manipulating the cell environment.

Already in 1997, the group of Eschenhagen developed a method to culture cardiomyocyte in a 3D collagen gel that allows for tissue contraction force measurement by attaching them to a force transducer. In the following years, this method has been used and optimized by many researchers (47, 48, 64, 65). However, since these tissue are relatively large, many cells are needed to create them, and studying cellular and intracellular components requires sacrificing the sample, followed by processing and sectioning of the tissues. Boudou et al. used the same principle but then at the microscale, which allows for real-time monitoring of the structural and functional properties of an engineered cardiac tissue, which is now cultured in a microfabricated tissue gauge (μTUG) system to quantify forces (54). The engineered cardiac tissues cultured in this system are hydrogel-based and therefore suitable to easily manipulate cell and matrix composition to systematically and quantitatively investigate the effects of cellular and extracellular perturbations of cardiomyocyte contractility.

Rationale and outline

Small perturbations in the cellular and extracellular environment of cardiomyocytes can trigger the onset of cardiac disease. A better understanding of the cues that affect cardiomyocyte contractility at the cellular level during the development of cardiac disease is crucial for the improvement of specialized treatments for heart failure. Therefore, the aim of this thesis is to unravel the effects of cellular and extracellular perturbations on cardiac tissue contractility. A systematic and quantitative analysis of cell and matrix organization and composition and their effect on cardiomyocyte contractility requires a controlled evaluation of cardiac tissue function in a manipulable 3D environment of an *in vitro* cardiac tissue model.

In the first section of this thesis, we explore the cellular and extracellular perturbations that occur during heart disease. In **chapter 2** we discuss the relevance of ECM structure and composition in engineering a diseased cardiac microenvironment. The main changes in cardiac structure and matrix composition during heart disease are described. Furthermore the implementation of these properties in currently available engineered cardiac tissue models is reviewed.

To obtain a more fundamental insight into the ECM of hearts suffering from cardiac disease, in **chapter 3**, we analyzed the ECM of mouse models with acquired and genetic heart

disease ourselves. In order to identify the most important disease characteristics, we used histology, biochemical assays, and mechanical tests to determine the composition and mechanical properties of the myocardium. Additionally, we compared the protein distribution found in the native myocardium to that of our engineered cardiac tissue model.

In the second part of this thesis, an *in vitro* model system is used to study the effect of the identified perturbations on cardiomyocyte contractility, as outlined graphically in figure 3.

Starting with model development in **chapter 4**, we chose the previously described µTUG system (54, 63) as our *in vitro* model system. By using flexible microposts, the model system allows us to culture, constrain and manipulate an engineered microtissue and at the same time measure its contractility. One of the main characteristics of cardiac fibrosis is deterioration of the aligned myocardial structure. To investigate if matrix disorganization affects cardiomyocyte contractility, we adapted the design of the µTUG system to manipulate the tissue architecture of our cardiac microtissues. This allowed us to study the influence of matrix (an)isotropy on cardiomyocyte contractility.

Next to disorganization, two other features of cardiac fibrosis are increased collagen content and increased fibroblast density. In **chapter 5**, we varied the composition of the microtissues in our µTUG system to mimic these two features *in vitro* to investigate if and how they affect cardiomyocyte contractility. By gradually increasing collagen concentration or fibroblast density, we systematically studied their effect on contractility of the cardiac microtissues.

Additionally, in **chapter 6** we used our model system to study the effect of genetic mutation on cardiac tissue contractility. To detect if a mutation in fibulin-4 leads to primary defects in the cardiomyocytes, we seeded cardiac cells with a fibulin-4 deficiency in our microtissue model. This allowed us to assess the contractility of cardiac tissues composed of wild-type or fibulin-4 deficient cells.

Finally, **chapter 7** provides a summary of the most important findings of this thesis as well as a general discussion and comparison of the results with their clinical relevance and implications for future research. Finally, we discuss the benefits and limitations of the model system used in this thesis, implications for their clinical translation and future perspectives.

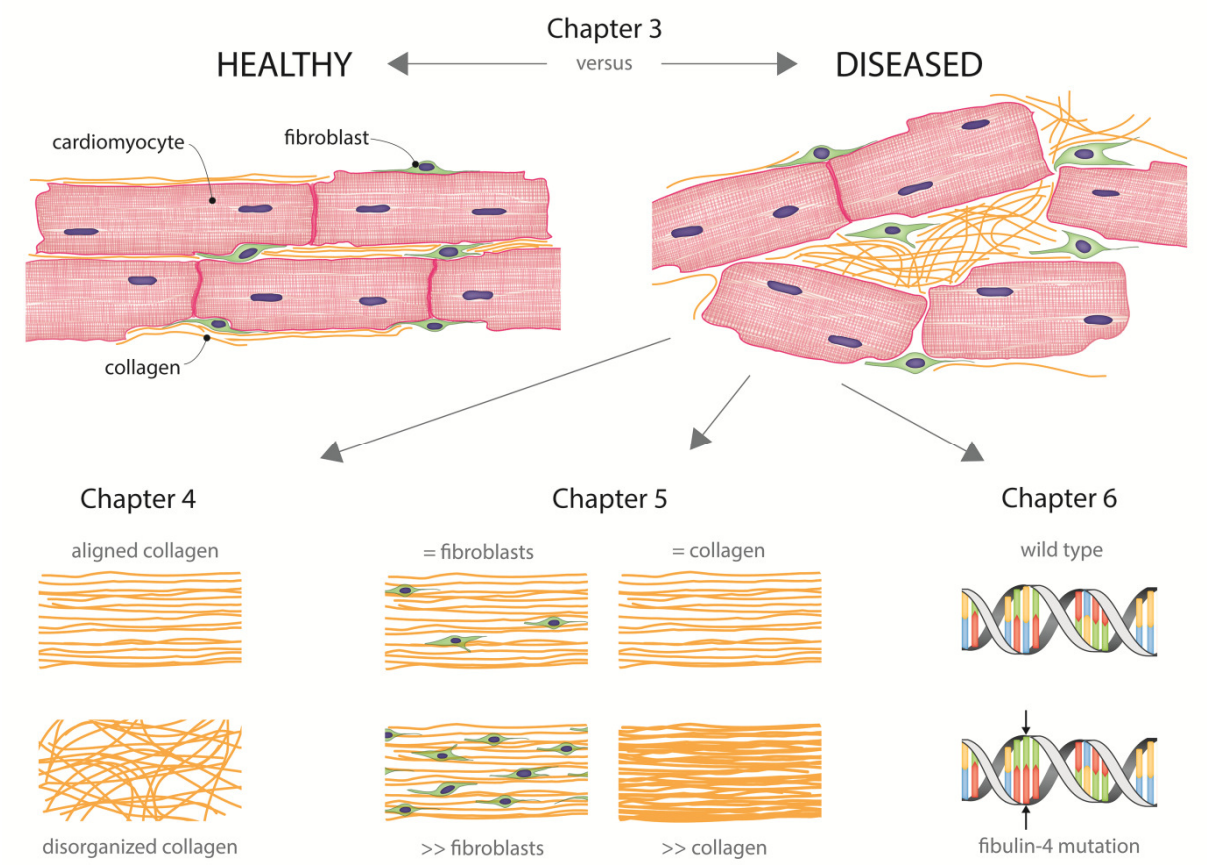

Figure 3 *Graphical abstract of the experimental investigations of cellular and extracellular perturbations on in vitro cardiac contractility. Native tissue analysis was performed in chapter 3, to identify tissue structure and composition in healthy and diseased hearts. Chapter 4, 5, and 6 concentrated on the effect of matrix disorganization, increased fibroblast density or collagen content, and a fibulin-4 mutation on cardiomyocyte contractility, respectively. Figure by Anthal Smits.*

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2

The relevance of extracellular matrix structure and composition in engineering the diseased cardiac microenvironment

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Abstract

Engineered cardiac tissues provide excellent tools to study cardiac (patho)physiology *in vitro*. These cardiac tissue models are also a platform to create disease in a dish, which can be achieved by manipulating either the cells or the matrix. During disease, not only the cells are affected, but matrix organization and composition are also disturbed. In the healthy heart, the extracellular matrix guides cellular orientation and organization, thereby facilitating efficient contraction, force transduction and electrical transmission of the cells. Pathological alterations in matrix structure or composition will therefore affect cellular function which in the end may lead to reduced cardiac output and eventually heart failure. Our knowledge about the effects of different changes in matrix composition or structure on cardiomyocyte function is still limited. Understanding how cardiomyocytes respond to these different microenvironments will support the improvement of cardiac regeneration therapies and facilitate discovery of new possible targets to treat cardiac disease. In this review, we will discuss the main changes in cardiac structure and matrix composition that occur during heart disease and how these matrix properties are implemented in engineered cardiac tissue models. Cardiac pathologies lead to alterations in structure and composition of the matrix, inhibiting normal cellular and tissue function. Systematically manipulating and quantifying these different matrix properties as well as their effects on cell and tissue function *in vitro* has revealed that both structure and composition provide important cues for cellular function. Analysis of diseased native tissue and improvements in the design of synthetic materials will lead to the development of the next generation cardiac tissue models.

Introduction

Cardiac tissue engineering comprises the seeding of cardiac cells in a 3D environment and the subsequent culture of the resulting construct under specified conditions. It has been performed for two main reasons: to create functional tissues for regeneration of the diseased or injured heart, and to develop *in vitro* model systems to study normal and diseased cardiac physiology. In addition, these tissue models have been used for the development of new regenerative therapies and drug screening. They aim to bridge the gap between two-dimensional (2D) cell models and animal models of cardiac (patho)physiology, often used to test novel therapies. It is hypothesized and indeed plausible that engineered cardiac tissues more closely mimic the three-dimensional (3D) native cardiac environment 2D cell cultures. In addition, they produce real time information with a high degree of experimental control that would never be possible in animal or human studies. Furthermore, when designed correctly, cardiac tissue models allow for high throughput screening, and quantification of tissue contractility, which is the most important measure of heart function. Another advantage of engineered cardiac tissue models is that each of the composing elements of the tissue can be modified separately to create disease models (figure 1). For example, disease-specific cells can be used, or cells can be manipulated using soluble cues to induce disease. Additionally, the extracellular matrix (ECM), which can be either native or synthetic, can be engineered to manipulate the microenvironment of the cells. In this way cardiac disease in a dish can be created to study aspects of the underlying pathophysiological mechanisms (figure 1).

Most of the currently used tissue models for cardiac disease focus on manipulating the cells by changing the culture conditions (1-3), adding proarrythmic drugs (4, 5), or using genetically affected cells (6, 7), while limited attention is paid to the cellular microenvironment. However, in disease, not only the cells are affected, but the composition and structural organization of the microenvironment changes as well. Normally, the cardiac ECM provides an anisotropic structural scaffold to guide aligned cellular distribution and organization. This accommodates contraction and relaxation of cardiomyocytes and facilitates force transduction, electrical conductance, intercellular communication and metabolic exchange within the myocardial environment (8). Despite the unequivocal influence of alterations in ECM structure and composition on cardiac function during disease, surprisingly little is known about the effects of matrix remodeling on cardiomyocyte function and survival. Understanding how cells respond to differences in matrix organization and composition will increase our insights in disease mechanisms and aid in the development and optimization of (regenerative) therapies.

This review concentrates on the implementation of ECM structure and composition in engineered cardiac tissue models and their relevance for disease modeling. To accurately mimic ECM properties, detailed knowledge of the native ECM under healthy and diseased conditions is required. Therefore, we first summarize the principle changes in structure and composition of cardiac ECM during disease development, followed by a discussion on current research related to ECM structure and composition in *in vitro* cardiac tissue models.

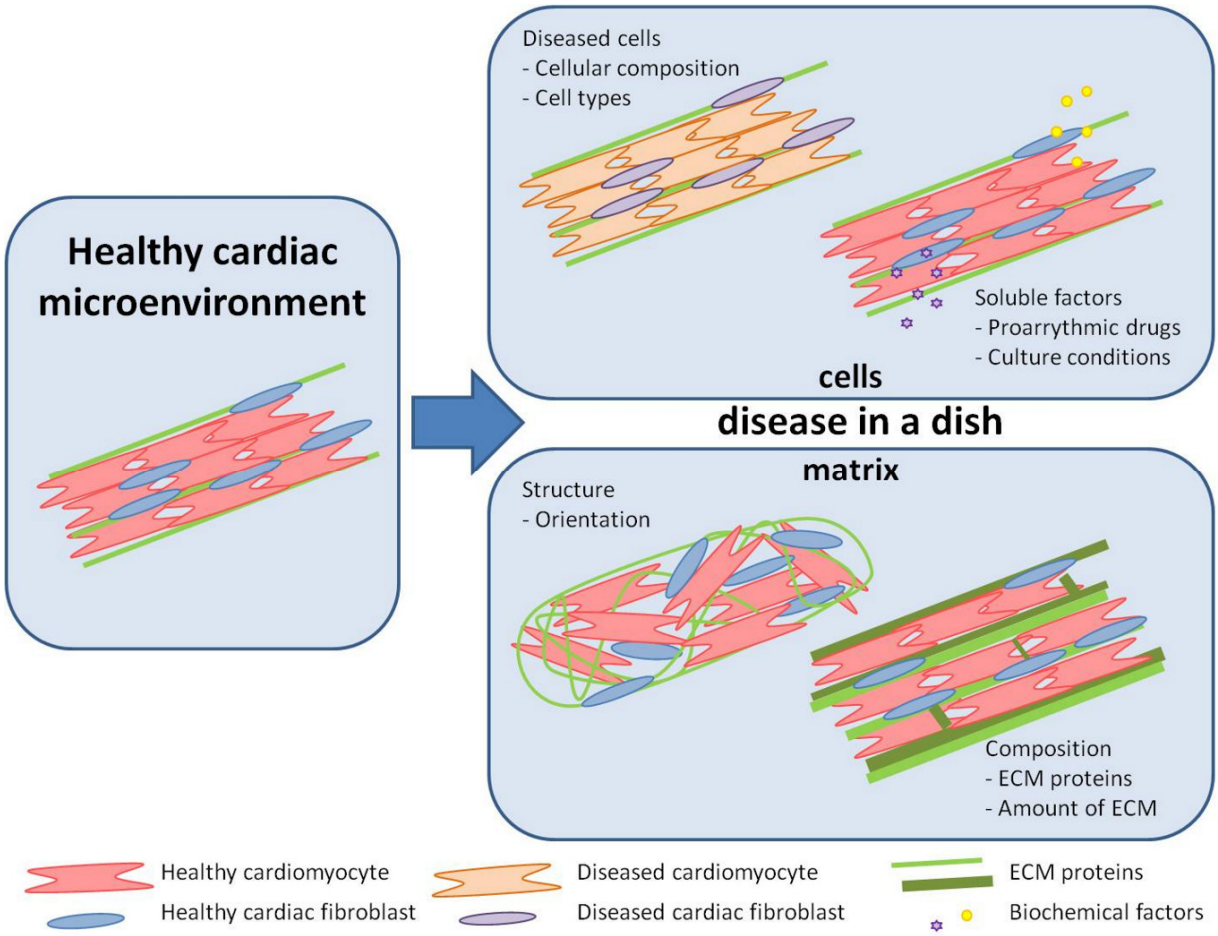

Figure 1 *Engineering the cardiac microenvironment. Diseased cell types or changing cell ratios as well as biochemical cues can be used to manipulate the cardiac cells directly, while matrix structure and composition can be manipulated to study their effect on cardiac cell and tissue function.*

Discussion

In vivo ECM structure and composition of the diseased heart

In the majority of cardiac pathologies, the accumulation of ECM or fibrosis is an integral part of the compensatory and repair mechanisms leading to chamber remodeling and functional adaptation (9). The distribution of myocardial fibrosis varies according to the underlying pathology. Replacement or scarring fibrosis occurs after cardiomyocyte loss, typically after a myocardial infarction and results in localized fibrosis. On the other hand, reactive fibrosis following myocardial stress or inflammation occurs in most cardiac diseases with pressure and volume overload. The latter type of fibrosis has a progressive onset and is characterized by a diffuse distribution (10).

Fibrosis has profound consequences for cardiac function due to adverse cardiac remodeling, changes in ventricular stiffness, functional deterioration and the possible development of heart failure. Under normal conditions, mainly cardiac fibroblasts maintain the ECM by producing and degrading the ECM proteins in a well-balanced manner. In heart disease,

changes in biomechanical cues related to mechanical stress induce fibroblast transformation into myofibroblasts (11). Furthermore, circulating macrophages, smooth muscle cells, endothelial cells and fibrocytes may also differentiate into myofibroblasts. These cells produce a different ECM, which is deposited in a chaotic network of collagen fibers, thereby altering the matrix architecture (8) (figure 2A, B). Additionally, myofibroblasts contribute to changes in collagen content, conformational changes in type of fibrillar collagen and an increase in cross-linking (11).

Collagen deposition in the extracellular compartment comprises mainly of collagen type I and III. Cardiac disease can cause a shift in the ratio of these collagen subtypes and thereby alter the composition of the matrix (12). A shift in the ratio of these collagen subtypes was thought to be responsible for increased chamber stiffness, although changes in total myocardial collagen concentration or shifting phenotypes do not necessarily translate into increased myocardial stiffness. A loss of collagen support due to increased degradation of mature cross-linked collagen which in turn is replaced by newly synthesized collagen with decreased cross-linking will lead to breaks or tears in the myocardial matrix (12). Other matrix components like fibronectin (figure 2C, D) and proteoglycans which are involved in cell-matrix interactions are also altered during cardiac disease and thereby affect cellular function and promote the formation of fibrotic tissue. The change in dynamic interactions between cells and ECM due to fibrosis is detrimental for contractile synchrony and cardiomyocyte function (13). The changes in ECM modify the microenvironmental signals that cardiomyocytes receive, leading to alterations in gene expression associated with cell morphology and contractile function (11). Furthermore, ECM modification contributes to arrhytmogenesis through impaired conduction and subsequent generation of reentry circuits (9).

Interruptions of the fibrillar collagen matrix alter cell support, geometric alignment and coordination of myocardial excitation-contraction coupling and weaken attachment to the ECM. Change in MMP activity in fibrosis influences bidirectional signaling via integrins and dystrophin (figure 2E, F), thereby weakening the cell-matrix association and alter the response of cardiomyocytes to stress or pressure-overload (14). Disruption of cell-matrix interaction also contributes to sliding displacement (slippage) of cardiomyocytes promoting cell death and leading to a decrease in the number of muscle layers (14). Given the impact of ECM changes on cellular function in cardiac disease, these characteristics should also be incorporated in *in vitro* cardiac tissue models.

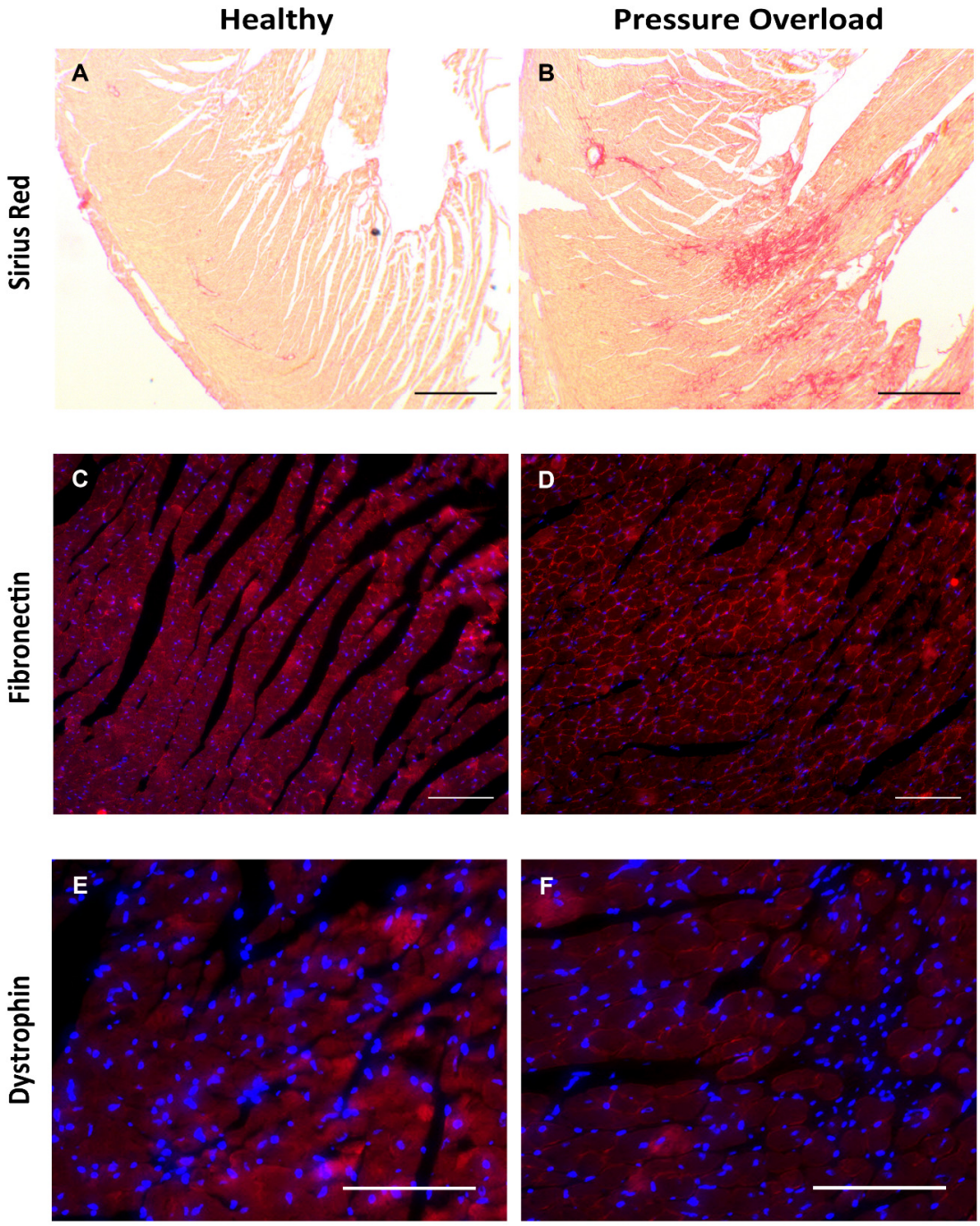

Figure 2 *Overview of changes in matrix composition during cardiac disease in mouse hearts due to pressure overload (9 weeks of transverse aortic constriction). Presence of collagen, visualized with Sirius Red, shows increase in collagen content in hearts with pressure overload (A, B). Fibronectin is induced in the myocardial matrix during pressure overload (C, D). Cell-matrix interaction via dystrophin is induced during pressure overload due to increase of dystrophin (E, F). Scale bars: A,B: 500 µm, C-F: 100 µm.*

Structure and composition of the ECM in engineered cardiac tissue

Engineered cardiac tissues are usually created between two anchoring points to obtain an aligned tissue representing the healthy anisotropic cardiac architecture (4-6, 15). The composition of most currently used engineered cardiac tissues is a combination of either collagen type I, fibrin and/or matrigel (16). Although mimicking essential parts of the ECM, this is of course a simplification of the complex native environment. In the next part, studies that specifically focused on mimicking at least part of the native cardiac structure or composition or studied the effect of changes in structure or composition on cardiac tissue function will be discussed.

Cardiac Structure:

The architecture of the collagen network in the healthy heart induces proper alignment of the cells, thereby enabling end to end coupling of the cardiomyocytes which is essential for the myocardium to act as a syncytium. During disease, disarray of the cells and matrix may occur. Systematic research using *in vitro* models has provided insight in the essential role for anisotropy in cardiac function. In studies with cardiomyocyte monolayers, anisotropy is mimicked by microcontact printed lines of ECM protein on a 2D substrate (17-19). These printed lines act as extracellular cues to create anisotropy in monolayers of rat neonatal cardiomyocytes thereby improving calcium handling compared to cells seeded on homogenous protein layers (17). Microcontact printing was also used on elastomeric thin films that bend upon beating of the cardiomyocytes, allowing for contractility measurements (18). Rat neonatal cardiac cells seeded on these elastic substrates with anisotropic orientation exerted higher forces randomly oriented cells (19). Wang et al. created alignment of cells in 2D by using wrinkled substrates to provide topological cues to human embryonic stem cell derived cardiomyocytes and showed that anisotropy significantly reduced the occurrence of arrhythmia in 2D cultures (20).

In 3D, matrix disarray which is frequently observed in diseased hearts has been mimicked by changing the anchoring points of the tissues. While anisotropic constraints on the tissue induce alignment of both cells and matrix, disarray can be achieved by using isotropic constraints. Our group has previously used collagen/matrigel microtissues cultured between PDMS microposts as constraints to induce both alignment and disarray of the matrix (21). We showed that alignment of mouse neonatal cardiomyocytes increased the homogeneity of force distribution in the tissues as compared to the isotropically constrained tissues. Another 3D study using fibrin gels created cardiac tissues in tubular molds and showed that anisotropy increased the contractile forces of engineered cardiac tissues (22). Furthermore, anisotropy in 3D network-like patches with rat neonatal cardiomyocytes also improved action potential propagation (23). Together these studies indicate that an anisotropic matrix structure is essential for proper cardiac tissue function.

Matrix composition:

Besides the structural role of ECM in guiding anisotropy, the complex composition of the cardiac environment plays an important nonstructural role in mediating cell-cell and cell matrix interaction. The cardiac matrix consist of fibrillar collagens (Type I and III), fibronectin, proteoglycans and basement membrane proteins (laminin and Col IV) which contribute to cell adhesion, cell survival, proliferation, differentiation and function via cell-matrix interactions. Since these interactions are important in regulating stem cell behavior (24), cardiac tissue models have been frequently used to study the effect of different microenvironments on differentiation and maturation of stem cell derived cardiomyocytes or to study stem cell therapy. To mimic stem cell therapy for cardiac regeneration, Valarmathi et al. engineered cardiac tissues using embryonic stem cell derived cardiomyocytes seeded on collagen tubes in co-culture with bone marrow stromal cells (25). The engineered cardiac environment induced differentiation of the stem cells into cardiomyocyte like cells. Similarly, Dengler et al. used neonatal rat cardiomyocytes to engineer a cardiac microenvironment which was used to examine the injection of embryonic stem cells and cardiac progenitor cells (26). After injection into the cardiac tissues, cardiac progenitor cells differentiated into the cardiomyogenic lineage, while the embryonic stem cells did not (26).

These *in vitro* models of stem cell injection recapitulate some of the in vivo results, but they lack a native like environment and more specifically a diseased environment. Researchers have tried to resolve this issue by the use of decellularized native matrices or matrix produced by cells from either healthy or diseased heart tissue. In a recent study performed by Castaldo et al. cardiac fibroblasts were isolated from healthy or end-stage heart failure cardiac tissues and used to deposit a layer of ECM proteins (27). Cardiac stem cells were seeded on this Biomatrix in order to assess its differentiation potential. Although both healthy and diseased Biomatrix prevented cells from apoptosis, only the healthy Biomatrix was found to stimulate their proliferation and migration (27). In another approach by Sullivan et al., decellularized healthy and infarct ECM was used as coating for 2D substrates (28). These substrates were seeded with mesenchymal stem cells to test the cardiac differentiation potential of the different ECMs. The healthy ECM was shown to promote early cardiac differentiation when compared to infarct ECM (28). In a 3D approach, native decellularized ECM powder mixed with collagen was used as a hydrogel to engineer cardiac tissues (29). Hydrogels containing 75% cardiac ECM promoted better differentiation of human embryonic stem cells into cardiomyocytes when compared to a collagen gel without cardiac ECM but with supplemental growth factors.

While these studies show that a more native like ECM composition enhances the differentiation of stem cells into cardiomyocytes, the effect of composition changes on contractility of matured cardiomyocytes is largely unknown. Boudou et al. showed that collagen concentration influences cell contractility (15). Cardiac microtissues were created using a collagen/fibrinogen hydrogel, and to examine the influence of matrix composition on contractility, the collagen concentration was varied. Both dynamic and static stresses were significantly lower in tissues with higher collagen concentration (15). While higher

collagen concentration led to denser and stiffer matrices, it is unclear whether these lower stresses are due to changes in composition or stiffness of the matrix.

Future challenges:

Current cardiac tissue models to study stem cell therapy mostly try to mimic the healthy cardiac microenvironment, while *in vivo* these cells will be injected in a diseased environment. Therefore, it would be relevant to mimic the diseased host environment *in vitro* when studying these processes.

As previously shown, the effect of ECM composition on cardiac tissue function has mainly been studied by using native decellularized matrices, although this decellularized native ECM is hard to control or manipulate. On the contrary, synthetic materials would provide the opportunity to manipulate all ECM components separately. Unfortunately, currently used synthetic materials fail to mimic the complete native environment. Future improvements of synthetic materials that will enable researchers to mimic of the different component of the ECM will help to extend our knowledge on the effect of diseased matrix composition. Furthermore, analysis of native tissue structure and composition could provide additional input for disease specific cardiac tissue models.

Manipulating either the structure or composition of the microenvironment, may result in a changed matrix stiffness, which in turn will influence cardiomyocyte contractility (15, 30, 31). Furthermore, it has been shown that changing only the constraints of the tissue to manipulate the anisotropy resulted in tissues with a different matrix composition after two weeks of culture (22), indicating that all these properties are interacting with each other. Studies that allow for systematic manipulation and quantification of different matrix properties, including matrix structure, composition and stiffness will aid in unraveling the complex dynamic interaction between cardiac cells and their microenvironment.

Conclusion

Both structure and composition of the native cardiac microenvironment provide important cues for normal cardiac function, which change upon development of heart disease. Research regarding the effect of changes in matrix structure and composition on cardiomyocyte functionality has highlighted some key functions of the different matrix properties. While the microstructure of the ECM was shown to be important for cardiomyocyte contractility, matrix composition seems to be essential for stem cell differentiation and cardiomyocyte maturation. Therefore, it is likely that regeneration of the heart can only be achieved when restoring both matrix structure and composition. Improvement in the design of synthetic materials and better knowledge of the in vivo diseased microenvironment will provide input for development of the next generation cardiac tissue models.

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3

Cardiac matrix analysis of genetic and acquired cardiac disease: Relevance for *in vitro* disease modeling

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Abstract

Fibrosis, a hallmark of cardiac disease, changes myocardial extracellular matrix composition that eventually may hamper ventricular function. Since therapeutic options for patients with ventricular dysfunction associated with fibrosis are limited, understanding the underlying pathology is crucial for the development of new therapies. Knowledge regarding the native extracellular matrix composition of the diseased ventricular wall can be used as guidelines to optimize *in vitro* 3D cardiac tissue models. Here, we investigate the changes in cardiac extracellular matrix properties of a genetic (*mdx)* and acquired heart disease (TAC) model and compare this to the matrix organization in currently available engineered cardiac microtissues. Both the *mdx* mouse cardiomyopathy as well as cardiac pathology after aortic constriction (TAC) showed patchy fibrosis in the left ventricle. Patchy fibrosis in *mdx* hearts was associated with decreased myocardial stiffness, while in TAC hearts the stiffness was unaffected. The *in vitro* microtissues showed homogeneous distribution and production of matrix proteins. Therefore, current cardiac microtissues need to be modified to incorporate the *in vivo* observed spatial distribution of fibrosis. This is necessary to translate the effect of fibrosis from a cellular to tissue level, which can be achieved by multiscale or macroscale approaches. These approaches will provide new insight in the influence of patchy fibrosis on cardiac contractility and will contribute to the improvement of treatments for heart disease.

Introduction

The cardiac extracellular matrix (ECM) is a complex meshwork of fibers which provides shape to the heart and support the cells that compose the heart. The ECM controls cell behavior by interacting with the cells and thereby regulates adhesion, proliferation, migration, performance and survival. Furthermore, the ECM mediates intracellular communication and metabolic exchange between cells (1, 2).

In the healthy heart, the ECM is a well aligned network which orchestrates contractility of the heart by facilitating force transmission between cardiac muscle cells (3). During cardiac disease, the aligned organization is deteriorated due to death of cells and/or a ceaseless imbalance in dynamic synthesis and breakdown of ECM proteins in the myocardium, resulting in excess production and accumulation of ECM proteins referred to as fibrosis (4, 5). Cardiac fibrosis is a hallmark of heart disease and two forms of fibrosis can be recognized; reactive and reparative fibrosis. Reactive fibrosis is an adaptive response of the heart aiming at preservation of the generative capacity to pump the blood through the body. This type of fibrosis starts by a diffuse increase of ECM around the coronary arteries (perivascular fibrosis) and is followed by distribution within the cardiac interstitial space (interstitial fibrosis). Reactive fibrosis has mostly been described in patients with hypertension and diabetes mellitus, but is also present in the aging heart and in hearts suffering from pressure-overload. An important characteristic of this type of fibrosis is the absence of cell loss (6, 7).

On the other hand, replacement or scarring fibrosis corresponds to the replacement of cardiomyocytes by fibrosis after cell death. As soon as cardiomyocyte integrity is affected, the lost cells are replaced by a fibrotic scar to preserve tissue integrity, and this is therefore referred to as reparative fibrosis. Reactive fibrosis will ultimately lead to replacement fibrosis in later stages of the disease where cellular damage and cell death appear. Both forms of fibrosis lead to changes in myocardial architecture and myocardial stiffness, thereby hampering ventricular contraction and leading to myocardial dysfunction (6, 7).

Cardiac pathology, including fibrosis, can have genetic as well as acquired causes, and several mouse models are available to study diseases of the heart. The *mdx* mouse is a model for the genetic disorder Duchenne muscular dystrophy. These mice have a premature stop codon in exon 23 of the *Dmd* gene, leading to the production of a truncated non-functional dystrophin protein. Dystrophin is expressed in skeletal and cardiac muscle fibers and provides mechanical stability during contraction. Lacking dystrophin, patients with Duchenne muscular dystrophy suffer from chronically damaged muscles after regular exercise, leading to loss of muscle tissue and function. Most patients also exhibit clinically relevant cardiac disorders such as depressed left ventricular function accompanied with electrocardiogram abnormalities and dilation of the left ventricle (8, 9).

Acquired heart diseases, on the other hand, develop secondary to another disease, condition or environmental factor. One of the most common forms of acquired heart disease is hypertrophic cardiomyopathy, as a result of pressure overload. Excessive pressure overload refers to the inability of the heart to pump sufficient amounts of blood through the

body due to an abnormal high arterial pressure. In mice, transverse aortic constriction (TAC) is a stable microsurgical technique, which induces left ventricular pressure overload leading to left ventricular hypertrophy and at a later stage to heart failure. The heart responds to the overload through a number of structural alterations on both the cellular and matrix level, including fibrosis. These alterations result in impaired myocyte contractility and thereby to dysfunction of the heart (10).

To date, cardiac fibrosis is still a cause of heart failure. Since only a few therapeutic options are available for patients with ventricular dysfunction associated with fibrosis, new approaches addressing the underlying pathology are urgently needed. First, knowledge regarding the ECM composition and architecture of the ventricular wall is fundamental and to our understanding this knowledge will improve tissue models of myocardial ECM thereby enabling the development of new treatment strategies. *In vitro* cardiac tissue models are the next generation approach since they bridge the gap between animal models and human patients (11). When properly designed, these tissue models mimic the 3D native cardiac environment more closely compared to 2D cultures, which is important for the translation of research outcomes to clinical applications. An advantage of engineered cardiac tissues is the high degree of experimental control. However, analysis of native heart tissue structure and composition is crucial to provide the necessary input to create disease specific cardiac tissue models. Therefore, in this study, changes in cardiac ECM properties during the development of heart pathology due to a genetic (*mdx)* or acquired heart disease (TAC) will be investigated in order to optimize current available engineered cardiac microtissues.

Materials and Methods

Ethical statement

All animal experiments were performed according to the Directive 2010/63/EU of the European Parliament and approved by the local Animal Care and Use committee of the Leiden University Medical Center and Eindhoven University of Technology. Animals were housed under standard laboratory conditions with 12-h light-dark cycles and received food and water *ad libitum*.

Animal models

C57Bl/10ScSnJ (control) and *mdx* (C57Bl/10ScSn-DMD^{mdx}/J) male mice at 10 months were sacrificed by cervical dislocation and hearts were isolated (8, 9). To induce pressure overload (TAC) in the left ventricle, 11 weeks old male C57BL/6 were anesthetized with 2.5%vol/isoflurane in 0.2 L/min O_2 and 0.2 L/min medical air and intubated for mechanical ventilation. To maintain the body temperature at 37 ^{o}C, animals were placed on a heating pad. Buprenorphine (0.1mg/kg subcutaneously injected) was administered for analgesia. Surgical procedures were performed as described previously (12). Briefly, a small incision was made above the first intercostals space and just lateral form the sternum. The aortic arch was tied off (6-0 silk suture) together with a 27G (Ø0.42 mm) needle between the brachiocephalic artery and the left common carotid artery. Blood flow was restored by

immediate removal of the needle (10, 12). Nine weeks after surgery hearts were excised as well as the hearts from age matched male controls (21 weeks).

Tissue preparation

After excision the hearts were perfused with PBS and cryopreserved in medium (DMEM High glucose) (Gibco, Paisly, UK) supplemented with 10% fetal bovine serum (Greiner Bio-one, Monroe, NC) and 10% dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany). The freezing process minimized the formation of ice crystals within the tissue by freezing at a rate of -1 ^{o}C/minute. Once frozen, hearts are stored below -80 ^{o}C before further examination. Before analysis, cyropreserved hearts were quickly thawed and washed with cold PBS (Sigma, St. Louise, MO).

Biaxial tensile testing

Strain at failure of healthy and diseased myocardium was characterized with a biaxial tensile tester (CellScale, Waterloo, Canada) (13). Briefly, hearts (n=3-5 per group) were split in a left and right side and each side was positioned between clamps such that the X-axis corresponded to the Apex-to-Base direction and the Y-axis to the anterior-posterior direction. Biaxial loading was applied to an area of minimally 3.75 mm by 3.75 mm to maximally 4mm by 4mm. Displacement-controlled equibiaxial protocols were implemented with a constant rate. For each sample, twenty-five preconditioning cycles were applied uniaxial in both directions to a maximum stretch of 120% to reduce tissue hysteresis. After preconditioning, a 120 sec rest period was included before the sample was stretched to 500% in both X and Y directions over a period of 240 sec. Testing was performed at ambient temperature and samples were kept in PBS throughout the test. The resulting force-strain curves were used to calculate strain at failure (%) in Matlab (The Mathworks, Inc.).

Micro-indentation

To determine local material stiffness, micro-indentation was performed. For this purpose, cryopreserved hearts (n=2-4 per group) were quickly thawed and cut parallel to the myocardial wall with a cryotome (Microm HM 550, Adamas, Rhenen, the Netherlands) in 500 µm thick slices. Due to considerable variation per location, 3 different locations per heart sample were investigated and two consecutive indentation tests were performed per location (14, 15). A spherical indenter with a diameter of 2 mm was used to indent the tissue slices. During indentation, force and indentation depth were recorded. The experimental force-depth curves were fitted to numerical simulations of indentation to determine the Young's Modulus using Sepran and Matlab as described before (14, 15). Isotropic neo-Hookean behavior and homogeneity of the heart samples was assumed.

Histological examination

For histology, hearts were fixed in 3.7% formaldehyde (Merck) in PBS (Sigma) for 24 hr at 4 ^{o}C and further processed for paraffin immunohistological investigation. Subsequently, 5 µm serial sections were mounted on polylysine coated microscope slides (Menzel-Gläser, Braunschweig, Germany). Serial sections were stained with picrosirius red according to standard histological procedures to confirm presence of collagen and analyzed using a light

microscope (Carl Zeiss, Oberkochen, Germany). Semi-quantification of picrosirius red staining was performed using ImageJ by comparing the percentage of fibrosis to the total amount of tissue within images. A color threshold was set and a minimum of 6 images per left and right ventricle throughout the heart was analyzed.

For immunofluorescence, sections were subjected to several antigen retrieval and blocking protocols depending on the primary antibody (table 1). Both blocking agents and primary antibodies were diluted in PBS (Sigma). Sections were incubated overnight and subsequently incubated with a fluorescent secondary antibody (table 1). After nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (Sigma), sections were mounted with Mowiol (Sigma). A fluorescent microscope (Axiovert 200; Carl Zeiss) was used to analyze the stained sections.

Table 1 *Antibodies used for immunohistochemistry*

Antigen	Source	Isotype	Species	Antigen Retrieval	Blocking agent	Dilution solution	Dilution	Label
Collagen I	Abcam (AB34710)	IgG	Rabbit	Citrate (pH6.0)	5% protifar	0.5% protifar	1:100	-
Collagen III	Abcam (AB7778)	IgG	Rabbit	Pepsin (pH2.0)	5% protifar	0.5% protifar	1:100	-
Fibronectin	SA (F3648)	IgG	Rabbit	Tris-EDTA (pH9.0)	10% HS	1% HS	1:200	-
α-Actinin	SA (A7811)	IgG1	Mouse	-	10% HS	1% HS	1:500	-
Phalloidin	SA (49409)	-	-	-	10% HS	1% HS	1:200	Atto 488
Rabbit IgG	IG (A31572)	IgG	Donkey	-	-	-	1:300	Alexa 555
Mouse IgG1	MP (A21127)	IgG1	Goat	-	-	-	1:300	Alexa 488

Abbreviations used in this table: SA, Sigma Aldrich; IG, Invitrogen; MP, Molecular Probes; HS, horse serum.

Biochemical assay for detecting fibrosis

Heart samples used for biaxial tensile testing were subsequently analyzed with biochemical assays for the amount of glycosaminoglycans (GAGs) and a major component of collagen, hydroxyproline (HYP). Left and right heart sides were divided in three parts and samples were lyophilized and digested overnight in papain solution (100 mM phosphate buffer [pH=6.5], 5 mM L-cystein, 5 mM EDTA, and 140 µg papain per mL (all Sigma)) at 60 ^{o}C. Digestion supernatant was collected, centrifuged for 10 min at 12000 rpm and stored at -20 ^{o}C for further analysis. Undigested heart tissue was lyophilized and subjected to a second digestion overnight. Samples were centrifuged and analyzed at the same time as the first digestion samples. To determine the amount of sulfated GAGs in the digestion supernatant,

a portion of the supernatant was subjected to a modification of the assay described by Farndale et al. (16). In short, 40 µl of diluted digestion supernatant and a reference of shark cartilage chondroitin sulfate (Sigma) was pipetted in duplicate in a 96-wells plate. Subsequently, 150 µl dimethylmethylene blue (DMMB) (Sigma) was added and the absorbance was measured at 540 nm. An assay based on the methods of Huszar et al. with trans-4-hydroxyproline (Sigma) was used to determine HYP quantity (17).

Cell isolation and culture

Cardiomyocytes and cardiac fibroblasts were isolated from neonatal mouse hearts as described previously (18, 19). Cells were cultured in high glucose DMEM (Lonza, Verviers, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (Greiner bio-one), 1% L-glutamine (Sigma), and 1% penicillin/streptomycin (Sigma). For depletion of most of the fibroblasts during isolation, cells were seeded in uncoated culture flasks. Non-attached cells were considered to be the cardiomyocyte-enriched cell population, containing cardiomyocytes and fibroblasts, which were plated at a concentration of $5x10^4$ cells/cm^2 in pre-coated well plates with fibronectin (10 µg/ml) (Tebu-Bio, Heerhugowaard, the Netherlands) dissolved in PBS (Sigma) containing 1% porcine gelatin (Sigma). After four days of culture microtissues were fabricated (18).

Microtissue Seeding

To create cardiac microtissues, a gel mixture of 50% collagen type I (BD Biosciences), 39% culture medium, 3% 0.25 M NaOH, and 8% growth factor-reduced Matrigel (BD Biosciences) was added to a previously designed µTUG system (18). Briefly, the hydrogel was spun into the microwells by centrifugation. After trypsinization, the cardiomyocyte-enriched population was resuspended in the hydrogel at a density of $1x10^6$ cells/ml. Samples were centrifuged again to spin the cells into the microwells. After spinning, the excessive gel was aspirated leaving biaxially constrained microtissues (18).

Immunofluorescence for confocal microscopy

Immunohistochemistry on microtissues was performed at day seven of culture with the same antibodies as described for the mouse heart tissue (table 1). Microtissues were fixed in 3.7% formaldehyde (Merck) for 10 min, followed by storage in PBS (Sigma) at 4 ^oC. Samples were permeabilized with 0.5% Triton-X-100 (Merck) and incubated with 10% horse serum (Gibco) prior to incubation with primary antibodies overnight. The next day, samples were washed with PBS (Sigma), incubated with secondary antibodies and counterstained with DAPI (Sigma). Microtissues were imaged on a two-photon confocal laser scanning microscope (Carl Zeiss).

Statistics

All data are represented as mean ± standard deviation. Statistical analyses were performed with GraphPad Prism software. Column statistics indicated that all data points had no Gaussian distribution therefore the nonparametric Mann-Whitney test and Kruskal-Wallis for multiple comparisons between groups with Dunns post-hoc test were performed. Differences were considered statistically significant when $P<0.05$.

Results

Cardiac morphology of mdx and TAC hearts

Mdx mice hearts had normal heart weight and fluid content and morphometric analysis of cross sections showed normal cardiac diameter compared to wild type littermates (table 2). The analysis of picrosirius red stained sections showed that fibrosis was present throughout the heart (figure 1). Most severe fibrosis was observed in the entire right ventricle. Interestingly, in the left ventricle patches of interstitial fibrosis were observed surrounded by areas of healthy myocardium (figure 1A-B, E-F). Furthermore, semi-quantitative image analysis revealed a significant higher area expressing collagen present in *mdx* hearts compared to healthy hearts (1.8x higher, P<0.001) (figure 1C). In the *mdx* right ventricle (RV) the area of collagen was 2.2x larger (P<0.01) (figure 1D), while in the left ventricle (LV) the collagen area is 1.7x larger compared with healthy hearts (P<0.01) (figure 1G). When only the patchy areas of fibrosis were considered in the *mdx* left ventricle, there was 2.8x more collagen present compared to healthy hearts (P<0.001) and 2.7x more compared to healthy areas in the *mdx* left ventricle were no fibrosis was present (P<0.01) (figure 1H). The amount of collagen present in the healthy areas in the *mdx* left ventricle was comparable to the amount found in the left ventricle of wild type hearts (figure 2H). Hearts of mice 9 weeks after aortic constriction had higher left ventricular weights (P<0.05) and larger, although not significantly, heart diameters (table 2) than sham-operated controls. Histology showed perivascular fibrosis and patchy interstitial fibrosis throughout the heart. Patchy fibrosis was especially found in the left ventricle and ventricular septum (figure 2A-B, E-F), while the right ventricles seemed normal.

Table 2 *Cardiac conditions of mdx, TAC and healthy hearts*

	Healthy	***mdx***	**P-value**
Heart weight (mg)	183.9±8.5	187.9±21.0	NS
Heart diameter (µm)	4050±150.0	3800±173.2	NS
Fluid content whole heart (%)	80.87±0.99	81.41±1.93	NS
	Healthy	**TAC**	**P-value**
Left ventricular weight (mg)	72.7±6.3	120.0±36.9	*P<0.05
Heart diameter (µm)	3750±450.0	4490±740.3	NS
Fluid content LV (%)	84.81±4.11	82.06±3.52	NS

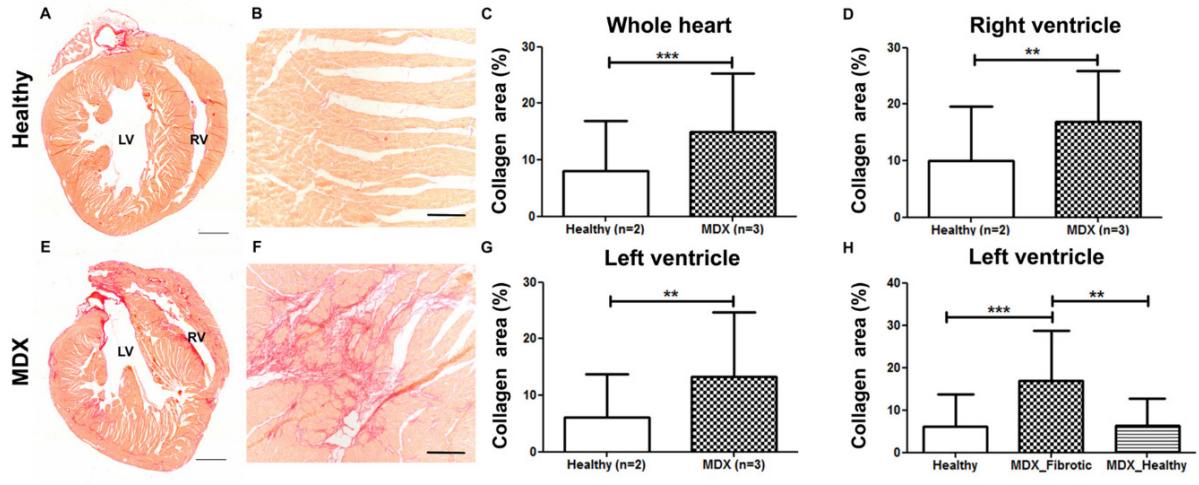

Figure 1 *Myocardial fibrosis in mdx mouse hearts. Low-power photomicrographs of picrosirius red-stained sections of healthy (A) and mdx (E) transgenic hearts from mice aged 10 months. Enlargement pictures of healthy (B) and fibrotic myocardium (F). Bar graphs represent areas of collagen (%) determined from the whole heart (C), right ventricle (RV) (D) and left ventricle (LV). Fibrotic areas in the left ventricle of mdx hearts were compared to "healthy" areas in mdx hearts and healthy control hearts (H). Scale bar A/E: 1000 µm and B/F: 100 µm. **P<0.01 and ***P<0.001.*

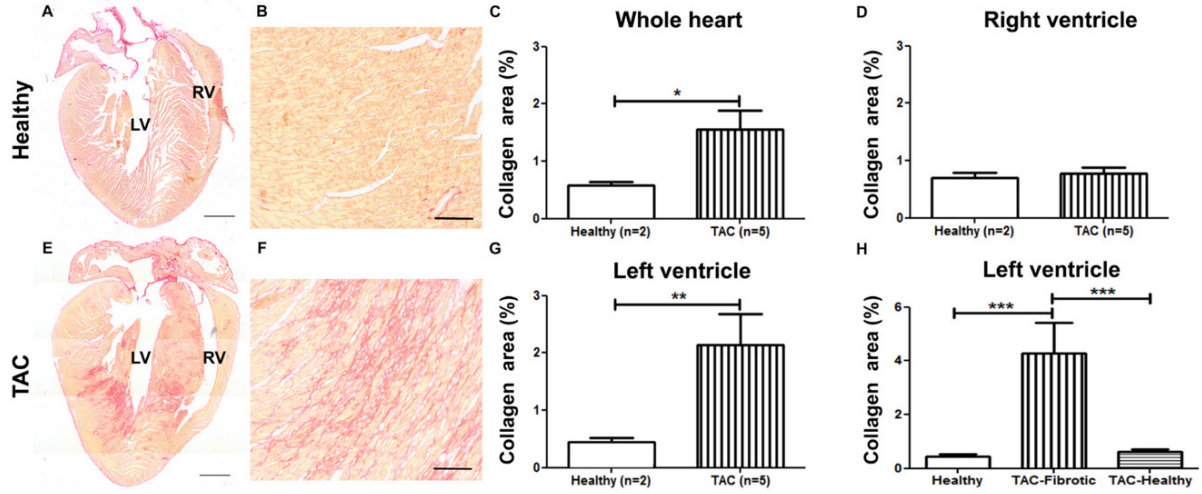

Figure 2 *Myocardial fibrosis in mouse hearts with transverse aortic constriction (TAC). Low-power photomicrographs of picrosirius red-stained sections of healthy (A) and TAC hearts 9 weeks after constriction (E). Magnification pictures of healthy (B) and fibrotic myocardium (F). Bar graphs represent areas of collagen (%) determined from the whole heart (C), right ventricle (RV) (D) and left ventricle (LV) (G). Fibrotic areas in the left ventricle of TAC hearts were compared to "healthy" areas in TAC hearts and healthy control hearts (H). Scale bar A/E: 1000 µm and B//F: 100 µm. *P<0.05, **P<0.01 and ***P<0.001.*

The area of collagen increased 1.8x in the whole heart (P<0.05) (figure 2C), there was no significant difference between the fibrotic areas in the right ventricle of the TAC hearts (0.78%±0.57%) compared to the right ventricle (0.70%±0.27%) of age-matched sham-operated animals (figure 2D). Therefore, for further analysis only the left ventricle of the TAC hearts were taken into consideration. Image analysis revealed 4.8x more collagen in the TAC compared to healthy left ventricles (P<0.01) (figure 2G). When only the patchy areas of fibrosis were taken into consideration in the TAC left ventricles, there was 9.6x more collagen present compared to sham-operated hearts (P<0.001) and 6.8x more compared to healthy areas in the TAC left ventricle where no fibrosis was present (P<0.01) (figure 2H). Immunohistological analysis of patchy fibrotic lesions in *mdx* left ventricles showed presence of collagen I, III and fibronectin (figure 3). In areas where there were no fibrotic lesions, the collagen I surrounding the cardiomyocytes was increased compared to healthy hearts. The distribution and presence of collagen III in *mdx* hearts resembled the healthy myocardium, while fibronectin was less organized in *mdx* heart. In contrast, the distribution of these matrix components in the fibrotic lesions in TAC hearts showed only the presence of collagen I (figure 4). In the myocardial tissue surrounding these fibrotic lesions, collagen I and III were increased while fibronectin was less organized in ventricles 9 weeks after TAC.

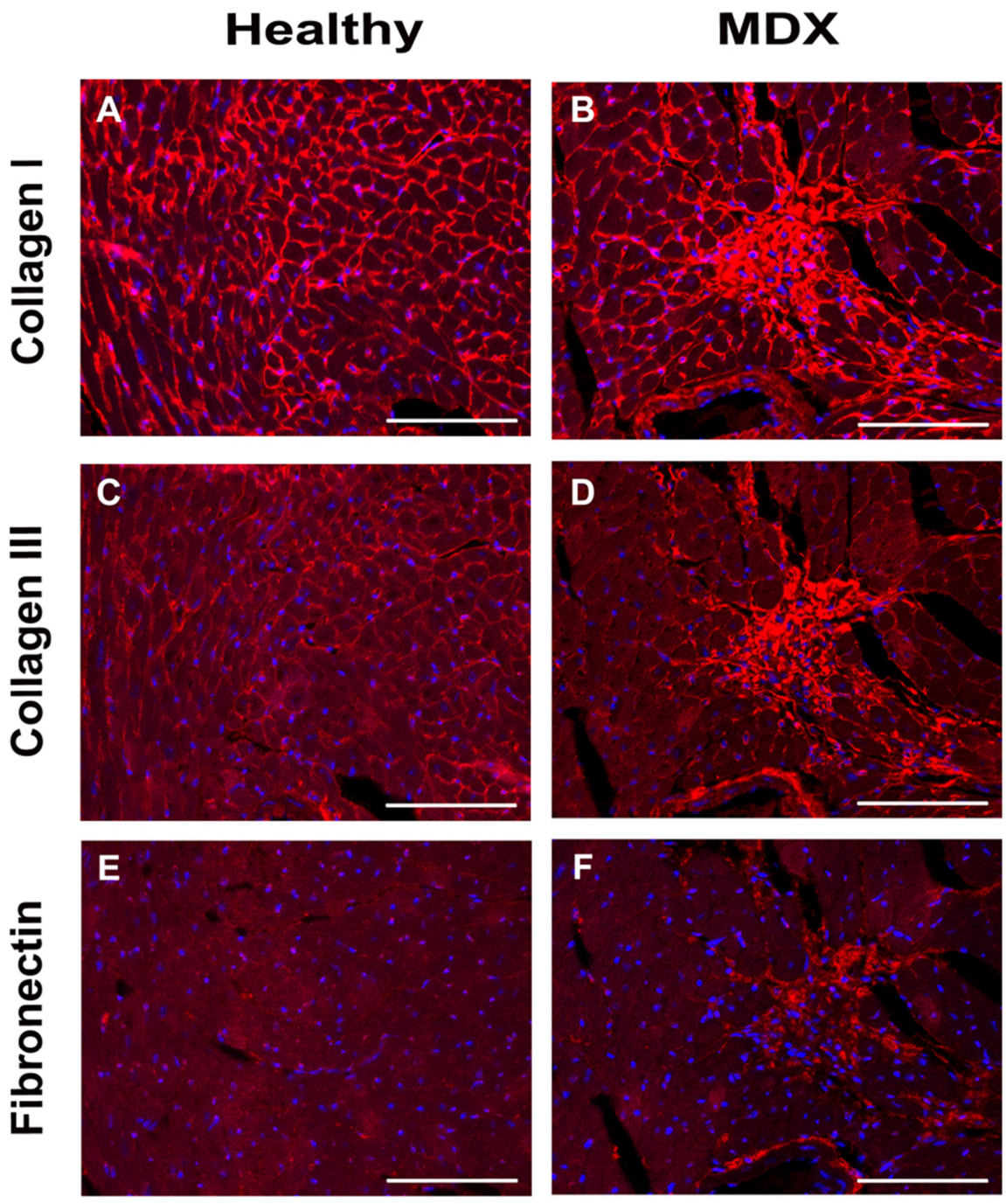

Figure 3 *Immunofluorescence staining for matrix components in healthy and MDX ventricles. Representative pictures of collagen isoform I in myocardial tissue of healthy (A) and (B) mdx left ventricles. In the fibrotic areas there is accumulation of collagen III (C-D) and fibronectin (E-F). Nuclei were stained in blue with Dapi. Scale bar: 50 µm.*

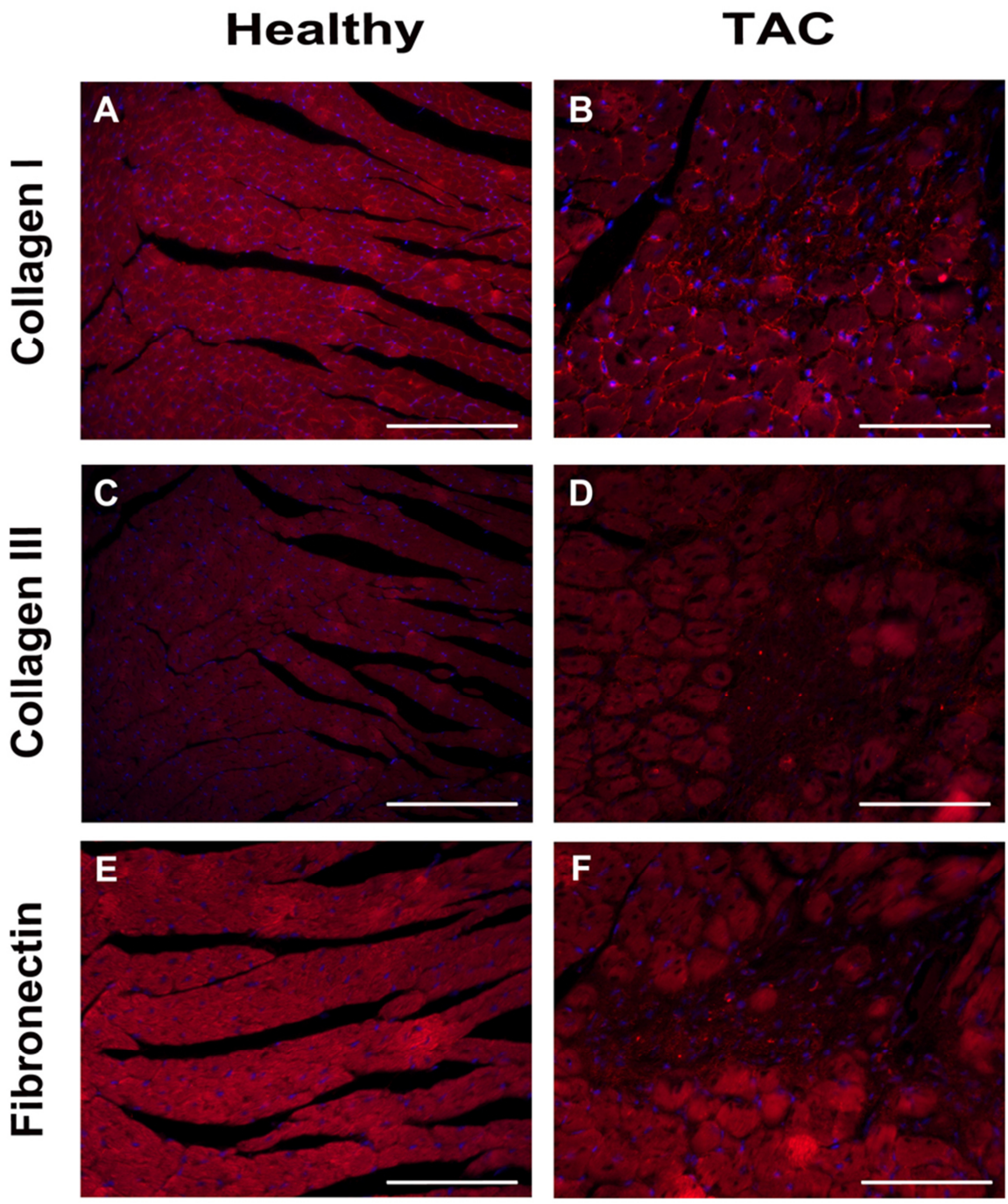

Figure 4 *Representative pictures of matrix components in healthy and TAC ventricles. Representative pictures of (A-B) collagen type I, (C-D) collagen type III and (E-F) fibronectin in myocardial tissue of healthy and (B) TAC hearts. Nuclei were visualized with Dapi and depicted in blue. Scale bar: 50 µm.*

Quantitative tissue analysis in tissue composition of mdx and TAC hearts

Tissue composition for collagen and GAGs was determined for *mdx*, TAC and age matched hearts from healthy animals (figure 5). In whole hearts of *mdx* mice there was a 1.6 fold induction of [HYP] per mg dry weight tissue (figure 5A), although this was not significantly different. No difference in the amount of HYP was found in the left (11.7±5.0 µg/mg) and right (12.6±1.9 µg/mg) ventricle of *mdx* mouse hearts compared to healthy hearts (LV: 7.5±0.7 µg/mg, RV: 7.7±3.2 µg/mg) (figure 5B, C). Analysis of the amount of GAGs in the whole heart (103.8±21.5 µg/mg) and both the left (54.4±17.8 µg/mg) and right (49.4±3.7 µg/mg) ventricle showed no significant differences compared to healthy hearts (80.6±6.4 µg/mg; 41.7±4.2 µg/mg; 39.0±9.7 µg/mg, respectively) (figure 5E-G). In the left ventricle of TAC hearts , a non-significant 1.6 fold induction of [HYP] per mg dry weight tissue was observed, similar to the *mdx* hearts (figure 5D). The total GAG content in left ventricles of TAC mice was not different from healthy ventricles (48.4±23.3 vs 57.0±22.7 µg/mg) (figure 5H).

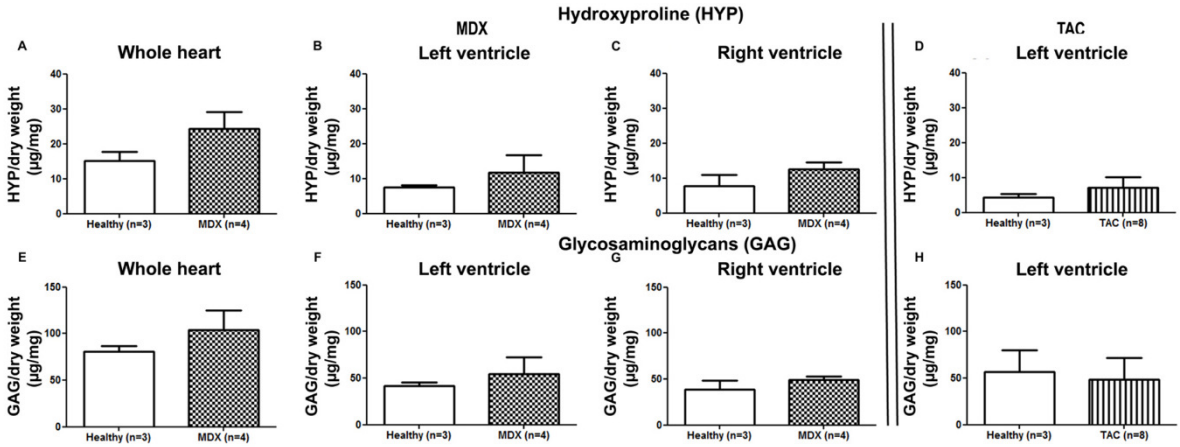

Figure 5 *Tissue composition of mdx and TAC hearts. Tissue composition (HYP and GAG) of mdx mouse hearts at 10 months of age (A-C, E-G) and heart 9 weeks after aortic constriction (TAC) (D, H) and age-matching healthy controls showed no significant differences.*

Biomechanical characteristics of mdx and TAC myocardial tissue

To determine the effect of matrix changes on biomechanical characteristics of cardiac tissue during disease, biaxial tensile and indentation tests were performed. In *mdx* mouse hearts, biaxial tensile testing was performed on both the left and right ventricle, but no significant difference in strain at failure (%) were found for both x- and y-directions compared to healthy hearts (figure 6A-B). The Young's modulus determined by indentation test was 12.4±4.5 kPa and 11.9±3.1 kPa for healthy left and right ventricle, respectively. Myocardial stiffness of the left ventricle in *mdx* hearts was 25% lower compared to healthy myocardium ($P<0.05$), while the stiffness of the right ventricle in *mdx* hearts was not significantly different from healthy myocardium (10.8±3.6 kPa) (figure 6D-E). Similarly to *mdx* mouse hearts no significant difference was found in failure strain for the left ventricle of TAC hearts (figure 6C). Young's modulus of the left ventricular myocardium after TAC (8.2±2.7 kPa)

revealed no significant difference in myocardial stiffness with the healthy left ventricle (11.1±7.4 kPa) (figure 6F).

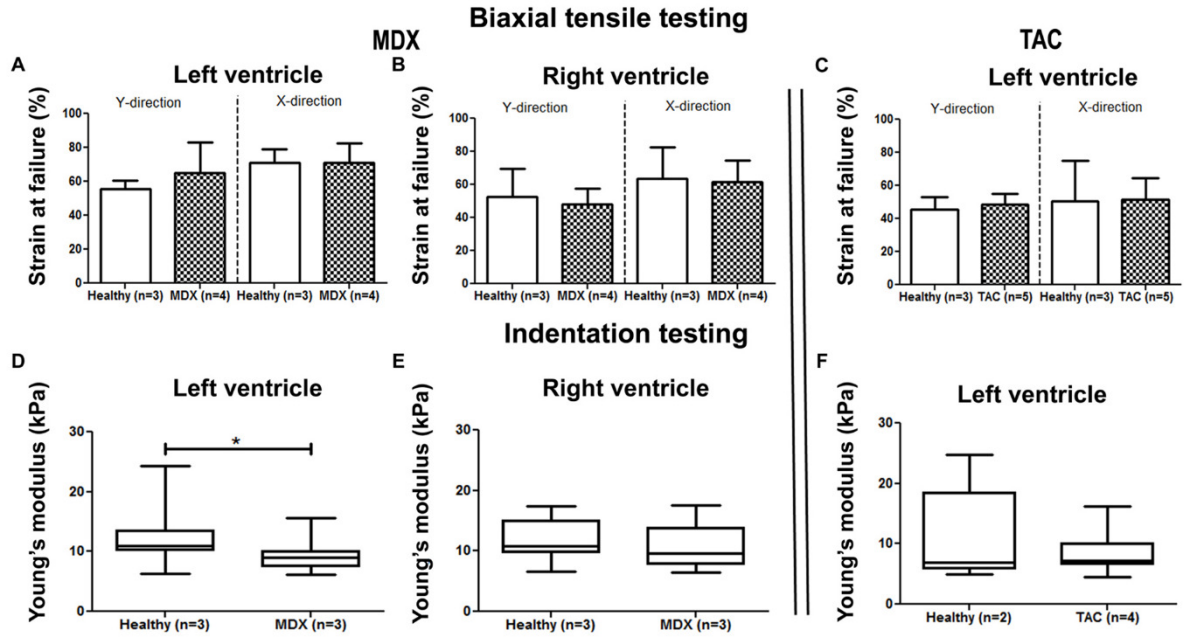

Figure 6 *Mechanical properties of healthy, mdx and TAC hearts. Failure behavior of healthy and mdx left ventricles (A) and right ventricles (A) in both X- and Y-direction showed no differences. The maximum strain of TAC hearts at the moment failure occurs is not different from healthy hearts (C). Box and whisker plots (minimum-maximum) of the Young's moduli of healthy and mdx left shows decrease in myocardial stiffness (D) and no difference in the right ventricle (E). Myocardial stiffness represented by Young's moduli of healthy hearts and hearts 9 weeks after TAC (F) showed no significant difference. *P<0.05*

ECM analysis in microtissues

Seeding cardiomyocytes and fibroblasts with exogenous rat tail collagen I and matrigel in the µTUG system led to formation of microtissues with equal distribution of cells and exogenous matrix components as described previously (18). After 7 days in culture confocal microscopy revealed presence of collagen I throughout the microtissues and also around the nuclei of the cells (figure 7A-B). Although no exogenous collagen III and fibronectin were added, both matrix components were homogeneously distributed throughout the microtissues and also present in the cytoplasm of the cells (figure 7C-F). Fibronectin was predominantly present in the cytoplasm of the sarcomeric α-actinin negative fibroblasts (figure 7E-F).

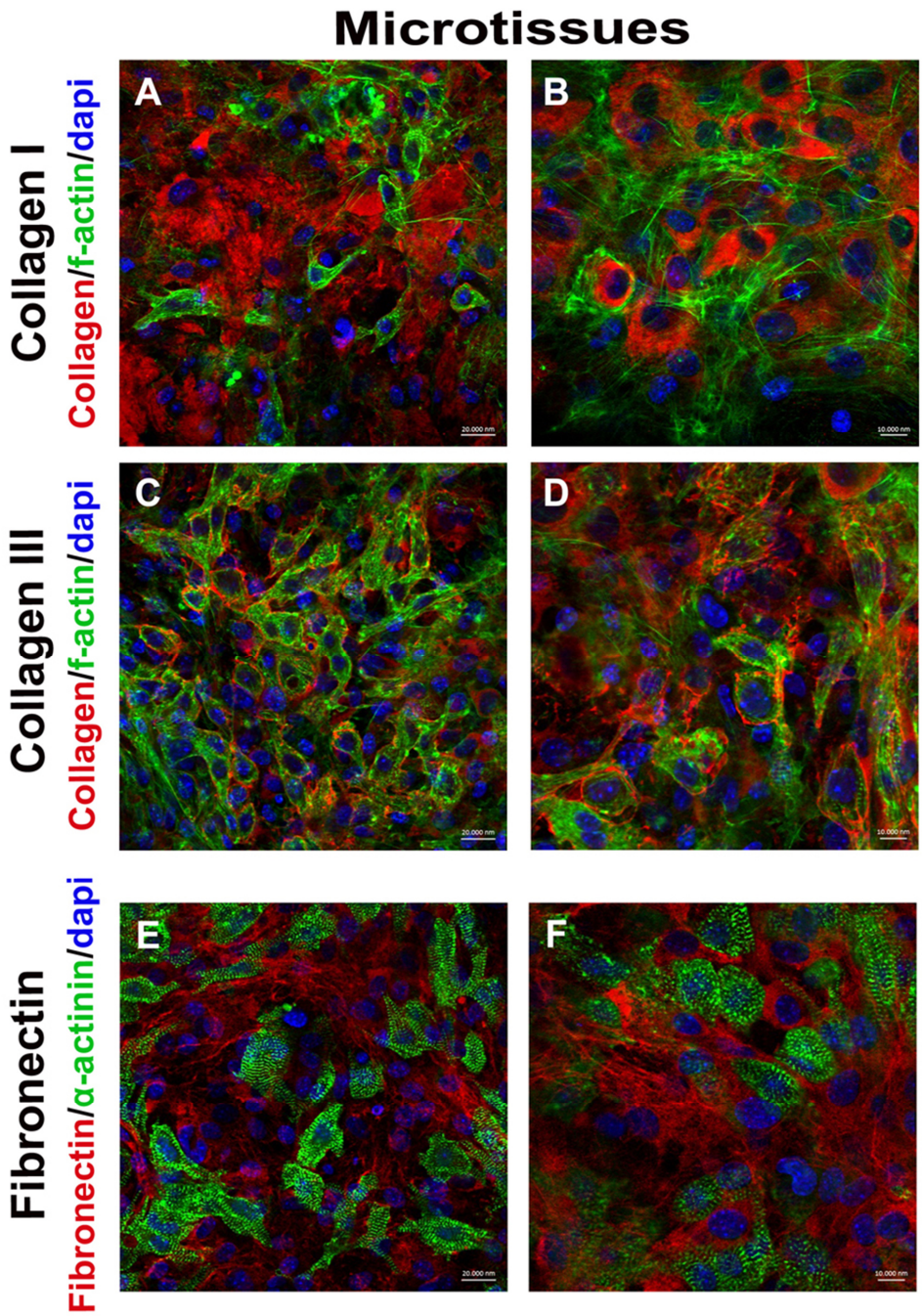

Figure 7 *Immunohistological characterization of mouse cardiac microtissues at day 7 of culture. Collagen I (red) is present throughout the microtissue since it was also present in the hydrogel (A) and the magnification shows presence of collagen I in the cytoplasm of the cells (f-actin in green) (B). Collagen III (red) is present surrounding the f-actin (green) positive cells (C) and the magnification shows presence of collagen III in the cytoplasm (D). Fibronectin (red) is present throughout the microtissue (E) and the magnification shows presence of fibronectin predominantly in the cytoplasm of sarcomeric α-actinin (green) negative cells (F). Scale bar: 20.000 nm (A, C, E) and 10.000 nm (B, D, F).*

Discussion

Heart failure remains one of the leading causes of morbidity and mortality worldwide. Although therapies improve progressively, no real cure is available for patients with fibrosis associated heart failure. For better understanding of the pathogenesis and improvement of treatment options, cardiac microtissues are an attractive new approach. Cardiac tissue engineering is one of the most challenging scientific adventures, since myocardial tissue is a complex composition of cells and their supporting ECM. The interplay between cells and cell-matrix interactions are crucial for proper cardiac function. In recent years it became clear that the key modulators of cardiac tissue engineering are not only cell composition, differentiation and orientation, but also embedding of matrix composition. Ideally, cardiac microtissues replicate key features of the *in vivo* heart, in particular in matrix organization and composition. Therefore, in the present study changes in the cardiac ECM properties in genetic (*mdx)* and acquired (TAC) diseased hearts were determined and compared to matrix organization in current engineered cardiac microtissues.

Histological analysis showed the presence of fibrosis in hearts from animals with both genetic and acquired heart disease. In *mdx* mouse cardiomyopathy, fibrosis in the left ventricle was characterized by a patchy distribution whereas fibrosis was homogeneously distributed throughout the entire right ventricle. In earlier studies, patchy fibrosis was shown to affect all regions of the left and right ventricle approximately equal (20), while others described fibrosis in *mdx* hearts for the whole heart or left ventricle, thereby omitting conclusions about the distribution pattern (8, 9, 21). Current analysis of the fibrotic lesions showed collagen I and III expression as previously described by Wehling-Henricks et al. (22). Besides collagens, fibronectin plays a pivotal role in the cardiac ECM as it provides the structural network for the cells. Furthermore, fibronectin is also required for the deposition of collagen type I (23, 24). While increase in fibronectin in fibrotic areas is well documented in skeletal muscle and diaphragms of *mdx* mice (25), to our knowledge this is the first time that the presence of fibronectin in fibrotic lesions in *mdx* hearts is described.

Although an increase in fibrosis was observed via qualitative immunohistochemistry and semi-quantitative image analysis, quantitative chemical analysis of hydroxyproline did not show any increase in collagen. A previous study by Wehling-Henricks et al. in older (12-14 months) *mdx* hearts showed significant increase in hydroxyproline content (22). It is well known that aging *mdx* hearts become more fibrotic while the collagen content in healthy aging hearts is constant (8, 20). This combined with the different techniques used in both studies to determine the hydroxyproline content, could be the cause of the contradiction between this study and Wehling-Henricks et al. (22). Furthermore, hydroxyproline is a major component of collagen, but only comprises 13.5% of the amino acid composition (26-28) which leads to lower total collagen content verified by biochemical assays since the affinity is less. Picrosirius red on the other hand binds with high affinity to the triple helix structure found in all collagen fibers leading to an over-estimate of the total amount of collagen (28), especially when patchy fibrosis is present. Next to the fibrous proteins, GAGs constitute a large fraction of the ECM. GAGs are important for the absorption of water within the tissue matrix, due to their negative charge and their inherent hydrophilicity (29). Biochemical

analysis of the amount of GAGs showed no differences between healthy and diseased hearts, which was in line with the comparable fluid content of both healthy and diseased hearts (*mdx* and TAC).

Changes in matrix composition may have an impact on the vulnerability to rupture of the tissue, which can be determined by investigating strain at failure. However, in both hearts of acquired and genetic disease models no differences were found. It is generally assumed that cardiac fibrosis causes an increase in myocardial stiffness. Unfortunately, differences in meaning of the word myocardial stiffness exist between clinicians and biomechanics, which confuse the field. In the clinic, the myocardial stiffening is appointed by the cardiologist when the left ventricle is not able to fill and relax as well compared to a healthy heart, and less blood than normal can be pumped out of the heart every heartbeat. This active measure of stiffness is physiologically relevant for tissue function. However, a more local tissue related measure is the Young's modulus of the myocardium, which is the ratio between stress and strain as measured under tension or compression of the tissue. To the best of our knowledge, this is the first study describing biomechanical characteristics of the *mdx* mouse heart by indentation tests to determine the Young's Modulus (15, 30-34). Biomechanical analysis showed a significant decrease in left ventricular stiffness, although patches of fibrosis with an increase in matrix proteins are detected in the left ventricle. Previous studies determined "stiff" myocardium in *mdx* mouse hearts by left ventricular pressure volume relationships only (31, 32). On the contrary, Pasternak et al. showed lower local stiffness of satellite cells from *mdx* mice by indentation with a glass probe (30). Goldmann et al. and Puttini et al. performed atomic force microscopy (AFM) on skeletal muscle in both *mdx* rats and mice, respectively. Both groups showed lower Young's moduli which represent less stiff muscles, while fibrosis in these muscles was also observed (33, 34). Goldmann et al. attributed this decrease in stiffness due to loss of actin filaments and/or microtubules (33). Another explanation for the observed decreased stiffness, despite the presence of fibrosis, could be cardiomyocyte death. In *mdx* hearts necrosis (35) and lysis of cardiomyocytes by macrophages (22) have been described. When cardiomyocytes die, this creates an empty space and the ECM surrounding these cells might collapse. Collapse of the ECM may result in a patchy fibrosis manifest with histology although there is no real increase in matrix proteins and thereby no increase in myocardial stiffness. Our data showed patchy fibrosis although biochemical analysis showed no increase in total collagen, which would support the fact that there is collapse of the ECM as source of fibrotic lesions in state of excessive matrix production in this area. Progressive pathology in *mdx* mouse hearts might be driven by a combination of fibrosis and/or cardiomyocyte loss (21) although the effect on biomechanical characteristics is still unknown. The fact that fibrosis in *mdx* mice can occur with or without cell death makes it difficult to determine whether there is reactive or reparative fibrosis.

An aortic constriction (TAC) in mice is a highly relevant model of pressure overload induced hypertrophy. In these hearts with acquired pathology we found reactive fibrosis following as a result of prevailing hemodynamic load, in line with earlier observations (36, 37). Perivascular fibrosis is observed throughout the left ventricle and interstitial patchy fibrosis is also present in the left ventricle. Patchy fibrosis in the left ventricle was previously

described from 16 weeks after TAC onwards, while in hearts in the early remodeling period (4-12 weeks after TAC), only diffuse fibrosis was reported (38, 39). On the contrary, in this study no fibrosis was found in the right ventricle of mouse hearts 9 weeks after induction of TAC. Chen et al. showed no induction of fibrosis throughout the heart at the same time-point (9 weeks), after TAC (37), while others described presence of fibrosis throughout the whole heart and also specifically in the right ventricle at different time-points after TAC (20, 40). The degree of fibrosis is dependent on the severity of TAC which is determined by the amount of pressure overload. Application of TAC is a technically challenging surgical procedure and the site of constriction determines the degree of overload on the left ventricle, therefore variety in severity of hypertrophy and remodeling are well accepted (41). In this study, the fibrotic lesions 9 weeks after TAC contained predominantly collagen type I. Research performed in nonhuman primate myocardium after TAC, showed an increase in collagen type I and III (42). Weber et al. distinguished between collagen type I and III by polarized light microscopy (42), while in this study a more specific antibody staining was used to detect both types of collagen. Using a specific staining for collagen III, little to no collagen III was found in the fibrotic lesions. Another study performed on rats both revealed an increase of collagen type I and III on DNA and protein level (43). The presence of fibronectin in the fibrotic areas, which was elucidated in this study, is supported by previous work of Grimm et al., who performed quantitative image analysis on fibronectin staining in rat hearts 12 weeks after TAC (43).

Although increases in collagen content were observed with histology, biochemical analysis of hydroxyproline did not show a significant increase. Like in *mdx* hearts this might be related to the sensitivity of the technique, but also to the large interindividiual variation. Previously in mouse hearts 8 weeks after TAC a 5-6 fold increase in hydroxyproline content was reported, although with a different method as described in this study (44). In nonhuman primates, Weber et al. showed a significant increase in hydroxproline already 4 weeks after induction of pressure overload (42), while in rats only differences in hydroxyproline concentration were determined when pressure overload ultimately induced heart failure (45). In other pressure overload studies in rat, aortic banding resulted in an increase in collagen concentration which was also considered responsible for the abnormal stiffness of cardiac muscle obtained from these hearts (46). In this study, no increase in Young's modulus was detected determined by indentation test. This is the first study which performed indentation tests on tissue slices to determine myocardial stiffness. Previous studies determined passive stiffness on a Langendorff set-up by calculating stress and strain which were dependent on left ventricular end diastolic pressure (mmHg) and end diastolic volume (ml) (46). Since pressure overload induced by aortic binding leads to an acquired cardiac pathology, which is dependent on several aspects such as operation skills, severity of TAC and timing of analysing fibrosis, scientific outcomes in this animal model show large variation and make it difficult to interpret and compare data between different studies.

Although the importance of ECM is pointed out in several publications on cardiac tissue engineering (47-49), none of these studies specifically looked at the spatial distribution of the matrix. Until now, engineered cardiac tissues have aligned healthy tissue architecture since constructs are created between anchor points (18, 48, 49). However during cardiac

disease, disarray of cells and matrix may occur. Recently this matrix disarray was mimicked in 3D by using isotropic constraints (18). The composition of engineered cardiac tissues usually consists of a combination of collagen I, fibrin and/or matrigel (18, 47-50). To preserve the composition of the native microenvironment, decellularized heart tissue can be emulsified and reconstituted as a gel (51). Another way to create a more specifically diseased environment is to use matrix produced by cells from diseased heart tissue. However, these attempts were either performed in 2D, or matrix architecture was not studied after preparation of the cardiac microtissues (51-53). In the current study, matrix composition in biaxial constrained microtissues was elucidated and compared to native *in vivo* matrix distribution. In a previous study, it was shown that collagen architecture in biaxial constrained microtissues is chaotic, comparable to diseased heart tissue (18). In this study, microtissues showed a homogeneous distribution of collagen I, collagen III and fibronectin and after 7 days of culture ECM components were also expressed in the cytoplasm of the cardiac cells. Previous work by Kelm et al. characterized the extracellular matrix of myocardial microtissues from primary neonatal mouse cardiomyocytes in hanging drop cultivations and showed presence of fibronectin but absence of collagen I after 7 days in culture (54). An explanation for the absence of collagen I in these purified mouse cardiomyocyte cultures by Kelm et al. was the predominant production of collagen I by fibroblasts (54). The microtissues used in this study are composed of both cardiomyocytes and fibroblasts and all cells show presence of cytoplasmic collagen I in both cell types.

Microtissues in this study showed homogeneous distribution of ECM components throughout the constructs which does not resemble the location and degree of patchy fibrosis characterized in native heart tissue of *mdx* and TAC hearts. The size of the microtissues used in this study (875x875 µm) is designed to investigate the direct effect of changes in the microenvironment on the cell performance and not on the whole tissue scale. Due to the size of the microtissues it will be difficult to induce patchy fibrosis and thereby resemble native cardiac tissue fibrosis. The microtissues allow obtaining knowledge on the microscale about the interaction between the microenvironment and the cells. By creating fibrotic and healthy tissues at the microscale, the collected data can be combined at the multiscale for e.g. with computational modeling which allows translation from cell to tissue level. Another approach to apply patchy fibrosis in cardiac engineered microtissues and thereby mimicking native tissue is by working with microtissues sizes at the macroscale.

Conclusion

Both *mdx* mouse cardiomyopathy as well as cardiac pathology in mouse hearts after TAC showed patchy fibrosis in the left ventricle. Changes in the cardiac ECM in both *mdx* and TAC hearts had no effect on the strain at failure. However, patchy fibrosis in the left ventricle of *mdx* mice decreased myocardial stiffness while in TAC hearts myocardial stiffness was not affected. Current engineered cardiac microtissues demonstrated homogeneous distribution and production of ECM proteins. Native cardiac tissue analysis provides directions for optimization of future cardiac microtissues, in which changes in the microenvironment from both acquired and genetic disorders need to be incorporated. Next generation tissue engineered approaches on the microscale as described in this study need

a multiscale approach to translate effect of the microenvironment on cell function to tissue function. Future optimization on a macroscale level should aim at introducing patchy fibrosis via local accumulation of ECM proteins. By working at the macroscale or multiscale level, the influence of this type of matrix remodeling on contractility can be studied in detail and provide new knowledge to improve current and develop new treatments for heart disease.

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The influence of matrix (an)isotropy on cardiomyocyte contraction in engineered cardiac microtissues

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Abstract

In the cardiac microenvironment, cardiomyocytes are embedded in an aligned and structured extracellular matrix (ECM) to maintain the coordinated contractile function of the heart. The cardiac fibroblast is the main cell type responsible for producing and remodeling this matrix. In cardiac diseases, however, adverse remodeling and cardiomyocyte death may lead to deterioration of the aligned myocardial structure. Here, we present an *in vitro* cardiac model system with uniaxial and biaxial constraints to induce (an)isotropy in 3D microtissues, thereby mimicking 'healthy' aligned and 'diseased' disorganized cardiac matrices. A mixture of neonatal mouse cardiomyocytes and cardia fibroblasts was resuspended in a collagen/matrigel hydrogel and seeded to form microtissues to recapitulate the *in vivo* cellular composition. Matrix disarray led to a stellate cell shape and a disorganized sarcomere organization, while cardiomyocytes in aligned matrices were more elongated and had aligned sarcomeres. Although matrix disarray has no detrimental effect on the force generated by the cardiomyocytes, it did have a negative effect on the homogeneity of contraction force distribution. Furthermore, proliferation of the fibroblasts affected microtissue contraction as indicated by the negative correlation between the percentage of cardiac fibroblasts in the microtissues and their beating frequency. These results suggest that in regeneration of the diseased heart, reorganization of the disorganized matrix will contribute to recover the coordinated contraction but restoring the ratio in cellular composition is also a prerequisite to completely regain tissue function.

Introduction

The myocardial microenvironment is composed of cardiomyocytes and non-myocytes embedded in an aligned and structured extracellular matrix (ECM), which is mainly produced by the cardiac fibroblasts (1). Cells and ECM proteins are connected via cell-cell and cell-matrix interactions to maintain this structural organization and enable coordinated contraction of the heart. During cardiac disease, such as ischemia and myocardial infarction, the myocardial microenvironment changes in response to pathological cues. The most common effects of heart disease are loss of cardiomyocytes, followed by tissue repair and remodeling, ultimately leading to fibrosis, due to adverse matrix remodeling (2, 3). It is generally believed that fibrosis leads to deterioration of the aligned myocardial tissue structure (1), especially when combined with massive cardiomyocyte death. While the influence of microenvironmental changes on cardiomyocyte function has been extensively studied at the cellular level (4-6), the impact of changes in structural organization on local tissue function is still unclear.

The importance of alignment of cardiomyocytes in coordinated contraction is not only proven by the native cardiac structure, but also in *in vitro* studies. Alignment of cardiomyocytes has been induced by microcontact printing parallel lines of matrix proteins onto 2D substrates (4-6). These studies demonstrated that alignment improves cardiomyocyte calcium handling and contractile properties when compared to randomly oriented cell monolayers (4-6). In 3D, researchers have mimicked matrix alignment by using electrospun scaffolds and showed that scaffold fiber alignment induced alignment of the cardiac cells (7, 8). Black et al. created fibrin based cardiac tissues using rat neonatal cardiomyocytes and measured twitch forces in aligned and disorganized 3D tissues (9). They reported that when paced at 1Hz, aligned cardiac tissues generated a higher contraction force compared to disorganized tissues, caused by improved gap junction formation (9). Desroches et al., used scaffold-free 3D microtissues to study cellular distribution, function and cell-cell interactions in a 3D environment (10). Interestingly, they demonstrated that neonatal rat cardiomyocytes and fibroblasts in these microtissues recapitulated the *in vivo* cellular distribution. Depending on their cellular composition, functional calcium handling as well as rhythmic contraction, indicating the importance of using additional cell types next to cardiomyocytes to create *in vitro* cardiac tissue models (10). The effects of other cell types, mainly (myo)fibroblasts, on cardiomyocyte function have also been extensively studied (11-13). Paracrine factors of cFBs (11) and mechanical (12) and electrical coupling (13) of cardiac fibroblasts and cardiomyocytes have all shown to affect the conduction velocity in cardiac cell monolayers.

Although these studies address the effects of different microenvironments on cardiomyocyte function, most studies did not provide insight into the evolution with time. As cells synthesize and remodel their own matrix, and matrix composition and organization in turn influence cell function, there is a dynamic interplay between cells and their microenvironment. We therefore aimed at monitoring changes in cell function in response to their environment in time to further understand the transient changes in cardiomyocyte contractile behavior in different microenvironments.

Currently available model systems do not allow for simultaneous manipulation of matrix architecture and monitoring of cardiac tissue composition and function over time. Therefore, we used a previously developed microfabricated tissue gauge (μTUG) design (14, 15) and adapted this to create microtissues with either uniaxial or biaxial constraints to manipulate matrix organization into an aligned (anisotropic) or disorganized (isotropic) organization. While alignment is chosen to represent a "healthy" tissue organization, the disorganized matrix structure is used to mimic a "diseased" tissue organization. The relatively small size of the tissues allows for real-time monitoring of the complete constructs using microscopy, whereas the use of arrays of tissues enables higher throughput data collection. Cardiac microtissues were created using a mixed cell population isolated from neonatal mouse hearts, representing a healthy cellular composition. The microtissues were expected to develop a more disease-like cellular composition with culture time, since cardiac fibroblasts proliferate with time and cardiomyocytes do not proliferate (13). To study the effect of matrix alignment we compared a number of features, i.e. cellular structure and composition, contraction force, beating frequency, and homogeneity of contraction between anisotropic and isotropic microtissues during one week of culture. Using this model system we showed that matrix disorganization affects the direction and homogeneity of cardiac tissue contraction although it has no detrimental effect on the force of contraction. Furthermore our results suggest a negative correlation between fibroblast proliferation and contraction frequency.

Materials and Methods

Ethical statement

Animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the National Institutes of Health.

Cell isolation and culture

Neonatal cardiomyocytes and cardiac fibroblasts were isolated from 1-3 day old C57/BL6 mouse hearts as described before (16). Cells were cultured in high glucose DMEM (Lonza), supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. To remove most of the cardiac fibroblasts, the isolated cells were first seeded in uncoated culture flasks to let fibroblasts adhere. Non-attached cells were considered to be the CM-enriched cell population (16). The enriched cardiomyocytes were plated at a concentration of $5x10^4$ cells/cm^2 on six-wells plates coated with 1% gelatin and 10 μg/ml fibronectin for four days before they were used for microtissue fabrication or staining.

μTUG fabrication

The design of the μTUG system was adapted from the system developed by Legant et al., which was fabricated using soft lithography (14). Each microwell contains polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning) microposts, providing biaxial (12 posts) or uniaxial (4 posts) constraint of the tissues (figure 1A-C). In short, multilayered

masters were manufactured by UV-induced polymerization of SU-8 photoresist (Microchem) spin coated on silicon wafers. Exposure and alignment was performed on a Karl Suss MJB3 mask aligner (Suss Microtec). The SU-8 masters were made non-adhesive via overnight silanization with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane under vacuum. Molds were created by casting a prepolymer to curing agent ratio of 10:1 w/w PDMS on the masters and incubating at 60°C for 48 hours. The PDMS molds were treated in a plasma oxidizer (1 min at 100 Watt) and made non-adhesive via overnight silanization to facilitate release of the molds from the µTUG system. PDMS was then casted on these molds and substrates were replicated on glass bottom cell culture dishes. After careful removal of the molds, µTUGs were sterilized in 70% ethanol followed by exposure to UV for 15 minutes. Furthermore, µTUGs were treated for at least 15 minutes with 0.2% Pluronic F127 (BASF) to reduce cell adhesion.

Microtissue seeding

A gel mixture was prepared by mixing 50% Collagen type I (BD Biosciences, 3.2 mg/mL), 39% CM culture medium, 3% 0.25 M NaOH, and 8% growth factor-reduced Matrigel (BD Biosciences), added to the well of the µTUG system, and centrifuged to spin the hydrogel into the microwells. After trypsinization, enriched cardiomyocytes were resuspended in the hydrogel at a density of $1x10^6$ cells/ml, and centrifuged again to spin the cells into the microwells. After spinning, excessive gel was aspirated leaving 0.14 µl or 0.25 µl of gel volume in the microwells with uniaxial or biaxial constraints, respectively. The cell seeded gel polymerized in an incubator at 37 °C, 5% CO_2 for 10 minutes, by inverting the petridish and adding sterile water to the lid to prevent dehydration of the tissues. Subsequently, culture medium was added to the microtissues and changed every two to three days. Cells compacted the matrix around the microposts creating uniaxially anchored microtissues of 875x375 µm and biaxially anchored microtissues of 875x875 µm (figure 1A).

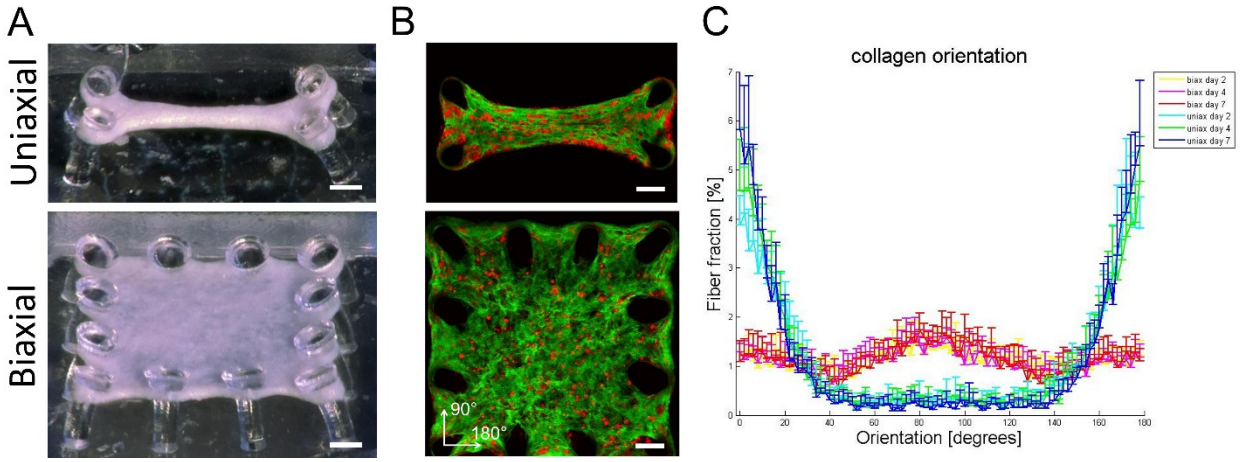

Figure 1 *µTUG system with uniaxial or biaxial constraints to manipulate tissue organization. (A) The cell seeded hydrogels compacted around the posts to from microtissues. (B) Collagen (CNA-35 OG, green) and cells (CTO, red) in uniaxially and biaxially constrained tissues had an aligned and random orientation, respectively. (C) Isotropic and anisotropic collagen organization was confirmed by image analysis showing the highest fiber fractions in the direction of the constraints and was consistent at day two, four, and seven. Scale bars represent 100 µm. Error bars represent SD for N=10 from one representative experiment.*

Immunofluorescence, confocal microscopy, and image analysis

Viable fluorescent probes, CNA35-OG (17) and Cell Tracker Orange (CTO, Invitrogen Molecular Probes), were used to visualize collagen and cells in the microtissues at day two, four, and seven, using two-photon confocal laser scanning microscopy (Zeiss LSM 510 META NLO). To determine the orientation and ratio of cardiomyocytes and fibroblasts and to study cardiomyocyte maturation, immunohistochemistry was performed using α-actinin as cardiomyocyte label and vimentin as fibroblast label on monolayers at day zero and microtissues at day two, four, and seven. Cells and microtissues were fixed in 10% formalin for 10 minutes, followed by storage in PBS. For double staining of α-actinin and vimentin, samples were permeabilized with 0.5% Triton-X-100 and incubated with 10% horse serum prior to incubation with antibodies against α-actinin (Sigma-Aldrich) and vimentin (Cell Signaling) overnight. The next day, samples were washed in PBS, incubated with secondary antibodies Alexa 488 (Invitrogen) and Alexa 555 (Molecular Probes), counterstained with DAPI, and then embedded in Mowiol. Monolayers were imaged on a fluorescence microscope (Zeiss Axiovert 200M) and microtissues on a two-photon confocal laser scanning microscope. The resulting images were analysed in Mathematica to quantify collagen and cell orientation as described before (18, 19). In short, the probability that a pixel belongs to a fiber-like structure was calculated, based on the vesselness measure defined by Frangi et al. (20). To determine the percentage of cardiac fibroblasts in the microtissues, α-actinin and vimentin positive cells were counted using ImageJ in images taken in the middle of the constructs. Sarcomere length was measured in ImageJ as the distance between adjacent α-actinin bands. Sarcomere width was measured in ImageJ as the length of individual α-actinin bands.

Force measurement

Micropost displacement caused by spontaneous beating of CMs, was monitored over time using a high speed camera mounted on a Zeiss observer microscope. Movies were recorded at day two, four, and seven for each experimental group. Micropost displacement was tracked and analyzed using feature detection software in Mathematica and Matlab, based on the use of the Hough transform. The Hough transform is a feature extraction technique used in image analysis to detect known shapes based on a voting procedure of the pixels in the image (21). More votes on one point means a greater probability of the point being the center of a circle. Once all microposts were detected in the first frame, they were tracked throughout all frames of the movie to get the displacement of the microposts over time. Finite element analysis was used to calculate the force-displacement relationship, leading to a spring constant of 0.7 μN/μm for our system (figure 2). Dynamic contraction forces were then calculated from displacements by using this displacement-force relationship. Contraction forces of all microposts were summed to obtain the total contraction force per microtissue. To account for the difference in cell number between anisotropic and isotropic microtissues, the measured forces were corrected for initial microwell volume, representative of the initial cell numbers. Beating frequency was deducted from the Fourier spectrum of the signal.

Computational strain analysis

The μTUG system was used to measure displacements at multiple microposts per tissue. This information was used to calculate local strains in the microtissues. In analogy with the finite element method, a polynomial approximation of the displacement field within each microtissue was determined from the measured displacements at the microposts and the shape functions. A 2D third-order quadrilateral Serendipity element (12 nodes) was used in case of a biaxial tissue, or a 2D first-order quadrilateral element (4 nodes) for a uniaxial tissue. Subsequently, the deformation gradient tensor **F** was determined at 16 equally distributed locations within each tissue from the spatial gradients of the displacement field. The deformation gradient tensor **F** was then used to calculate the Green-Lagrange strain ($\mathbf{E} = 0.5(\mathbf{F}^T.\mathbf{F} - \mathbf{I})$) at each location. The standard deviation (SD) of the normal strain in x- (E_{xx}) and y-direction (E_{yy}) were calculated as a measure of the homogeneity of force distribution.

Statistical analysis

All data is represented as mean ± standard deviation. Statistical analysis was performed with GraphPad Prism software. Comparisons of parameters between two groups were made using unpaired T-test, multiple comparisons between groups were made using two-way analysis of variance (ANOVA) with Bonferroni post-hoc test, and comparisons of variance between two groups were made using the F-test of equal variance. Differences were considered statistically different when $P<0.05$.

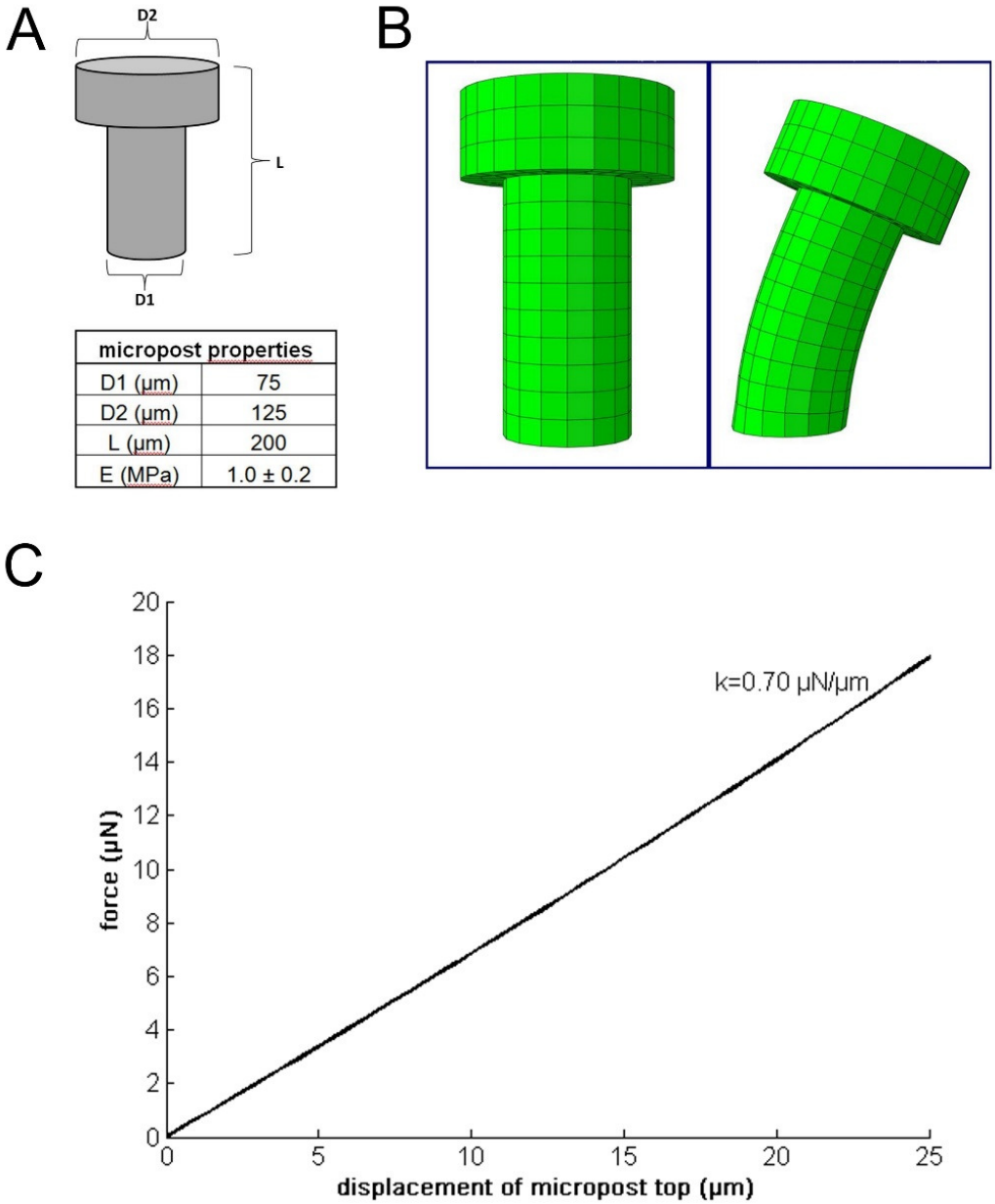

Figure 2 *Micropost bending properties. (A) Image of micropost with corresponding micropost dimensions and mean E-modulus ± SD of the PDMS as calculated from tensile test data (N=12 from 2 batches of PDMS). (B) Finite element model of the micropost in its initial and deformed shape. (C) Plot of displacement vs. force as was calculated from the finite element model, resulting in a spring constant k=0.70 µN/µm.*

Results

Microtissue constraints determine collagen organization

In this study, both healthy aligned and diseased disorganized cardiac tissues were successfully mimicked by seeding cardiac cells in µTUGs with uniaxial or biaxial constraints to manipulate tissue organization (figure 1A). By using a viable probe to stain the collagen (17), matrix organization could be followed over time in the same sample. Confocal microscopy on microtissues stained with CNA-35 OG and CTO at day two, four, and seven showed that cells were embedded in a collagen matrix and distributed homogenously throughout the microtissues in both designs (figure 1B). Orientation analysis of the collagen network showed that uniaxial anchoring resulted in anisotropy of the collagen with the highest fiber fraction oriented at 180°, which is the direction of the constraints. Biaxial anchoring did not result in a preferred fiber orientation and this tissue architecture is therefore considered isotropic. The collagen organization was present from day two and did not change over time (figure 1C). Thus, constraining the tissues uniaxially or biaxially using the microposts resulted in the desired anisotropic and isotropic collagen organization, mimicking healthy and diseased myocardium.

Matrix (an)isotropy has no effect on cellular composition in microtissues

Cardiomyocytes and cardiac fibroblasts were equally distributed throughout all microtissues (figure 3A). Despite purifying the cardiomyocyte population due to adherence of fibroblasts to plastic, at the day of microtissue seeding, the cell population contained 35 ± 9% cardiac fibroblasts. At day two of culture microtissues consisted already of 43 ± 7% cardiac fibroblasts and this increased to 52 ± 10% at day four and 65 ± 11% cardiac fibroblasts at day seven (figure 3B). Tissue organization did not have any influence on the percentage of fibroblasts in the microtissues as no differences were observed between anisotropic and isotropic microtissues (figure 3B).

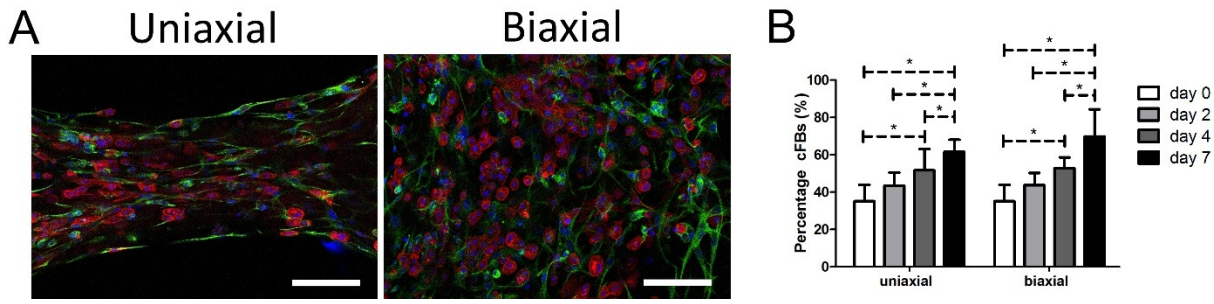

Figure 3 *The percentage of cFBs in the microtissues increased over time. (A) CMs (α-actinin, red) and cFBs (vimentin, green) were distributed throughout the microtissues in uniaxially and biaxially constrained microtissues, here shown at day two of culture (nuclei in blue). (B) Percentage of cFBs increased equally in both uniaxially and biaxially constrained microtissues. Scale bars represent 100 µm. Error bars represent SD of N≥8 from two independent experiments. *P<0.05*

Matrix anisotropy enhances cell and sarcomere alignment in microtissues

For efficient contraction, cardiomyocytes need to mature and develop an organized sarcomere structure. Maturation of cardiomyocytes was investigated by staining the microtissues for α-actinin, which is a protein that organizes into a striated pattern when the sarcomeric proteins develop into a mature structure. Although cardiomyocytes still had a round shape, α-actinin was visible from day two, indicating a developing sarcomere structure (figure 3A). After seven days, cardiomyocytes were more spread and obtained an organized sarcomere structure in both anisotropic and isotropic microtissues (figure 4A, B). However, cardiomyocytes in anisotropic microtissues (figure 4A, E) were more aligned compared to cardiomyocytes in isotropic microtissues that obtained a more stellate shape and random orientation (figure 4B, E). The alignment of cells in anisotropic microtissues also resulted in a better alignment of the sarcomeres (figure 4). Next to sarcomere alignment, sarcomere length and width are important for the contractile function of the cardiomyocytes (22-24). Determination of the sarcomere length using ImageJ, indicated a length of ~1.9 µm in both anisotropic and isotropic tissues. Sarcomere width was found to be ~1.8 µm in both groups. Although the exact values may not be as accurate as measured with other techniques (25, 26), this indicates that matrix organization did not influence sarcomere length or width in our microtissues. Together, matrix alignment improved organization of the sarcomeres and alignment of the cells.

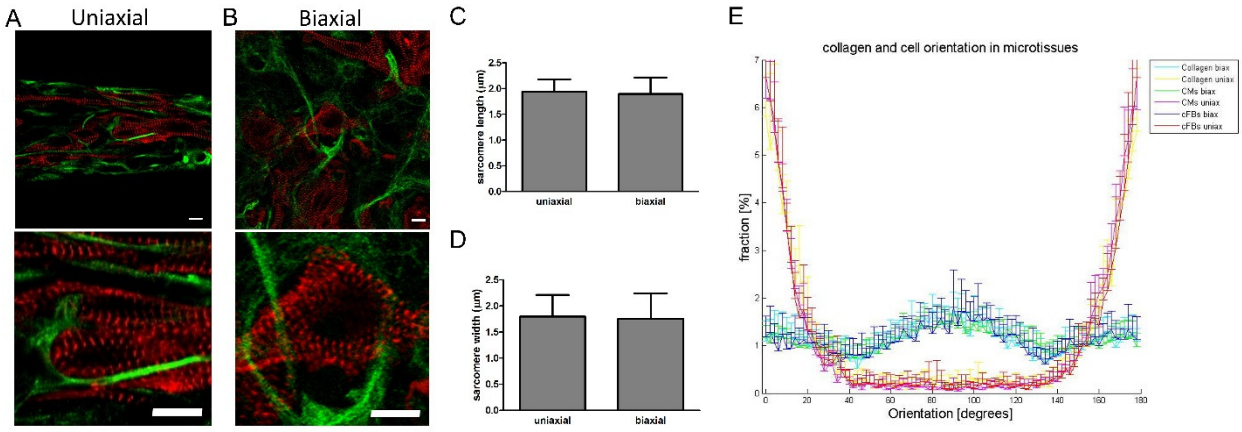

Figure 4 *Sarcomere organization in anisotropic and isotropic microtissues. (A) Matrix alignment induced alignment of the cardiomyocytes and fibroblasts and concomitant sarcomere organization. (B) A disorganized matrix induced random cell orientation and a stellate cell shape. Although cardiomyocytes developed a mature sarcomere structure, the individual sarcomeres do not have the same orientation throughout the cell. Scale bars represent 10 µm. (C) Sarcomere length was not significantly different between uniaxially or biaxially constrained microtissues. (D) Sarcomere width was not affected by matrix organization. Error bars represent N=100 sarcomeres measured in 20 different cardiomyocytes. (E) Image analysis of immunofluorescent staining showed that cardiomyocytes (green and purple lines) and cardiac fibroblasts (blue and red lines) adopt the same orientation as the collagen fibers (turquoise and yellow lines) in both anisotropic and isotropic microtissues. Errorbars represent SD for N=10 from one representative experiment.*

Microtissue contraction frequency is not affected by matrix organization

The µTUG system containing arrays of flexible microposts was used to measure the contractile properties in anisotropic and isotropic tissues. To investigate the effect of matrix organization on microtissue contractile behavior, displacement of microposts caused by spontaneous beating of CMs was monitored for seven days. Single beating cells were observed within the microtissues from day one of culture while synchronized beating that caused micropost displacement (figure 5A) could be observed from day two of culture in both groups. At day two, the beating frequency of anisotropic microtissues (3.5±0.7 Hz) was not significantly different from isotropic microtissues (3.6±0.5 Hz). At day 4, the beating frequency of both groups did not significantly change compared to day 2, although the frequency of the anisotropic tissues (3.1±0.6 Hz) was significantly lower than of the isotropic tissues (3.8±0.5 Hz). While the beating frequency remained rather constant over the first four days of culture, at day seven, a large decrease in frequency was observed in both groups (65% decrease in anisotropic microtissues, 70% decrease in isotropic microtissues). This resulted in a frequency of 1.0±0.9 Hz for the anisotropic microtissues and a frequency of 1.1±0.9 Hz for the isotropic microtissues (figure 5B).

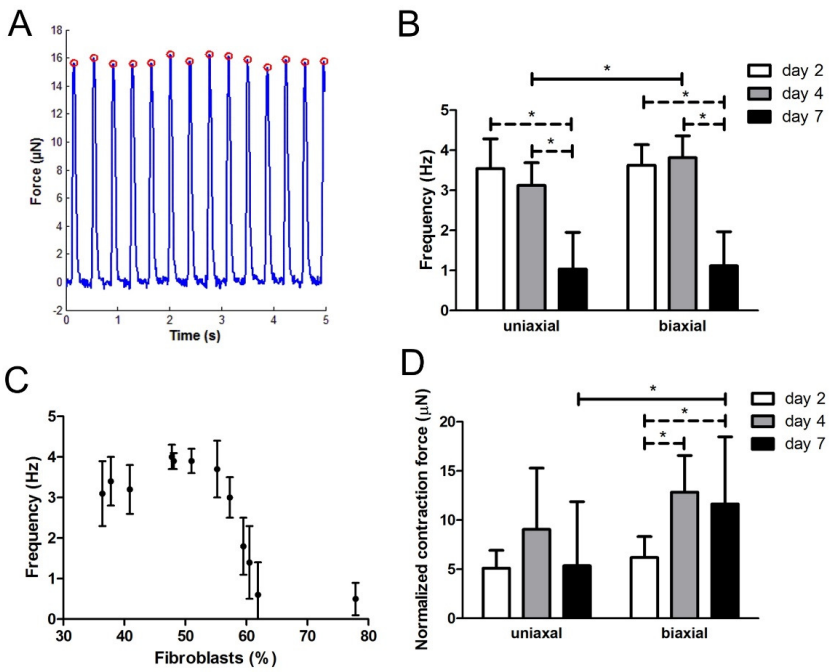

Figure 5 *Contraction force and frequency of uniaxally and biaxially constrained microtissues. (A) Total absolute contraction force over time for a representative microtissue, peaks indicate the dynamic contraction force. (B) Frequency decreased on day seven in both groups. (C) Beating frequency versus percentage of cardiac fibroblasts shows that after reaching a threshold of 55% cFBs the frequency decreased rapidly. (D) Dynamic contraction force normalized for microtissue volume increased over time in biaxially constrained microtissues. On day 7 the contraction force in biaxially constrained microtissues was significantly higher compared to uniaxially constrained microtissues. Error bars represent SD of N≥18 from two independent experiments. *P<0.05*

Interestingly, despite the small significant difference at day 4, matrix organization did not have a strong effect on the beating frequency of the microtissues. Apart from tissue organization, cellular composition may also affect microtissue contractile properties. To

assess for a possible correlation between fibroblast numbers and microtissue beating frequency, the percentage of fibroblasts per experimental group was averaged and plotted against the frequency measured in the same experiment. Interestingly, a threshold value for the percentage of cardiac fibroblasts of around 55% was found to correlate with a reduction of the frequency from 3.5 Hz to almost 0 Hz (figure 5C).

Matrix disarray does not decrease the ability of CMs to generate contractile forces

Next to beating frequency, also the dynamic contraction force was calculated from micropost displacements. While contraction force in isotropic microtissues increased over time, no significant increase was found for the anisotropic microtissues. On day seven, contraction force was even lower in anisotropic microtissues (5.4±6.5 µN) compared to isotropic microtissues (11.6±6.8 µN) (figure 5D). To check for an effect of fibroblasts numbers on microtissue contraction force, the average percentage of cardiac fibroblasts per experimental group was also plotted against the contraction force measured in the same experiment, but no correlation was found. Due to the alignment of matrix and cells in the uniaxially constrained tissues, it was expected that contraction in these tissues is mainly in the direction of the alignment. In the anisotropic microtissues, contraction force in the direction of alignment was 59±10% of the total contraction, while in the isotropic tissues the distribution was 50±6% in both directions. So even if we take just one direction into account, the anisotropic microtissues were not able to generate higher force compared to isotropic microtissues. Together, these results suggest that in 3D, a disorganized matrix organization alone does not decrease the ability of the CMs to generate contractile forces.

Direction of contraction force and homogeneity of contraction force distribution is disturbed by matrix disorganization

The µTUG system was used to measure displacements at multiple microposts per tissue, to calculate local strains. This enabled the investigation of contractile behavior of the microtissues in more detail than by just looking at total contraction force. The standard deviation (SD) of the strains in the microtissues (figure 6A, B) was calculated as a measure of homogeneity of force distribution. If all cardiomyocytes in the microtissues generate the same force, the strain throughout the tissue will be equal, resulting in a very small SD. On the other hand, a heterogenous force distribution will be represented by a large SD of the strain. The SD of strains in both x- and y-direction was significantly different between the two groups on all time points (figure 6C, D). This indicates that the contraction force is much more homogenously distributed in anisotropic microtissues, compared to isotropic microtissues. Surprisingly, this homogeneous contraction did not lead to higher contractile forces. This suggests that matrix disorganization only influences the direction and homogeneity of contraction and not the total contraction force or frequency of the microtissues.

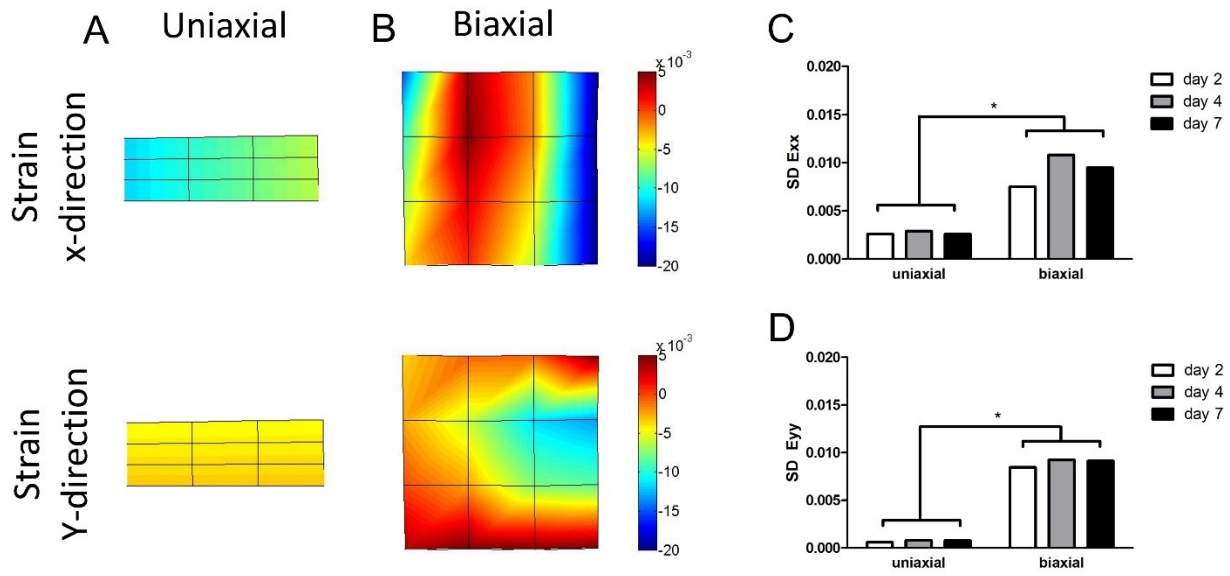

Figure 6 *Distribution of strains in anisotropic microtissues was more homogeneous compared to isotropic microtissues. (A) Strains in x- and y-direction in an anisotropic microtissue were homogenously distributed. (B) Strains in x- and y-direction in an isotropic microtissue were very heterogeneously distributed. (C, D) Mean SD of N≥16 from two independent experiments showed a higher standard deviation of the strains in both directions in the biaxially constrained microtissues compared to the uniaxially constrained microtissues, reflecting the heterogeneity in force distribution. *P<0.05*

Discussion

Knowledge on CM contractile behavior in response to changes in their microenvironment is still limited. Although recent research has shown the importance of matrix alignment for cardiomyocyte function, the dynamic interplay between cells and their environment makes this a complex phenomenon which is not yet fully understood. As the presence of cardiac fibroblasts helps to recapitulate the *in vivo* cellular distribution (10), we used enriched cardiomyocytes isolated from neonatal mouse hearts to create engineered cardiac tissue. As the fibroblasts are expected to proliferate during culture and the cardiomyocytes do not proliferate (13), depletion of fibroblasts was performed before microtissue seeding. Despite this approach, 43 ± 7% cardiac fibroblasts were found in our samples at day two, which is comparable to the amount of non-cardiomyocytes in neonatal murine hearts (27), thereby indicating the presence of a healthy cellular composition in our microtissues at this time point. Where the percentage of fibroblasts in the adult heart stabilizes over time, in our study it increased to more than 60% at day seven. The high proliferation of cardiac fibroblasts was previously described in 2D in vitro cultures using rat cardiac cells in which the percentage of fibroblasts increased from 16% at day one of culture to 37% at day four and 62% at day nine (13).

The cardiomyocytes and cardiac fibroblasts that were seeded in a collagen/matrigel mixture compacted around the micropost and self-sorted into a heterogeneous cell distribution similar to the cardiac microtissues of Desroches et al. (10). After two days of culture, the cardiomyocytes in the microtissues started to beat synchronously, resulting in displacement

of the microposts. Interestingly, matrix disarray did not negatively affect the beating frequency. The average microtissue beating frequency of 3.5 Hz at day two and four was relatively higher than the frequency between 2 Hz and 3 Hz that has been previously reported for mouse engineered cardiac tissues (28). However, the explored *in vitro* frequencies do not reach the *in vivo* frequency of a mouse heart, which is around 10 Hz (29), probably caused by the differences in organization, composition and maturation of the CMs.

At day seven, we measured a large decrease in frequency in both groups (65% decrease in anisotropic microtissues, 70% decrease in isotropic microtissues). The increase in cFB number during culture and the decrease in frequency at day seven raised the suggestion of a relation between these two effects. Indeed we found a negative correlation between the percentage of cFBs and the beating frequency. Interestingly, our results suggest a threshold of around 55% cFB which correlates with the sudden reduction in frequency from 3.5 Hz to almost 0 Hz. At day 4 of culture, microtissues contained already 52 ± 10% cFBs. In cardiac cell monolayers it has been shown that proliferation of cFBs reduces the conduction velocity probably by inhibiting contraction signal propagation between cardiomycoytes (13). Surprisingly, we did not see a decrease in frequency at this time point, suggesting that the CMs can still propagate their conduction signals. Only when the percentage of fibroblasts increased even more, the frequency drastically decreased to almost 0 Hz. More cardiac fibroblasts could also lead to a higher matrix production, which in turn may stiffen the matrix over time and may affect beating of the cardiomyocytes. For single cardiomyocytes in 2D, it has been shown that higher substrate stiffness lead to a decrease in beating (30).

Next to beating frequency, we also investigated the effect of matrix (an)isotropy on the force of contraction in microtissues. Our anisotropic tissues did not show higher contraction forces than isotropic tissues, while previous studies showed that alignment of cells improved the contractile strength of the tissues (5, 6, 9). The contradiction between our data and previous data could be caused by the fact that previous studies all used electrical stimulation, while our study concentrated on spontaneous contraction. Furthermore, where previous studies (5, 6) used curvature of the substrate in just one direction as a measure of contraction force, in our study the displacement of the microposts in all directions was used. So to compare our results to these studies (5, 6), only forces in one direction should be considered. However, our data showed that even if we considered forces in just one direction, alignment of the matrix did not lead to higher contractile forces.

A limitation of our study is that the alignment of collagen in the anisotropic microtissues may cause local increases in stiffness that could possibly affect cardiomyocyte contraction. Previous 2D studies have showed that cardiomyocyte contraction force increased on stiffer substrates (23, 31). Although the exact effect of stiffness on sarcomere properties remains unclear, most studies found differences in sarcomere length and/or width when changing substrate stiffness (23, 24, 32).

To the best of our knowledge, this is the first study describing strain distributions in cardiac microtissues. Our data showed a more homogenous contraction force disctribution in

anisotropic microtissues compared to isotropic microtissues, probably caused by the improved organization and morphology of cardiomyocytes in these tissues. A quantitave analysis of cell orientation showed that matrix alignment induced alignment of cells. In the cardiomyocytes, this also led to alignment of the sarcomeres, while a more stellate cell shape resulted in disarray of the sarcomeres. However, alignment or disarray of sarcomeres did not affect sarcomere length and width, which were found to be similar in anisotropic and isotropic microtissues. Although a previous study in 2D showed that cardiomyocyte contractility is a function of cell shape which coincides with sarcomere organization (22), our data did not confirm this for a 3D environment. In 2D, myocyte shape regulates alignment of sarcomeres and thereby cardiomyocyte contractility (22). Our data showed similar sarcomere lengths for the different cell shapes found in anisotropic and isotropic tissues, indicating that in 3D, cell shape does not coincide with sarcomere length. Although the heterogeneity of force distribution that we showed in isotropic microtissues did not negatively affect total contraction force and frequency, in the native heart this inhomogeneity might decrease efficient pump function.

Conclusion

Our data showed that the described µTUG system was suitable to mimic (dis)organization of the cardiac microenvironment. Disorganization of the microenvironment did not negatively affect the force that could be generated by cardiomyocytes, although it did have a negative effect on the direction and homogeneity of contraction force distribution. This is probably caused by the differences in cell shape and sarcomere organization dictated by the matrix organization. Cellular composition however, did influence microtissue contraction as indicated by the negative correlation between the percentage of cardiac fibroblasts and the beating frequency of the microtissues. This suggests that in regeneration of the diseased heart, not only reorganization of the disorganized matrix will contribute to restore the coordinated contraction but to completely restore tissue function, repair of the cellular composition is also of importance.

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5

Fibroblast density and not collagen content affects cardiomyocyte contractility in *in vitro* tissue models of cardiac fibrosis

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Submitted for publication

Abstract

Myocardial fibrosis is associated with increasing collagen content and fibroblast density. While it is well known that fibrosis has a detrimental effect on cardiac function, it remains unclear what the effect of increasing collagen content and fibroblast density have on cardiomyocyte function. Here, we present an *in vitro* tissue model of the myocardium, which was used to mimic the different features of cardiac fibrosis. Cardiac cells were seeded in microtissues with increasing collagen concentration or fibroblast density. Our results showed that although collagen content did affect overall tissue forces, fibroblast density was most detrimental for cardiomyocyte contractility. Furthermore, we revealed a fibroblast density threshold after which cardiac microtissues beating was severely reduced. Together, these results suggest that for regeneration of the diseased heart, it is more important to restore the balance between the different cell populations than to decrease the collagen production.

Introduction

One of the most common features of heart disease is myocardial fibrosis. Depending on the underlying pathology, this can either be characterized as interstitial or replacement fibrosis, with a diffuse or localized increase in collagen content respectively (1). Cardiac fibrosis is accompanied by cardiomyocyte loss and/or (myo)fibroblast activation and proliferation, which disturbs the cellular composition of the diseased myocardium (2, 3). As a result, myocardial function is impaired, leading to development of heart failure. To reverse this process, anti-fibrotic therapies are being investigated and developed (4). However, this remains difficult due to the complex nature of this pathology and the lack of basic knowledge about the effects of fibrosis at the cellular level. Whereas it is clear that both cellular and extracellular changes are involved, it remains uncertain what is most detrimental for cardiomyocyte contractility. In order to achieve therapeutic advances, it is critical to identify the cellular and extracellular features that impair cardiomyocyte function and thereby contribute to the development of heart failure.

While cardiomyocytes are the contractile units of the heart, cardiac fibroblasts are responsible for producing and maintaining the extracellular matrix (ECM), which mainly consists of collagen type I. The collagen fibers provide structural cues for alignment and organization of the cardiomyocytes, and thereby facilitate efficient contraction of the heart. Upon development of heart disease, fibroblasts become activated, increase their proliferation, and change their matrix production and degradation.

In 2D cultures, an increase in the number of fibroblasts was shown to decrease the conduction velocity (5). Upon activation from a fibroblast to a myofibroblast phenotype, these cells become more contractile than in a quiescent state. This has been shown to increase activation of mechanosensitive channels and decrease conduction velocity even further (6). The effect of increase in collagen content has mainly been studied indirectly, assuming that increase in collagen leads to a higher tissue stiffness. These monolayer studies showed that cardiomyocytes increase their contraction force in response to higher environmental stiffness (7, 8). Cardiac fibroblasts are also affected by substrate stiffness, and are suggested to migrate to stiffer regions in two-dimensional (2D) cultures (9). However, increase in collagen content alone does not necessarily lead to an increase in tissue stiffness, as was recently shown by different groups (10, 11).

Although this data from previous research show a clear effect of fibroblasts on cardiomyocyte conductivity, their impact on contractility of the cardiomyocytes remains unclear. Furthermore, the impact of increased collagen production is unknown, as only the effect of stiffness was extensively studied. Next to that, only few studies have addressed cardiomyocyte functionality in their more native-like 3D environment, while it has been shown that proliferation, attachment to the extracellular matrix and maturation are different in 2D than in 3D (12).

Recently, we established a 3D in vitro tissue model of the myocardium, which allows for measurement of both frequency and force of beating cardiac microtissues (13). In the present study, the model system was adapted to mimic the various features of fibrosis by

increasing collagen content or cardiac fibroblast density. We demonstrated that contraction force of the tissue is decreased with an increasing fibroblast density or collagen content. Interestingly, beating frequency is only affected by fibroblast density and not by increasing collagen content. Strikingly, a threshold of around 55% fibroblasts was revealed, above which synchronized beating of the cardiac microtissues was inhibited.

Materials and Methods

Neonatal cardiac cell isolation and culture

Animal experiments were approved by the Animal Experiments Committee of The Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the National Institutes of Health. Mouse neonatal cardiomyocytes and cardiac fibroblasts were isolated as described previously (13, 14). Briefly, neonatal mouse hearts were incubated overnight in Trypsin (Sigma) before using three-minute collagenase A (Sigma) incubation steps to dissociate the tissue. The obtained cell suspension was seeded in an uncoated T75 flask for 60 minutes. Non-attached cells were seeded in a new uncoated T75 flask for another 30 minutes. Non-attached cells from this flask were considered as enriched cardiomyocytes and were cultured in six-well plates coated with 1% gelation (Sigma) and 10 µg/ml fibronectin (Tebu-Bio) at a density of $5x10^4$ cells/cm^2. Adherent cells after 60 minutes of depletion in a T75 flask were considered cardiac fibroblasts and were cultured until confluent before they were split 1:3. When cultured until confluent, the cells were frozen and stored for next experiments. Culture medium consisted of high glucose DMEM (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (Greiner Bio), 1% L-glutamine (Sigma), and 1% penicillin/streptomycin (Sigma). Freezing medium for cFBs consisted of culture medium with an additional 45% heat-inactivated fetal bovine serum and 5% DMSO. After thawing, fibroblasts were seeded in culture flasks coated with 1% gelatin and 10 µg/ml fibronectin.

Microtissue seeding

Microfabricated tissue gauges (µTUGs) with microposts as uniaxial tissue constraints were fabricated using soft lithography as described previously (13, 15). We created microtissues with increasing fibroblast density and with increasing collagen concentration. To create microtissues with increasing fibroblast density, hydrogel composition was the same as described previously (13) and was prepared by mixing 50% collagen type I (BD Biosciences, 3.2 mg/ml), 39% culture medium, 3% 0.25M NaOH, and 8% growth factor-reduced Matrigel (BD Biosciences), resulting in a final collagen concentractin of 1.6 mg/ml. For microtissues with increasing collagen concentration, the final concentration was varied between 0.5, 1.5, 2.5 and 3.5 mg/ml collagen type I. For both groups, cells were trypsinized and suspended in the hydrogel at a concentration of $1x10^6$ cells/ml. For microtissues with increasing collagen content, only enriched cardiomyocytes were used, while for microtissues with extra cardiac fibroblasts, this cell suspension consisted of enriched cardiomyocytes mixed with 0%, 15%, 30% and 45% cardiac fibroblasts. The cell-hydrogel suspension was centrifuged into the microwells before aspirating excessive hydrogel. After 10 minutes of polymerization in an incubator, culture medium was added to the constructs.

Tissue compaction and force measurement

After seeding, cells compacted the matrix around the microposts. Beating of the cells caused displacement of the microposts. Movies were recorded at day two using a high speed camera mounted on a Zeiss observer microscope with a 10x magnification. Tissue width was measured for each sample using the first frame of the recorded movie. Compaction was calculated as the percentage of decrease in tissue width in the middle of the tissue. The distance between the micoposts (325 µm) was used a reference for 0% compaction.

Dynamic contraction force and beating frequency of the microtissues was calculated from the micropost displacements as described previously (13). Displacement of the microposts was tracked using feature detection software in Mathemica and Matlab. The displacement-force relationship of the microposts with a spring constant of 0.7 µN/µm (13) was then used to calculate the dynamic contraction forces of the microtissues. Beating frequency was deducted from the Fourier Spectrum of the signal.

Immunofluorescence staining and image analysis

Microtissues were fixed in 10% formalin for 10 minutes and stored in PBS before immunofluorescence staining. Samples were stained with antibodies against α-actinin (Sigma-Aldrich, 1:800) and vimentin (Cell Signaling, 1:400) with secondary antibodies Alexa 555 conjugated anti-mouse igG1 (Molecular Probes, 1:300) and Alexa 488 conjugated igG (Invitrogen, 1:300), respectively. Confocal microscopy was performed on a Zeiss 2-photon confocal laser scanning microscope. To determine the percentage of cFBs in the microtissues, α-actinin and vimentin positive cells were counted in ImageJ using two images per sample with a 40x magnification.

Stiffness measurement

Stiffness of the different hydrogel compositions with extra cFBs and extra matrix were determined using a Piuma nanoindenter (Optics11, The Netherlands). Tissues consisted of 100 µl of hydrogel with cell densities and collagen concentrations as described for microtissue seeding. After 30 minutes of gelation, medium was added to the constructs. Nanoindentation was applied at day two of culture using a glass spherical indenter with a radius of 44 µm and a spring constant k=0.52 N/m. Samples were indented for 10 µm on three different spots per sample. The Young's modulus was calculated from the force-displacement curves by the Piuma software based on the method by Oliver and Pharr (16, 17).

Statistical analysis

All results were expressed as mean±SD. Statistical analysis was performed with GraphPad Prism software using a one-way ANOVA with Bonferroni post-hoc test for multiple comparisons with a Gaussian distribution. Otherwise, a non-parametric Kruskal-Wallis test with Dunns post-hoc test was used. Differences were considered statistically different when $P<0.05$.

Results

Engineering fibrotic microtissue constructs

Fibrosis was successfully mimicked by manipulating cardiac fibroblast density and collagen content of microtissues seeded in a previously developed µTUG system (13). After cell isolation, a mixed population of cardiomyocytes and fibroblasts was obtained. To create microtissues with increasing fibroblast density, the ratio between cardiomyocytes and cardiac fibroblasts was changed by adding cardiac fibroblasts to the cell suspension before seeding the microtissues, resulting in four significantly different groups (figure 1). Microtissues without any additional fibroblasts contained 68 ± 5% cardiomyocytes and 32 ± 5% cardiac fibroblasts and are further referred to as the 0% extra cFBs group. Adding extra fibroblasts resulted in microtissues with 42 ± 6%, 62 ±7%, and 66% ± 8% fibroblasts, which are further referred to as the 15%, 30%, and 45% extra cFB groups. Increased fibroblast density caused more compaction of the tissues and resulted in more elongated cells (figure 1D) when compared to microtissues without extra cardiac fibroblasts (figure 1A).

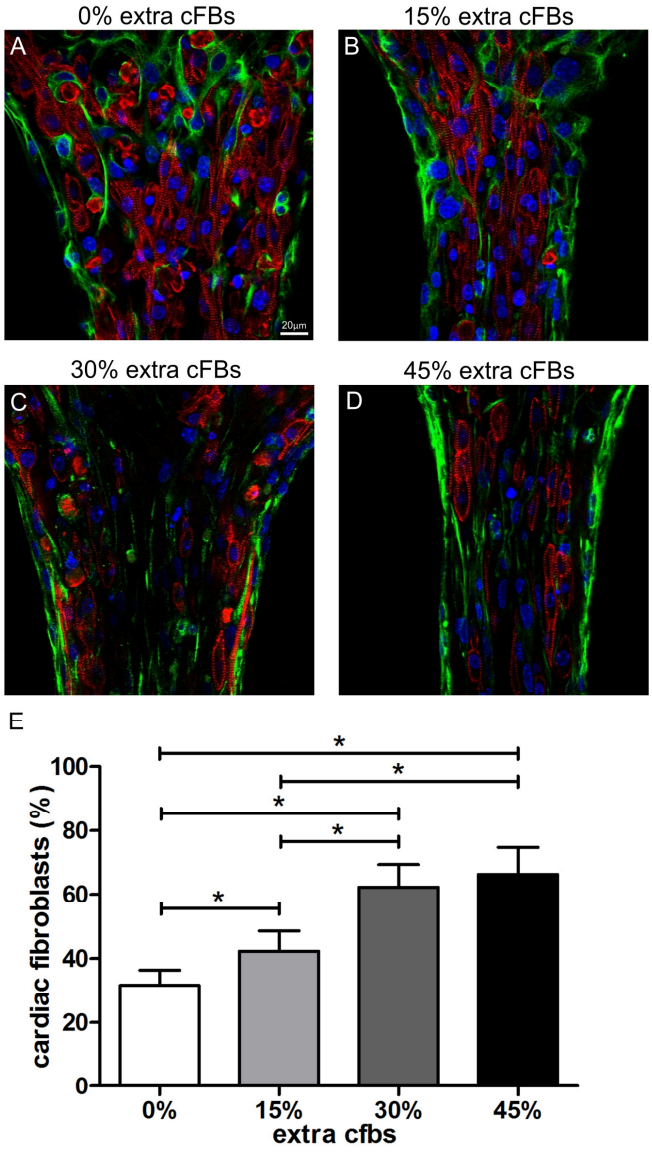

Figure 1 *Engineering microtissues with increasing fibroblast density. Increasing cardiac fibroblast density (E) was confirmed using immunohistochemistry markers for cardiomyocytes (alpha-actinin, red) and cardiac fibroblasts (vimentin, green) with nuclei in blue at day two of culture (A-D). Errorbars represent SD of N≥30 from three independent experiments. *P<0.05*

Increasing fibroblast density inhibits cardiac microtissue contractility

Increasing cardiac fibroblast density of the microtissues mimicked another feature of fibrosis. The increased fibroblast density had negative consequences for the contractility. Both beating frequency and dynamic contraction force significantly decreased upon increase in fibroblast density (figure 2). Without additional fibroblasts, beating frequency of the microtissues was 2.2 ± 0.7 HZ. Adding 15% extra fibroblasts did not significantly affect the beating frequency. However, beating frequency of the microtissues significantly reduced to 1.0 ± 0.9 Hz and 0.3 ± 0.5 Hz upon addition of 30% and 45% extra fibroblasts, respectively (figure 2A).

Dynamic contraction force was determined at 5.2 ± 2.7 µN for the control group without extra fibroblasts (0% extra fibroblasts) and reduced to 3.5 ± 1.9 µN by adding 15% extra fibroblasts, although this decrease was not significant. Interestingly, further increase in fibroblast density did cause a significant decrease in contraction force to 1.2 ± 1.1 µN and 0.4 ± 0.8 µN, resulting in almost no contraction in microtissues with 45% extra fibroblasts (figure 2B).

The fact that beating frequency did not decrease upon addition of 15% extra cardiac fibroblasts, while it did decrease in groups with 30% and 45% extra fibroblasts, raised the suggestion that there is a threshold fibroblast density after which beating of the microtissues is drastically reduced. Indeed, when analyzing frequency and fibroblast percentage of each individual microtissue a clear threshold value was observed at 50% cardiac fibroblasts above which average beating frequency dropped significantly (figure 2C). Microtissues with less than 50% fibroblasts had an average frequency of 2.3 ± 0.9 Hz with only 8% non-beating tissues compared to 0.6 ± 0.9 Hz with 62% non-beating tissues in microtissues containing more than 50% fibroblasts.

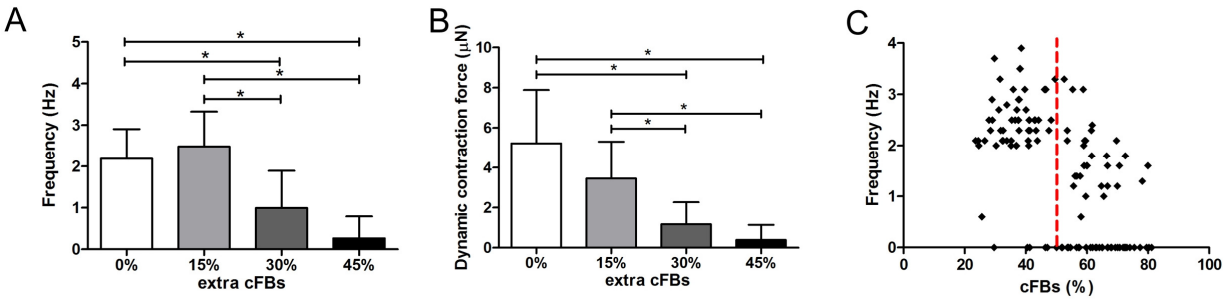

Figure 2 *Effects of increasing fibroblast density on microtissue contractility. Beating frequency (A) and dynamic contraction force (B) decreased with increasing cardiac fibroblast density. A threshold of 50% cardiac fibroblasts was found (indicated by the red line), after which beating of the microtissues was severely hampered (C). Errorbars represent SD of N≥30 from three independent experiments. *P<0.05*

Increasing collagen concentration decreases cardiac microtissue contraction force

Fibrotic cardiac microtissues mimicked by an increase in collagen concentration, the increased collagen content had negative consequences for the dynamic contraction force, but not for the beating frequency. Beating frequency of the microtissues fluctuated between 1.9 ± 0.9 Hz and 2.7 ± 0.7 Hz with significant differences but no increasing or decreasing trend (figure 3A). However, dynamic contraction force of the microtissues showed a significant decrease with increasing collagen concentration. Dynamic contraction force was 6.1 ± 2.6 µN in microtissues with 0.5 mg/ml collagen type I. Increasing the collagen concentration in the microtissues to 1.5 mg/ml caused a significant decrease in contraction force to 4.7 ± 2.3 µN. Contraction force was even further reduced to 4.0 ± 2.0 µN and 3.6 ± 2.0 µN in microtissues with 2.5 mg/ml and 3.5 mg/ml collagen (figure 3B).

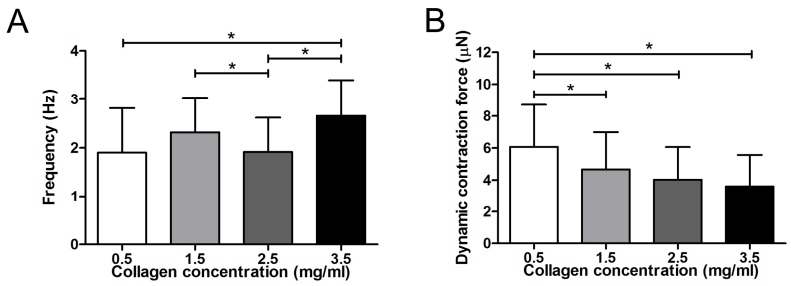

Figure 3 *Effects of increasing collagen concentration on microtissue contractility. Beating frequency (A) fluctuated while dynamic contraction force (B) decreased with increasing collagen content. Errorbars represent SD of N≥39 from three independent experiments. *P<0.05*

Tissue stiffness is not affected fibroblast density or collagen content

To assess whether the decrease in contraction force with increasing fibroblast density or collagen concentration was due to higher tissue stiffness, indentation tests were performed on tissues with increasing fibroblast density or collagen content. Tissues without any additional fibroblasts had an E-modulus of 3.9 ± 0.3 KPa. Increasing the fibroblast density had no effect on tissue stiffness (figure 4A). Tissues with the lowest concentration of 0.5 mg/ml collagen had an E-modulus of 3.7 ± 0.5 kPa. Interestingly, increasing the collagen concentration did not change the stiffness of the tissues (figure 4B).

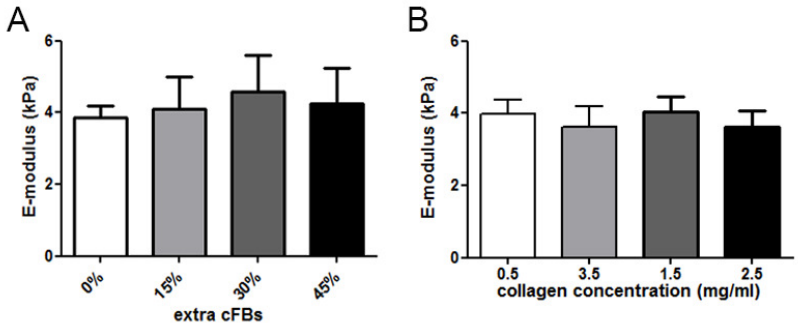

Figure 4 *Stiffness measurements for fibrotic tissues. Stiffness remained constant for increasing fibroblast density (A) or collagen content (B). Errorbars represent nine measurement spots on three samples per group in one experiment.*

Fibroblast density and not collagen content affects cardiomyocyte contractility

By changing the cellular and extracellular composition of the cardiac microtissues, tissue compaction also changed. Increasing fibroblast density, resulted in higher compaction of the tissues. Cardiac microtissues without extra fibroblast compacted by 52 ± 4%. Adding extra fibroblasts to the microtissues, caused a significant increase in compaction up to 64 ± 2% for microtissues with 45% extra fibroblasts (figure 5A). On the other hand, increasing the collagen concentration of the microtissues caused a reduction in compaction. Cardiac microtissues with 0.5 mg/ml collagen compacted for 67 ± 5% and increasing collagen concentration reduced the compaction to 47 ± 5% for microtissues with the highest collagen concentration (figure 5B).

To exclude the effect of these changes in compaction on the dynamic contraction force of the different groups, forces were corrected for changes in tissue width. For microtissues with extra fibroblasts, this correction did not change the overall results, as there is still a significant decrease in contraction force with increasing fibroblasts density (figure 5C). However, for microtissues with extra matrix, this correction did change the outcomes: while the dynamic contraction force showed a decrease with increasing collagen content, the corrected contraction force was now similar for the different groups (figure 5D).

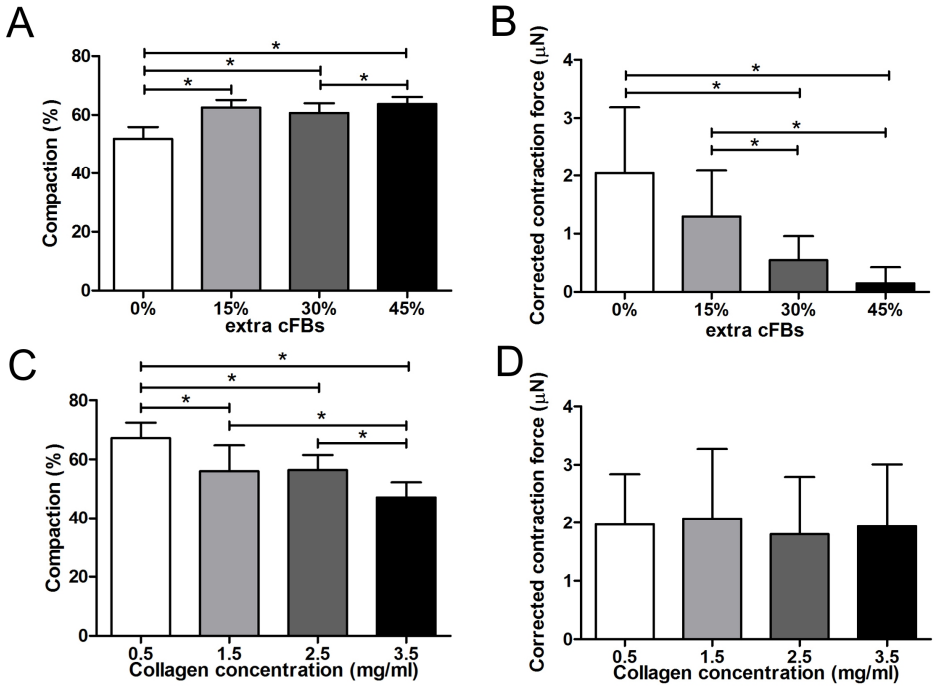

Figure 5 *Tissue compaction and dynamic contraction forces corrected for changes in tissue width. Compaction of the microtissues increased with increasing fibroblast density (A), while it decreased with increasing collagen concentration (B). Dynamic contraction forces corrected for compaction decreased with increasing fibroblast density (C) but remained constant with increasing collagen concentration (D). Errorbars represent SD of N≥30 from three independent experiments. *P<0.05*

Discussion

In the fibrotic cardiac microenvironment, both cellular and matrix composition are disturbed due to cardiomyocyte death, fibroblast proliferation and activation, and excessive matrix production (1, 3). In order to systematically asses the effect of fibroblast density and collagen content on cardiomyocyte contractility, we mimicked these features of fibrosis by increasing fibroblast density or collagen concentration in cardiac microtissues seeded in a previously developed μTUG system (13). To mimic the aligned tissue organization, tissues were uniaxially constrained, by which an anisotropic cell and collagen orientation is obtained (13). The cardiac cells that were isolated from neonatal mouse hearts consist of a heterogeneous population of both cardiomyocytes and cardiac fibroblasts. However, since fibroblasts do proliferate in culture, while cardiomyocytes do not (5), preplating was performed to remove excess fibroblasts from the cell population. These enriched cardiomyocytes were then used to create cardiac microtissues for the 0% group, resulting in

tissues with 68 ± 5% cardiomyocytes and 32 ± 5% cardiac fibroblasts. This is comparable to the cellular composition found in neonatal murine hearts (18). Furthermore, fibroblasts are essential for proper functioning of the cardiomyocytes (19). Therefore, using this mixed cell population is more closely mimics the native cardiac tissue composition, compared to other studies that use a pure cardiomyocyte population to engineer cardiac tissues (20, 21).

While it is clear that the number of fibroblasts increase during development of fibrosis (2), exact numbers are lacking. Therefore, in this study we chose to increase fibroblast density stepwise by adding cardiac fibroblasts from a previous isolation, resulting in microtissues with a range of fibroblast densities from 32 ± 5% up to 66 ± 8% fibroblasts. We hypothesized that increasing fibroblast density in our 3D cardiac microtissues would result in a decreased beating frequency. Indeed, our results demonstrate a significant decrease in beating frequency when the number of fibroblasts increased. This correlates with previous 2D research, which demonstrated a decreased conduction velocity with increased fibroblast proliferation (5). Furthermore, studies by other have shown that fibroblasts can electrically connect to cardiomyocytes via gap junctions (22, 23). Although the gap junction protein connexin43 was present in all cardiac microtissues, the distribution was random and not structured in intercalated discs (data not shown). Therefore, it was not possible to quantify the amount and location of these junctions. Therefore, our results could not be directly linked to gap junction formation between cardiomyocytes and fibroblasts. Nevertheless, in 2D it was shown that cardiomyocytes beat much faster when cultured on fibroblasts devoid of connexin43 than on wild-type fibroblasts that do express these gap junction proteins (24). Although it has frequently been reported that fibroblast proliferation inhibits the contractility of cardiomyocytes, in our study, for the first time we demonstrate a threshold value for the fibroblast density after which synchronized beating is severely hampered. Cardiac microtissues with less than 50% fibroblasts are rarely non-beating and have a frequency higher than 2 Hz, while microtissues with more than 50% fibroblasts are mostly non-beating or have a reduced frequency. Our results not only show a decrease in beating frequency, but dynamic contraction force was also decreased in cardiac microtissues with increasing fibroblasts density.

While increasing fibroblast density decreased both beating frequency and dynamic contraction force, increasing collagen content of the microtissues only affected contraction force. As many studies assume that tissue stiffness increases when collagen content increases, we verified this by determining the E-moduli of our cardiac tissues using nano indentation. Our data indicate that increasing fibroblast density or collagen concentration does not affect tissue stiffness. Recently it has been shown that not collagen content but collagen cross-linking determines tissue stiffness (10, 11). Due to the relatively short culture time of the tissues in this study, we assume that cross-linking is still limited and therefore has no effect on tissue stiffness. Even though tissue stiffness did not change with increasing amounts of collagen, cardiac microtissues were not able to produce to same force. However, if the cardiomyocytes exert the same contraction force independent of the amount of matrix around them, of course the whole tissue will be less contracted when they contain more matrix.

In both mimicked fibrotic environments in this study, cardiomyocyte performance is determined by beating frequency and contractile force, independent of tissue size and cellular composition. Since our results showed that increasing the collagen concentration affected tissue compaction. Microtissues with the highest collagen concentration had 46% less tissue compaction than microtissues with the lowest collagen concentration. To judge if cellular contractility is hampered by increased collagen content, independent of tissue size and cellular composition, it would be more accurate to calculate stresses instead of forces. However, due to the very small size of the tissues, tissue thickness could not be quantified in an accurate and reliable way. Therefore, we corrected the forces for differences in tissue width only. When dynamic contraction forces were corrected for these differences in dimension, indeed there was no longer a difference between the groups. This implies that the cardiomyocytes were still able to exert the same force of contraction, even though there was more collagen in between the cells. On the contrary, adding more cardiac fibroblasts, led to higher compaction of the microtissues. However, corrected dynamic contraction forces still showed the same decreasing trend and significant differences with increasing fibroblast density. This suggests that increasing the number of fibroblasts in the cardiac microtissues has a detrimental effect on cardiomyocyte functionality next to lowering their beating frequency. The exact mechanism still needs to be unraveled, but could be of electrical (5), mechanical (6), or paracrine origin (25).

Altogether, we showed that increased fibroblast density and not collagen content is most detrimental for cardiomyocyte contractility. For development of treatments for cardiac disease, our results indicate that at the onset of fibrosis, it is more important to focus on restoring the healthy cardiomyocyte-fibroblast ratio than to decrease collagen production.

Acknowledgments

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6

Fibulin-4 deficiency affects cardiomyocyte organization and leads to decreased contractility in engineered cardiac microtissues

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In preparation

Abstract

Fibulin-4 is essential for elastic fiber formation, and mutations in this gene lead to cardiac remodeling and dysfunction. While this has mainly been attributed to aortic valve problems, recently, it has been suggested that fibulin-4 deficiency also leads to direct myocardial dysfunction. To elucidate whether fibulin-4 directly affects the performance of cardiac cells, we isolated wild type (WT) and heterozygous (+R) and homozygous (RR) fibulin-4 deficient cardiac cells from neonatal mouse hearts. Subsequently, we cultured them in our previously developed microTUG system to measure tissue contractility, resulting in the first *in vitro* cardiac tissue model for fibulin-4 deficiency. Our results showed that fibulin-4 deficiency resulted in an increase of cardiac fibroblasts and disturbed cardiomyocyte organization for both +R and RR microtissues when compared to WT cardiac tissues. Together, this led to a decrease of both contraction force and beating frequency of +R and RR cardiac microtissues when compared to microtissues seeded with WT cardiac cells. These data indicate that next to its role in elastogenesis, mutations in fibulin-4 also directly affect cardiac function.

Introduction

The cardiac extracellular matrix (ECM) provides important structural and mechanical cues for the cells that reside in the heart. While collagen is the main load bearing protein of the ECM, the elastin network gives the heart elasticity to maintain its shape during contraction. The formation of the elastin network starts with secretion of tropoelastin proteins by the cardiac cells, which then mature and crosslink into a network of elastic fibers. In humans, impaired elastic fiber formation is associated with several heritable diseases such as the Marfan syndrome and Loeys-Dietz syndrome (1, 2). Impaired elastogenesis is caused, among others, by mutations in the fibulin genes. Fibulins are a familiy of proteins involved in the assembly of the elastic fiber network. The family consists of 7 members, with Fibulin-4 and -5 being the most important ones (3). These disorders affect the connective tissue in many parts of the body and both diseases are often associated with aortic aneurysms and heart valve regurgitation.

These malformations in the cardiovascular system were also observed in mouse models with a fibulin-4 deficiency. Fibulin-4 is a crucial factor in elastic fiber assembly and homeostasis. Recently, it has been shown that a two (fibulin-$4^{+/R}$) or four-fold (fibulin-$4^{R/R}$) systemic reduction in fibulin-4 expression also leads to cardiac remodeling and dysfunction (4, 5). In mice with a four-fold decrease in fibulin-4 expression, this has mainly been attributed to changes in the elastin formation and resulting aortic valve regurgitation. However, aortic valve leakage was not observed in mice with a two-fold fibulin-4 reduction even though mild cardiac dysfunction was present (5). These results suggest that fibulin-4 deficiency might lead to primary myocardial dysfunction (4, 5). This implies that Fibulin-4 also has functions outside of elastogenesis, which has already been shown for other cell types. For example, mouse smooth muscle cell specific knockout of Fibulin-4 resulted in an immature phenotype and increased proliferation compared to wild type smooth muscle cells (6).

To identify possible direct effects on cardiac pathology due to fibulin-4 deficiency, more knowledge is needed about contractile function and phenotype of fibulin-4 deficient cardiac cells. Investigation of this research question in the living heart is difficult, since the cells reside in a complex environment, which is affected by fibulin-4 deficiency and thereby might influence the cells. To elucidate whether fibulin-4 directly affects performance of cardiac cells, it is crucial to study these cells outside the *in vivo* environment at an early time point. Therefore, i*n vitro* cardiac tissue models could serve as an excellent tool to study the cardiac cells in a well-defined and controlled environment.

In this study, wild type and fibulin-4 deficient cardiac cells were isolated from neonatal mouse hearts and cultured in our previously developed microTUG system (7), which is capable of measuring contractile performance of cardiac microtissues. Here, we demonstrate that reduced fibulin-4 expression affects cardiomyocyte organization and leads to decreased contractility of the cardiac microtissues.

Materials & Methods

Experimental animals

Heterozygous Fibulin-$4^{+/R}$ mice in a mixed C57B1/6J;129Sv background were mated to obtain Fibulin-$4^{+/+}$ (WT), Fibulin-$4^{+/R}$ mice (+R), and Fibulin-$4^{R/R}$ (RR) littermates. Fibulin-$4^{+/R}$ genotype resulted in a 2-fold reduction and Fibulin-$4^{R/R}$ genotype in a 4-fold reduction of Fibulin-4 expression. All animals received care in compliance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals published by the NIH.

Cell isolation and culture

After sacrificing 1-3 day old neonatal mice and isolation of the hearts, each heart was individually incubated in trypsin overnight. A piece of the tail from each mouse was digested for genotype determination by performing a PCR for Fibulin-4, as described previously (8). The next day, hearts were pooled according to their genotype and cells were isolated using 3 minute collagenase incubation steps, as described previously (9). Most fibroblasts were removed from the cell culture by preplating in T75 flasks. Afterwards, cardiac cells were cultured in 6-well plates coated with 1% gelatin and 10 µg/ml fibronectin. Culture medium consisted of high glucose DMEM (Lonza), supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin.

Microtissue seeding and contractility analysis

Cardiac microtissues were seeded in a previously developed µTUG system with uniaxial constraints (7). Briefly, cardiac cells from each genotype were trypsinized and resuspended in a hydrogel consisting of 50% collagen type I (BD Biosciences, 3.2 mg/ml), 39% culture medium, 8% growth factor reduced Matrigel (BD biosciences), and 3% 0.25 M NaOH. This cell-gel mixture was then centrifuged into the microwells of the µTUG system. Excessive gel was aspirated before the microtissues were put in an incubator at 37 °C and 5% CO_2 to polymerize the hydrogel. After 10 minutes, culture medium was added to the microtissues and medium was changed every two days. At day two and four of culture, movies of the microtissues were recorded using a high-speed camera on a Zeiss observer microscope. Micropost displacement of beating microtissues was tracked with previously developed feature detection software in Mathematica and Matlab (7). Dynamic contraction force and beating frequency were calculated from the micropost displacements over time.

Immunohistochemistry and image analysis

After fixation in 10% formalin for 10 minutes, microtissues were stored in PBS. Samples were stained with antibodies against α-actinin (Sigma-Aldrich, 1:800), and vimentin (Cell Signaling, 1:400), with secondary antibodies alexa 555 conjugated IgG (Molecular Probes, 1:300) and alexa 488 conjugated IgG (Invitrogen, 1:300) respectively. Confocal microscopy was performed on a 2-photon confocal laser scanning microscope (Zeiss). To determine the percentage of cFBs in the microtissues, α-actinin and vimentin positive cells were counted in ImageJ using images with a 63x magnification. Sarcomere length was measured in ImageJ as

the distance between two α-actinin bands and sarcomere width as the width of a single α-actinin band.

Statistical analysis

All results were presented as mean ± SD. Statistical analysis was performed with GraphPad Prism software. Comparisons of a parameter between the three phenotypes were made using a non-parametric Kruskal-Wallis test with Dunns post-hoc test. Comparisons of two time points for one parameter were made using the Mann-Whitney test. Differences were considered statistically different when $P<0.05$.

Results

Reduced Fibulin-4 expression affects cellular composition and organization

At day 4 of culture, cardiac microtissues of wild type cells consisted of 26 ± 4% cardiac fibroblasts and 74 ± 4% cardiomyocytes. Cardiac cell populations from +R and RR hearts consisted of significantly more cardiac fibroblasts, with 35 ± 3% and 33 ± 4% cardiac fibroblasts in +R and RR microtissues respectively (figure 1D).

WT cardiomyocytes had an elongated cell shape and oriented in the direction of the constraints at day 4 of culture. Furthermore, they developed a mature sarcomere structure, as indicated by the striated pattern of α-actinin (figure 1A). Cardiomyocytes in +R and RR microtissues had a rounder cell shape and were therefore less oriented than in WT microtissues (figure 1B, C). However, sarcomere length was not affected by these structural changes, as no significant differences were found between the different groups. In all groups, the average sarcomere length was 1.8 ± 0.2 µm (figure 1E). Sarcomere width was only significantly reduced in +R microtissues (1.7 ± 0.6 µm), compared to WT (2.7 ± 0.8 µm) or RR (2.4 ± 0.8 µm) microtissues (figure 1F).

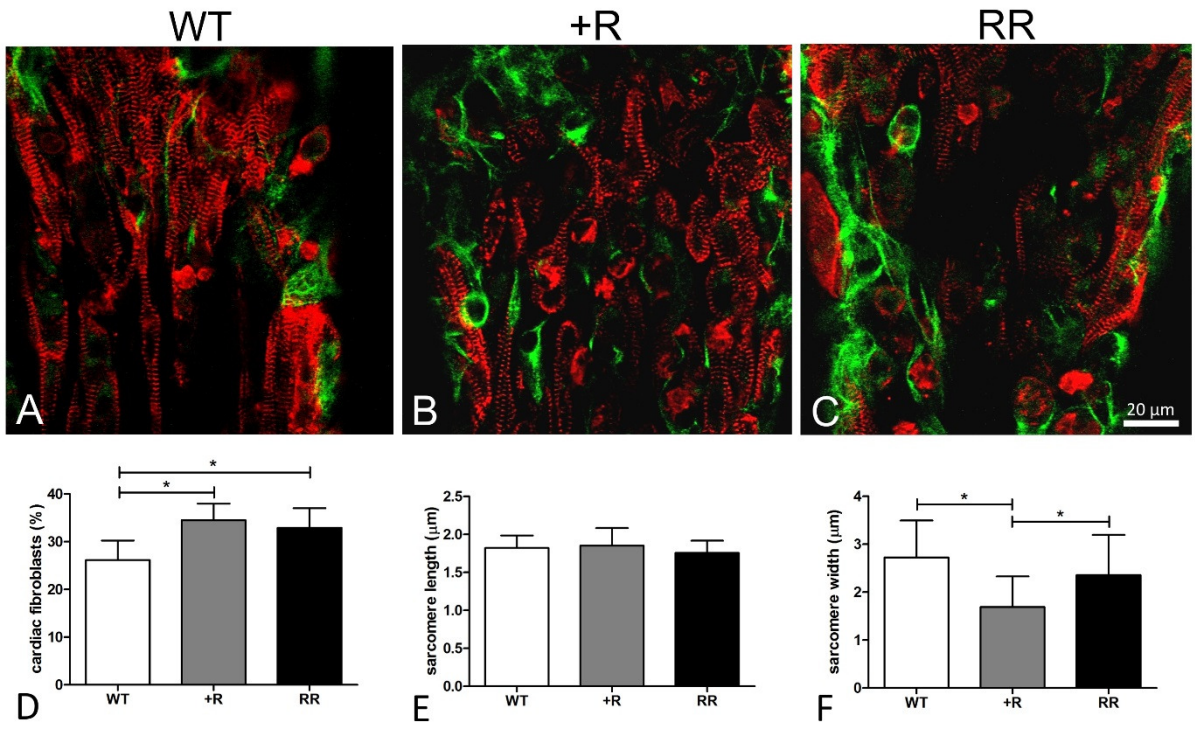

Figure 1 *Cell distribution and sarcomere organization in WT, +R and RR cardiac microtissues at day 4 of culture. The percentage of cardiac fibroblasts was significantly higher in +R and RR microtissues than in WT microtissues (D). WT cardiomyocytes were elongated with a mature sarcomere organization (A), while reduced Fibulin-4 expression resulted in a rounder and less structured cardiomyocyte organization (B-C). However, sarcomere length was not affected by these structural differences (E), and sarcomere width was only significantly reduced in +R microtissues (F). Error bars represent SD of N≥6 of two independent experiments. *P<0.05.*

Reduced Fibulin-4 expression leads to decreased contractility

To investigate the effect of Fibulin-4 deficiency on contractility, beating frequency and dynamic contraction force was measured for microtissues of all genotypes at day 2 and 4. At day 2, beating frequency of WT microtissues was 2.3 ± 0.8 Hz, while fibulin-4 deficiency resulted in a significant decrease in beating frequency with 1.4 ± 0.9 Hz and 1.0 ± 0.7 Hz for +R and RR microtissues respectively. Interestingly, beating frequency of WT and RR microtissues remained constant at during culture, frequency of +R microtissues decreased even further to 0.8 ± 0.9 Hz at day 4 of culture (figure 2A).

Next to the beating frequency, analysis of dynamic contraction force revealed forces of 3.3 ± 1.4 µN in WT microtissues at day 2 of culture. Contraction force of +R microtissues was not affected (3.6 ± 2.0 µN), while RR microtissues showed a significantly decrease in contraction force (2.4 ± 1.3 µN). Similar to the beating frequency, only the contraction force of +R microtissues reduced significantly to 2.3 ± 2.1 µN at day 4 of culture (figure 2B).

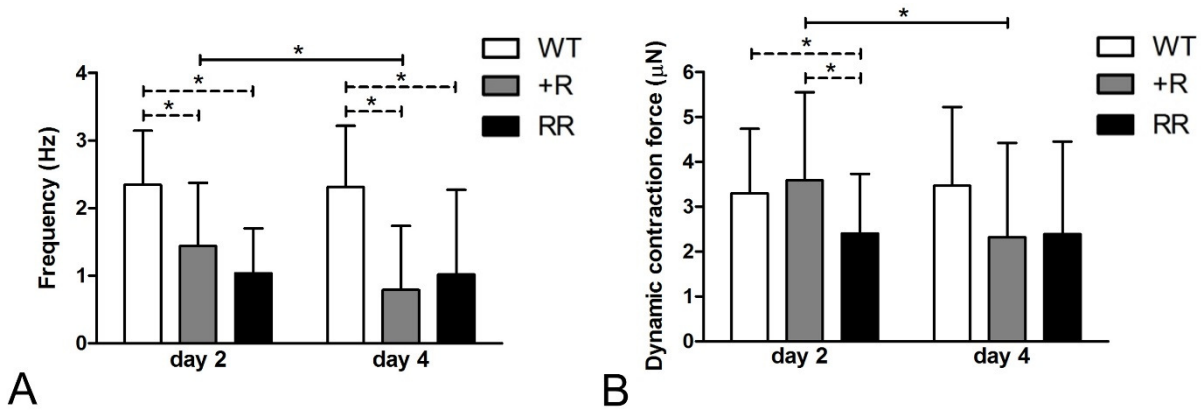

Figure 2 *Beating frequency and contraction force for WT, +R, and RR cardiac microtissues at day 2 and 4 of culture. Frequency of +R and RR microtissues was significantly lower than frequency of WT microtissues at day 2 and day 4 (A). Dynamic contraction force was significantly reduced in RR microtissues compared to WT and +R microtissues on day 2 (B). Frequency and contraction force of +R microtissues decreased over time (A, B). Error bars represent SD of N≥24 of two independent experiments. *P<0.05.*

Discussion

Complete knockout of Fibulin-4 in mice results in perinatal lethality, which has been attributed to impaired elastogenesis (10). To examine the role of fibulin-4 in elastic fiber assembly and cardiovascular disease, a mouse model which underexpresses fibulin-4 was generated (8). For the heart, reduction in Fibulin-4 expression leads to cardiac remodeling and dysfuction (5). While this has been mainly attributed to changes in the elastin network and resulting aortic valve regurgitation, a primary myocardial dysfunction has also been suggested (4, 5). In mice with a Fibulin-4 deficiency, an increase in cardiomyocyte size was observed, but it remains unclear whether this is directly caused by Fibulin-4 deficiency or a secondary effect of disturbed matrix deposition. Therefore, in this study we investigated a possible primary effect of Fibulin-4 deficiency on contractility of cardiac microtissues. While *in vitro* models have been used to study other genetic causes of heart disease (11, 12), this is the first *in vitro* model using fibulin-4 deficient cells.

Cardiac cells were isolated from WT, +R and RR hearts and seeded in a collagen/matrigel hydrogel in our previously developed μTUG system (7) with uniaxial constraints to mimic an aligned and structured ECM composition and organization. The isolated cells are a heterogeneous population composed of cardiomyocytes and cardiac fibroblasts. After 4 days of culture, WT microtissues contained 26 ± 4% cardiac fibroblasts while +R and RR microtissues contained more cardiac fibroblasts with 35 ± 3% and 33 ± 4% respectively. Although this is a significant increase compared to the WT microtissues in this study, these percentages are similar to what has previously been found for WT microtissues (7) and for neonatal murine hearts (13). Furthermore, they do not reach the threshold of 55% fibroblasts that was previously found to inhibit the beating frequency of cardiac microtissues (Chapter 5). Nonetheless, this slight increase in fibroblasts compared to WT

microtissues might indicate a difference in proliferation between the different groups. Previous work by Huang et al. using smooth muscle cells, showed an increased proliferation in cells that lack Fibulin-4 compared to wild type cells (6).

Previous work of our group showed that uniaxial constraints resulted in orientation of both cells and matrix in the direction of these constraints (7). Also in this study, WT cardiomyocytes elongated and oriented in this direction and developed a mature sarcomere organization, which is essential for contraction. However, cardiomyocytes in +R and RR microtissues appeared more round and less elongated than WT controls. We quantified sarcomere length and width, which are both important for proper contractility of the cell (14). Despite the differences in cell shape, sarcomere length was equal in all groups, with an average length of 1.8 ± 0.2 µm, which is similar to the length we and others have measured previously (7, 15, 16). However, sarcomere width of cardiomyocytes in +R microtissues was significantly decreased compared to WT controls. Surprisingly, this was not observed for RR microtissues. Although not completely captured by the sarcomere properties, the cytoskeleton in cardiomyocytes in both +R and RR microtissues appeared to be less structured than WT cardiomyocytes. Structural differences were also found for smooth muscle cells without fibulin-4, where a decrease in stress fibers was observed when compared to WT smooth muscle cells (6).

Contractility of the cardiac microtissues was assessed during spontaneous contraction, which started from day 2 of culture. At this time point, WT microtissues had a beating frequency of 2.3 ± 0.8 Hz. +R microtissues showed a 39% decrease in beating frequency. RR microtissues were even more affected and showed a 56% decrease in frequency compared to WT microtissues. While beating frequency of WT and RR microtissues remained constant during culture, frequency of +R microtissues decreased even further at day 4 of culture. This difference in contractility was not found in *in vivo* studies, where no differences in heart rate were found between WT, +R and RR hearts of 16 week old mice (4). These contradicting results might be explained by the fact that compensation mechanisms to keep a constant heart rate *in vivo*, are missing in our *in vitro* cardiac tissues. This also indicates that our model system is capable of detecting the earliest defects, before development of secondary changes lead to heart failure.

Next to beating frequency, dynamic contraction force was also affected by Fibulin-4 deficiency, although less severe. A significant reduction in contraction force was only observed in RR microtissues but not in +R microtissues, compared to WT controls at day 2. Even though average contraction force of +R (2.3 µN) and RR (2.4 µN) microtissues were lower than WT microtissues (3.5 µN), this was not significantly different, probably due to the large standard deviation.

Since the cardiac cells were seeded in a hydrogel and cultured for a period of only two days, most likely only a little amount of endogenous ECM has been produced. Therefore it is unlikely that impaired elastic fiber assembly affected the adhesion and contractility of the cardiomyocytes at these early time points. These results raise the suggestion that Fibulin-4 deficiency causes a primary defect in cardiomyocytes rather than a secondary effect, which caused the reduction in contractility of our cardiac microtissues. Together, we showed that

reduced Fibulin-4 expression resulted in a disturbed cardiomyocyte organization, which is most likely the underlying cause for the decreased contractility that was found in this study. Furthermore, our results confirm that Fibulin-4 has essential functions outside of elastogenesis as a direct contributor to cardiac function.

Acknowledgements

The authors thank Joyce Burger of the Erasmus Medical Center in Rotterdam for assistance with cardiac cell isolation and genotype determination. Luuk te Riet of the Erasmus Medical Center in Rotterdam and Elza van Deel of the VU Medical Center in Amsterdam are acknowledged for fruitful discussion of the results.

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7

GENERAL DISCUSSION

The aim of this thesis was to elucidate the impact of cellular and extracellular perturbations on cardiomyocyte contractility. A systematic analysis of cell and matrix organization and composition as well as their effect on cardiomyocyte contractility was carried out using an *in vitro* cardiac tissue model. In this chapter, we first review and discuss the main findings of the research presented in this thesis. Furthermore, we will consider the clinical relevance of these results and identify directions for future research. In the second part, benefits and limitations of our μTUG system will be discussed, before considering potential clinical applications and future perspectives of our model system.

Main findings and perspective

Cardiac disease leads to alterations in structure and composition of the matrix, inhibiting normal cellular and tissue function. In chapter 2, we explored how cardiac structure and matrix composition are implemented in current *in vitro* cardiac tissue models. We concluded that systematic manipulation and quantification of the different tissue properties is needed to unravel the complex dynamic interaction between cardiac cells and their environment.

To obtain more knowledge about the *in vivo* characteristics of cardiac disease, in chapter 3, we started by exploring the mechanical and biochemical properties of healthy and diseased myocardium, using an acquired and a genetic mouse model. Patchy fibrosis was found in the left ventricles of both mouse models. Further analysis revealed that the deposited matrix in the fibrotic lesions were mainly composed of collagen type I. Since we want to mimic the *in vivo* characteristics in our *in vitro* models, immunohistochemistry was used to analyze the composition of our engineered cardiac microtissues. These results confirmed the presence of important cardiac ECM proteins such as collagen I, III, and fibronectin.

To study cardiomyocyte contractility in a 3D environment, a model system that meets specific criteria is required. Most importantly, the system should allow for quantitative assessment of contraction force and frequency of the cardiomyocytes while they reside in a 3D environment that can be manipulated according to our research question. Therefore, in chapter 4, we developed a model system capable of measuring contractility of cardiomyocytes in a manipulable 3D environment and used this to study the effect of (an)isotropy on cardiac contractility. Matrix anisotropy induced alignment of the cardiomyocytes and concomitant sarcomere organization, while isotropy induced a random cell orientation and a stellate cell shape. As a result, the individual sarcomeres were also not properly aligned in these cells. However, the resulting chaotic organization did not result in a decreased contraction force or beating frequency compared to anisotropic tissues. Nevertheless, matrix disorganization did influence the direction and homogeneity of the contraction.

Most cardiac diseases, whether the cause is acquired or genetic in origin, are associated with fibrosis, as was also described in chapter 3. Therefore, in chapter 5 we decided to mimic two important features of fibrosis in our *in vitro* model by manipulating either cardiac fibroblast density or collagen content. Fibroblast density and not collagen content proved to be detrimental for cardiomyocyte contractility. Collagen content did affect overall tissue

force of contraction, which was not related to tissue stiffness. Importantly, this study also revealed a fibroblast density threshold of 50% fibroblasts above which beating of the microtissues was severely hampered.

Chapter 6 was dedicated to a genetic cause of ECM perturbation, due to fibulin-4 deficiency. While it is known that fibulin-4 is involved in formation of the elastin network, we tested the hypothesis that fibulin-4 also has a direct effect on cardiac contraction. Indeed, in our microtissues we observed structural changes in the sarcomeric organization of the cardiomyocytes in +R and RR microtissues. Here we showed for the first time that contractility decreased due to fibulin-4 deficiency, which was most prominent for the contraction frequency. This indicates that fibulin-4 is not only important for extracellular assembly of the elastic fiber network, but that this protein is also involved in cardiac contractility.

In this thesis we investigated the effect of different cellular and extracellular disturbances on cardiac tissue contractility. The model system developed in chapter 4 was used throughout the different studies, providing the same readouts for each study. This allows for a unique comparison of the collected results, although statistical comparison is not possible due to the fact that in each chapter different cellular or tissue properties were manipulated.

Comparing all data of the different chapters (figure 1) showed that increasing the fibroblast density resulted in the most detrimental effect on tissue contractility. Both contraction frequency (figure 1A) and force (figure 1B) decreased severely with increasing amounts of cardiac fibroblasts. Next to that, fibulin-4 deficiency also had a strong negative effect on contractility, which was most prominent for beating frequency (figure 1A). By manipulating the alignment and collagen content of the cardiac microtissues, only the passive tissue properties changed, while by increasing the amount of fibroblasts or by introducing a fibulin-4 deficiency, the cardiomyocytes itself are affected. By increasing fibroblast density, the ratio of contracting versus non-contracting cells decreased. Furthermore, fibroblasts most likely have an additional active contribution in decreasing contractility due to their connection to the cardiomyocytes, whether this is electrical (1), mechanical (2), or paracrine (3). For the fibulin-4 deficiency, we showed that this affected sarcomere organization and thereby tissue contractility.

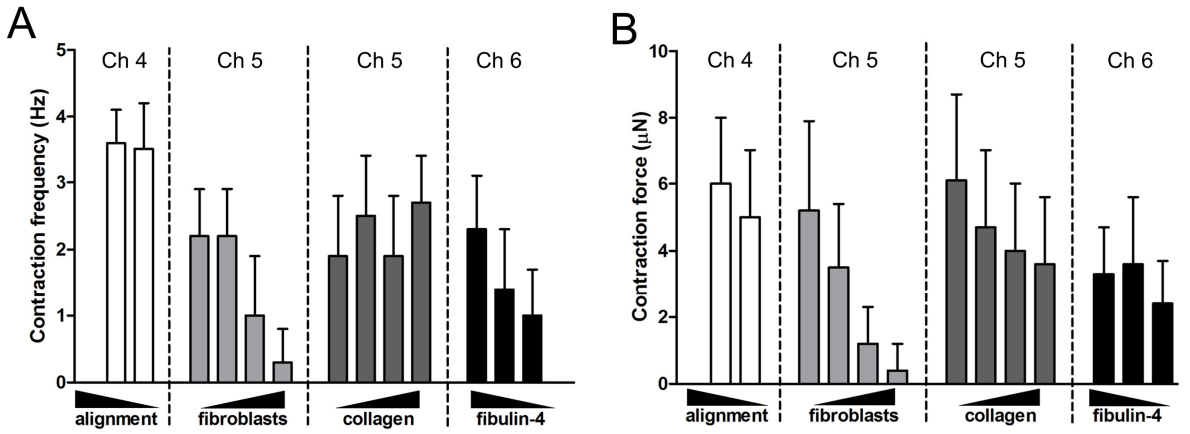

Figure 1: Overview of the contraction frequency (A) and contraction force (B) measured in chapters 4, 5, and 6 shows that increasing fibroblast density has a more detrimental effect on contractility than manipulating ECM structure or composition or introducing a genetic mutation.

The µTUG system was used to provide insight on the effects of cellular and extracellular perturbations on cardiomyocyte contractility that would be hard to measure *in vivo.* In summary, the most important finding of this thesis are:

- A chaotic matrix organization does not decrease cardiomyocyte contractility.
- Increased collagen content, without increasing tissue stiffness, does not affect tissue contraction.
- A fibroblast density above 50% inhibits spontaneous beating.
- Mutations in fibulin-4 directly affect cardiomyocyte contraction force and frequency.

Clinical relevance and indications for future research

From a clinical perspective, the findings of this thesis indicate that restoring the balance of the different cell populations in the heart is most effective to improve the original function of the heart. However, since the heart has only a limited regenerative capacity, increasing the number of cardiomyocytes is still a challenge. A possible strategy to achieve this is via stem cell therapy. Injection of different stem cell sources have been proposed to regenerate the cardiomyocyte population in the heart (4-7). Even though treatment effects are still limited in both animal and human studies (7, 8), this could be a promising technique to restore the balance between cardiomyocytes and fibroblasts and thereby preserve cardiac function.

Of course, to completely recover efficient pump function of the heart, reducing collagen accumulation and re-establishing the anisotropic organization are also important. Especially when you realize that each small perturbation in the cardiac microenvironment may trigger adverse remodeling leading to a negative spiral of events that eventually results in heart failure.

Future research regarding cardiomyocyte contractility could help to answer some of the following questions that still remain. While we showed that stiffness of the cardiac tissues is

not affected by collagen content, it is still unclear what the effect of alignment or fibroblast density is on tissue stiffness. Furthermore, to better understand the inhibiting effect of cardiac fibroblasts on contractility, it is important to unravel the exact mechanism(s) by which they influence cardiomyocyte contractility. In chapter 6, we showed that cardiomyocyte contractility is directly affected by a mutation in fibulin-4. But we do not know yet if the ECM, which is also affected by this mutation, also influences cardiomyocyte contractility and to what extent.

In vitro tissue model approach

Studying cardiomyocyte contractility in the living heart is challenging, and unraveling the effect of different stimuli on cardiac contractility is even more difficult in this complex and dynamic environment. Therefore, *in vitro* studies are extremely useful to systematically alter and study the effect of different parameters on contractility in a controlled way. Of course, an in vitro tissue model is always a simplification of the *in vivo* reality. However, by including the right parameters, it can be an efficient tool to answer a research question. In this section we will discuss the benefits and limitations of our µTUG system regarding the 3D environment, cell source and model readouts, before we consider the implications for clinical translation and future perspectives.

Ethical considerations

During the research performed for this thesis, mouse neonatal cells were used to create engineered cardiac tissues. All animal experiments performed for this research were approved by the Animal Experiments Committee of the involved institute and in conformation with the Experiments on Animals Act established under European guidelines, which also has been indicated in the relevant chapters. Nevertheless, it is important to discuss the ethical considerations regarding the use of animals for our research purposes.

Heart failure is associated with high mortality and morbidity with frequent and long-term hospitalization, resulting in high healthcare costs and a decreased quality of life for the patients. The research in this thesis presented some new insights into the onset of heart failure and therefore provided options for the development of new therapies for heart failure. While setting up our research plan, we considered the three R's concerning the use of animals in testing, which are: Replacement, Reduction, and Refinement.

Only few cell lines for cardiomyocytes that could replace cells isolated from animals are available. However, our results with these alternatives showed that they lack a proper sarcomeric organization and consequent rhythmic beating in 3D culture. Since our research focused on contractility, these cell lines were not suitable for our purpose and hence we decided to isolate primary cells from animals. To reduce the amount of animals needed for our experiments, we used previously optimized and tested protocols to obtain the maximum amount of cardiac cells from each mouse heart. The miniaturized design of our µTUG system ensures high sample numbers for statistical comparison using a minimal amount of cells; and thus animals. The animals used for this research did not undergo any treatment before they were sacrificed, which minimized the discomfort for the animals.

Cardiac microenvironment

Traditionally, *in vitro* studies were performed on cells cultures on 2D substrates while *in vivo* these cells are embedded in a 3D environment of ECM proteins and neighboring cells. It has been shown that cell morphology, proliferation, cell-cell, and cell-matrix interactions are different in 2D and in 3D environments (9, 10). To more closely mimic the native 3D environment, while allowing for controlled and systematic analysis of cellular processes, numerous 3D *in vitro* models have been developed. The choice for the right *in vitro* model is crucial, since it determines the possible input parameters you can manipulate and the output parameters you can measure. For the research in this thesis, our model system had to meet three important criteria: (I) The ability to measure contractility of cardiomyocytes in (II) a manipulable 3D environment, while (III) allowing the possibility to study cellular and sarcomere properties of the cardiomyocytes.

To achieve this, we adapted a microfabricated tissue gauge system (μTUG) previously developed by Legant et al. and Boudou et al. (11, 12). This model system consisted of two flexible PDMS micropillars which were used to simultaneously constrain the hydrogel and measure force generated displacement. The uniaxial constraints guide the collagen and cell orientation in one direction. To manipulate tissue architecture and create an isotropic cell and collagen organization, we adapted the design and varied the position of the microposts. This resulted in a biaxial design with four posts on four sides of the tissue. Initially, removal of the posts on two sides was meant to result in anisotropy of cells and matrix. However, reduction of the tissues aspect ratio was needed to induce proper alignment, resulting in the final μTUG design that was presented in chapter 4 of this thesis.

The cardiac cells are seeded in a collagen-matrigel hydrogel, which gives ample opportunities to change the cellular or matrix composition of the cardiac tissues. It is therefore a frequently used hydrogel in many studies related to cardiac tissue engineering (13-16). By using this hydrogel, containing proteins of the cardiac ECM such as collagen I, IV and laminin, we provided the cells with a native-like environment. At the same time, this limits the possibility to study altered matrix production by the cardiac cells in the different microenvironments. Protein production by the cells will be difficult to quantify, since these proteins are already present at the time of seeding. Therefore, we also explored the use of fibrin in our μTUG system, but this proved to be practically challenging due to the rapid gelation.

In our search for specific disease characteristics that could be implemented in our μTUG system, we set out to investigate native tissue properties of an acquired and genetic mouse model of heart disease. With the currently used techniques, such as biochemical assays and mechanical tests, it proved to be difficult to reveal different properties between healthy and diseased tissues, since these can be subtle changes. Most likely, these techniques are too global to measure the local differences that occur due to the patchy fibrosis that was detected with immunohistochemistry. Furthermore, our biochemical assays only detected collagen and glycosaminoglycans, while obviously the cardiac ECM is much more complex than that. A promising technique to overcome this problem is mass spectrometry, which can detect the presence and amount of all cardiac ECM components (17, 18).

Cells and cell source

All studies described in this thesis were performed with mouse neonatal cardiac cells. Although the mouse is a frequently used model for both *in vitro* and *in vivo* studies (14, 19-22), translation to human applications is not straightforward because mouse and human cardiomyocytes and the heart itself possess some explicit differences. Obviously, heart rate of a human being (1 Hz) is much slower than of a mouse (10 Hz). But also at the cellular level, the cells are different. Mouse and human cells do not have exactly the same ion channels, causing species dependent repolarizing characteristics (23). The ratio of cardiomyocytes and fibroblasts is also species dependent (24, 25), so especially the results from our fibroblast density study cannot be directly translated to human cardiac tissue. Although I anticipate that similar effects will occur if the experiments are repeated with human cells, the exact values will most likely not be the same.

The microwells of the µTUG system had dimensions of 1125x625 µm for the uniaxial design and 1125x1125 µm for the biaxial design. Due to these small dimensions, only a million cells are required to seed a complete µTUG with 64-80 microtissues. This allows for high numbers of samples to be included per experiment. Furthermore, oxygen and nutrients can easily reach the cells inside the tissue via diffusion. In our case, the microscale also has a disadvantage, since we have less precise control over the amount of cells seeded per individual tissue. Nevertheless, miniaturization is a great benefit, as it allows for microscopic screening of the tissues without additional sectioning to study the cellular and intracellular components of the microtissues. The possible variability in cell number might have increased the standard deviation of our results. Furthermore, each experiment was performed with cells from a different isolation, which may introduce even more variability due to small differences in mouse age, fibroblast density and viability of the cells.

A considerable problem with *in vitro* studies of cardiac cells is the continuing proliferation of cardiac fibroblasts. Because neonatal cardiomyocytes withdraw from the cell cycle, they hardly grow after cell isolation. Therefore, a co-culture of cardiomyocytes and cardiac fibroblasts will be rapidly overgrown by fibroblasts. This was most prominent in our anisotropy study, were we monitored the cardiac microtissues over a time course of 7 days. The ratio of cardiomyocytes versus fibroblasts completely turned around from 65%-35% at day 0 to 35%-65% at day 7 (Chapter 4). Additionally, to obtain beating cardiac microtissues in the first place, two preplating steps were necessary to reduce the amount of fibroblasts in the obtained cell mixture after isolation. Askar et al. successfully inhibited the proliferation of rat fibroblasts in their monolayer cultures by chemical treatment with mitomycin-C (1). However, we observed severely affected sarcomere organization and viability of the cardiomyocytes after using the same treatment, which inhibited beating of the microtissues (figure 2).

Next to proliferation, differentiation of fibroblasts into myofibroblasts is also a concern during *in vitro* studies. Upon isolation, fibroblasts were taken out of their native environment to be cultured on plastic before seeding in our microtissues. This rigorous change from a soft 3D to a stiff 2D substrate will trigger activation of the fibroblasts and induce differentiation into myofibroblasts. Even though seeding in a soft hydrogel will

diminish this effect (26), it is most likely that these cells will not become fully quiescent anymore. Since, it is known that differentiation to myofibroblasts lead to increased contractile, proliferative, and migratory properties of the cells (27), this might influence results obtained in *in vitro* studies. It will be hard to prevent differentiation *in vitro*, so we should keep this in mind while translating *in vitro* results towards *in vivo* applications.

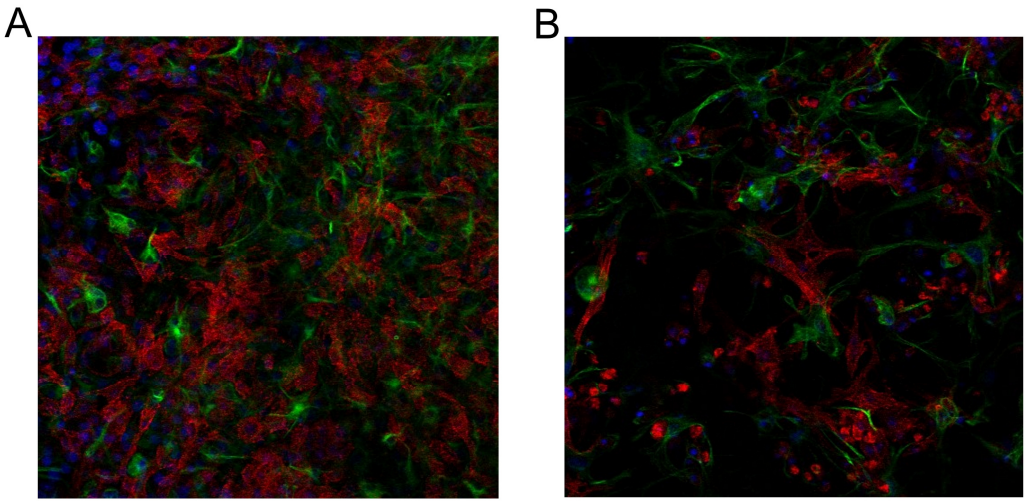

Figure 2 *Effect of mytomycin-C treatment on cardiac microtissues. Cardiomyocytes (red) and cardiac fibroblasts (green) were homogeneously distributed throughout biaxially constrained microtissues (A). Mytomycin-C treatment severely affected cardiomyocyte organization and viability, as shown by the reduced cell number and high amount of rounded cardiomyocytes in the microtissues (B). Pictures by Alex Bastiaens.*

Model readouts

Overall, the frequencies observed in our microtissues are similar to the research of Stohr et al. who found frequencies between 2 and 3 Hz for *in vitro* mouse cardiac tissues (14). Nevertheless, all *in vitro* studies (14, 20, 28) on cardiac beating frequency report lower values than frequency *in vivo* mouse heart, which can be up to 10 Hz (21, 22). Also the contraction forces measured in our microtissues, fail to reach the *in vivo* values. In small papillary-like cardiac muscle tissues obtained from mouse hearts, a tension of ~30 mN/mm^2 was measured (29). If we estimate the cross section to be 7.5x10^{-3} mm^2, for our microtissues this would be a contraction force higher than 200µN, instead of 5 µN that was measured. Most likely, this deviation from *in vivo* observations is due to differences in organization, composition, maturation of the CMs, or the absence of innervation.

The µTUG model has dozens of microwells in one system, allowing for high throughput seeding and screening of the samples. While seeding is performed at once for all samples in one dish, analysis still limits real high throughput screening since video recording and microscopy was performed manually for each tissue. Furthermore, video and image analysis that was performed in the research of this thesis also required some manual input for each sample. Of course, this becomes very time-consuming for large sample numbers. Therefore, improvement for automated screening and analysis could advance *in vitro* tissue models towards real high throughput systems.

Analysis techniques that require a certain amount of tissue, such as biochemical assays, cannot be applied to our small scale microtissues. The only option to apply such techniques, would be to use the entire well with all samples. However, incomplete or broken tissues that might be present in the well could easily influence the results. Furthermore, all microtissues in one system are seeded with the same cell and gel composition and cultured under the same conditions. This increases the sample number per condition but it restricts the possibility of high throughput screening of the tissues under different conditions. The addition of a microfluidic device to locally manipulate the culture conditions by varying certain concentrations could be an option to solve at least part of this problem.

In the end, we want to use the outcomes of our studies to expand our knowledge about clinically relevant problems and to improve currently available therapies. But how can we translate findings of a micrometer scale cardiac tissue towards a four-chambered heart? Correlating the outcomes of *in vitro* and *in vivo* studies is very difficult due to differences in measurement techniques. Whereas we used displacement of the microposts to assess contractility of the cardiac tissues, physicians use measurements like ultrasound or MRI to assess the pump function of the heart. Where beating frequency (measured in hertz) and heart rate (measured in beats per minute) are relatively easy to convert, correlating contraction force (measured in micronewton) and cardiac output (measured in volume per minute) is not straightforward.

Implications for clinical translation

Some components of the heart are not replicated in our *in vitro* model of the myocardium. We only mimicked a small part of the myocardial wall, which does not include blood vessels or innervation. For transplantation purposes, the presence of blood vessels is extremely important and has therefore been studied by many researchers (30, 31). Since our microtissues are very thin, they do not need blood vessels to supply the whole tissue with nutrients. However, the endothelial cells of the vasculature have been shown to communicate with and influence the function of other tissues (32).

Next to that, due to the lack of innervation in our microtissues, there is no external trigger for contraction of the cells. As such, we only considered spontaneous contraction by the cells itself. Many other studies have applied electrical stimulation to mimic this external trigger (33-36). In our µTUG system, pacing can also be implemented, which was observed in one of our pilot studies (figure 3), although we chose to measure spontaneous contraction. Of course, the outcomes of our studies would be quite different when pacing was applied. First, the variation in beating frequency would be much lower, since all tissues are forced to beat at the same frequency. Second, I think that a decreasing trend, such as occurred with increased fibroblast density, cannot be measured while pacing the tissues. Rather, you would measure a maximum capture rate at which the microtissues cannot follow to the applied frequency anymore, which would then be lower for higher fibroblast densities. An example of such maximum capture rate measurements can be found in the study by Turnbull et al., who showed that their engineered cardiac tissue could capture the imposed frequency from 1 Hz up to 3.3 Hz. When paced at higher frequencies, the action potential signal became irregular (15).

Even so, innervation does more than just providing a trigger for contraction, as it is also responsible for maintaining a constant cardiac output. Of course, this feedback mechanism is not present in our microtissues. However, in my opinion, the absence of this feedback mechanism makes our system more sensitive to changes that affect cardiomyocyte contractility. It might pick up changes in contractility that will never be perceptible in the living heart, because it will constantly try to compensate for it. This was actually demonstrated in chapter 5 of this thesis, where our microtissue model revealed a decreased beating frequency due to fibulin-4 deficiency, while *in vivo* no differences in heart rate were observed.

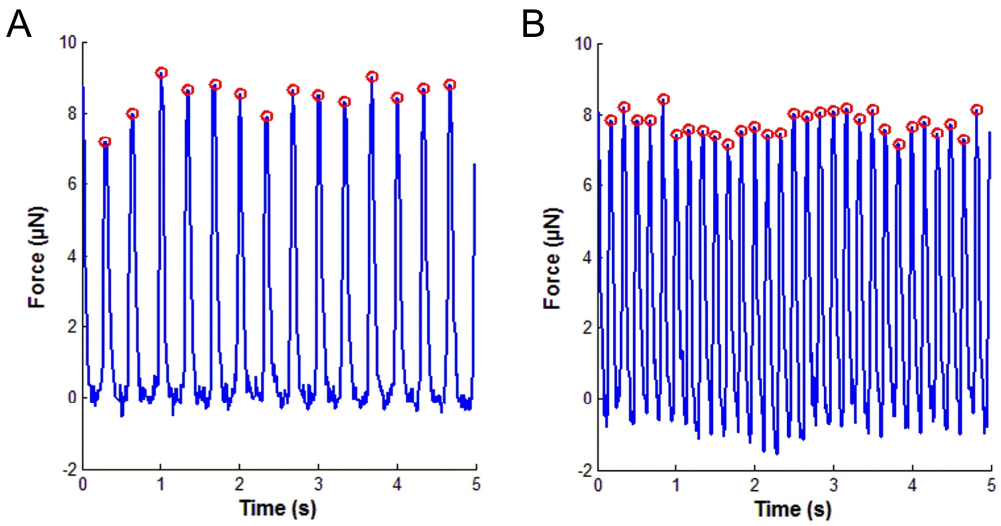

Figure 3 *Electrostimulation of biaxially constrained cardiac microtissues at day 2 of culture. Contraction force was measured in the same cardiac microtissue when stimulated at 3 Hz (A) or 6 Hz (B) using an IonOptix culture pacing system.*

While our model system is now only used to study questions related to cardiac contractility, it is a versatile research tool that could be used for other fields of interest as well. Cellular forces play an important role in all processes of the human body, from embryonic development to the development of diseases. Furthermore, understanding the complex interactions between cells and their environment is also essential to develop regenerative therapies. For example, the development of successful tissue engineered heart valve replacements is hampered by tissue retraction of the leaflets, most likely caused by increased cellular traction forces (37, 38). The model system described in this thesis provides an excellent tool to study such processes at the microscale. This could lead to new insights that can be implemented in heart valve tissue engineering protocols to prevent leaflet retraction. By choosing the appropriate cell source(s), matrix composition and µTUG design, the model can be tailored to mimic different types of tissues. It presents a unique approach to measure the forces exerted by the cells and at the same time study the cellular processes in the tissue.

Future perspectives

One of the most common applications of microtissues systems, or organs-on-chips, is their use in drug screening. To advance our model system towards a screening tool for drugs or

therapies for human heart diseases, the incorporation of a human cell source is necessary. The first reported human *in vitro* cardiac tissues were created by Caspi et al. using embryonic stem cell-derived cardiomyocytes (31). Of course, the use of embryonic stem cells raises some ethical issues. The discovery of a reprogramming technology to convert somatic cells into stem cells has provided a less onerous cell source, called induced pluripotent stem (IPS) cells (39, 40). Since these cells can be derived from an adult cell source instead of an embryonic cell source, it gives the opportunity to create patient specific tissue models by capturing the complete genetic background of this person. However, since this is no longer a controlled cell line with a single mutation, it will be more complicated to find the right controls for studies with these cells. Furthermore, batch-to-batch variability and limited maturation of the cardiomyocytes are also concerns that need to be addressed before a "patient-in-a-dish" can be created (41).

Nevertheless, I believe that if these problems are solved, heart-on-a-chip can greatly contribute to the development of personalized medicine. From all possible organs-on-chips, heart-on-a-chip can be particularly important as many drug withdrawals are caused by cardiotoxicity problems. Withdrawal from the market happens because many drug-induced toxicities occur only in diseased or genetically predisposed patients and not in healthy test subjects. By using patient-derived cells, it would be possible to screen for toxicities that are specific to certain patient populations. This means that in the future, a drug which is safe for 95% of the entire population and toxic for 5% of the population, does not have to be withdrawn from the market. By using patient-derived cells to create myocardium in dish, it can be tested if this drug is truly safe for each specific patient.

In my opinion, the field certainly needs to move in this direction in order to keep on finding new cures for diseases. Because the scientific knowledge about certain diseases is continuously expanding, they are further divided in subclasses with specific symptoms and thus specific options for treatments. It is unlikely that new drugs will be discovered which are able to cure all different subclasses of one disease. So, as the diseases and possible treatments become more and more specific, I believe that screening assays should develop accordingly. This would also provide a huge economical saving, because a relatively cheap *in vitro* test can be used as a first screening assay before spending large amounts of money on animal tests and clinical trials.

Of course, heart-on-a-chip can only predict possible toxicity of a drug for the heart and not for other organs, so if you really want to approach the clinical trial-on-a-chip, multiple organs-on-chips would have to be used. However, I think (or even hope) that this is out of reach, because the complex interactions of all tissue and organs in the human body can never be fully mimicked in a dish.

Conclusion

In this thesis, a model system was developed to measure the contractility of cardiomyocytes embedded in a 3D environment. Our results provided insight into the cellular and extracellular cues that influence cardiac tissue contractility at the onset of cardiac disease. Matrix disorganization did not result in a decreased contraction force or beating frequency.

Increased collagen content affected overall tissue force of contraction, but fibroblast density proved to be most detrimental for cardiomyocyte contractility. We even revealed a fibroblast density threshold of 50% fibroblasts, after which beating of the cardiac microtissues was severely reduced. For the first time, we showed that a genetic fibulin-4 deficiency resulted in structural changes of the sarcomere organization, which caused a decrease in contraction force and beating frequency. From these finding, we conclude that restoring the cardiomyocyte population is most important to regain cardiac function. Our model system proved to be a valuable and versatile research tool, as it can be manipulated according to the researchers' needs by varying the design, cell source or matrix composition to fit multiple research questions. While in this thesis our model system was only applied to scientific research questions, it has high potential as a screening tool for drugs or therapies.

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Samenvatting

De invloed van cellulaire en extracellulaire verstoringen op de contractiliteit van hartspiercellen in een model voor hartspierweefsel

Weefselmodellen van het hart worden onder meer ontwikkeld om de fysiologie van het hart *in vitro* te bestuderen. Verschillende eigenschappen van het weefsel kunnen met een hoge controleerbaarheid worden nagebootst waardoor de bijdrage van deze eigenschappen aan het ontstaan van hartziekten ontrafeld kunnen worden. Een kleine verstoring in de micro-omgeving van een hartspiercel kan namelijk een kettingreactie van remodelering tot gevolg hebben, wat weer kan leiden tot het ontstaan van hartziekten. Huidige behandelingen voor hartziekten richten zich vooral op het bestrijden van symptomen in plaats van de oorzaak zelf aan te pakken. Om nieuwe behandelingen te ontwikkelen, is er meer kennis nodig over het effect dat deze kleine verstoringen van cellen of matrix hebben op het functioneren van hartspiercellen. Om het effect van deze verstoringen op lokaal niveau te bestuderen, worden er gekweekte hartspierweefselmodellen gebruikt.

Het onderzoek in dit proefschrift beschrijft het effect van verstoringen in de micro-omgeving van het hart op de contractiliteit van hartspiercellen. Voor dit doel is een microweefselmodel van het hart ontwikkeld, bestaande uit hartspiercellen en fibroblasten in een extracellulaire matrix. In dit model kunnen het aantal cellen, de verhouding tussen de beide cel types, de matrix organisatie, en de matrix samenstelling gemanipuleerd worden. Daarnaast kan het modelsysteem worden gebruikt om de kracht die het microweefsel uitoefent te meten.

Literatuuronderzoek liet zien dat er in de huidige weefselmodellen maar weinig aandacht wordt besteed aan de cellulaire micro-omgeving en het gedrag van hartspiercellen bij veranderingen in matrix organisatie en samenstelling. Gedetailleerde kennis van de natieve micro-omgeving van het hart, in zowel gezonde als zieke situaties, is echter essentieel om *in vitro* modellen te ontwikkelen voor het nabootsen van specifieke ziekte kenmerken. Daarom werden er muismodellen met aangeboren en later ontwikkelde hartaandoeningen bestudeerd, om zo de veranderingen in matrix eigenschappen in kaart te brengen. De onderzochte harten vertoonden allemaal lokale opeenhoping van matrix, oftewel fibrose, en op deze plekken was de anisotropie van cellen en matrix verstoord. Om de invloed van matrix (an)isotropie op hartspiercel contractie verder te onderzoeken, werd de collageen oriëntatie van de hartspier microweefsels gemanipuleerd in het laboratorium. Het microweefsel werd door paaltjes aan twee of aan vier kanten vastgehouden om zo de matrix organisatie te beinvloeden. Op deze manier werd een anisotrope en isotrope matrix organisatie gemaakt die daarmee een gezonde en een zieke weefsel organisatie van het hart nabootsten. De resultaten toonden aan dat verstoring van de matrix organisatie niet leidt tot vermindering van de kracht en frequentie die het weefsel kon genereren, hoewel de verdeling van de krachten wel verstoord werd.

In het hart gaat fibrose vaak samen met fibroblast proliferatie en toename van de matrix productie. Om het effect van deze factoren op de contractilteit van hartspiercellen te kwantificeren, werd zowel het aantal fibrobasten als de hoeveelheid collageen in de hartspier microweefsels gevarieerd. In deze studie werd een drempel gevonden van 50% fibroblasten, waarboven de frequentie van de microweefels drastisch daalde. Naast de frequenctie, nam ook de kracht van de microweefsels af bij toename van het aantal fibroblasten. Het verhogen van de collageen concentratie had alleen een negatief effect op de kracht die de microweefsels genereerden, maar niet op de frequentie.

Naast collageen is ook elastine een belangrijk eiwit in de extracellulaire matrix van het hart. Een verstoorde vorming van het elastische netwerk is de onderliggende oorzaak van hartspierziekte in muizen met een verminderde fibuline-4 expressie. Het is alleen nog onbekend of deze mutatie, naast een effect op de matrix, ook een direct effect heeft op de functie van hartspiercellen. Om dit te onderzoeken werden microweefsels gezaaid met hartcellen van wild-type muizen en van muizen met een verminderde fibuline-4 expressie. Onze resultaten lieten zien dat de contractie van fibuline-4 deficiënte microweefsels verminderd was ten opzichte van wild type microweefsels. Deze resultaten suggereren dat een fibuline-4 mutatie niet alleen de matrix productie beïnvloed, maar ook een direct effect heeft op de hartspiercellen.

Vergelijking van alle data uit de verschillende hoofdstukken laat zien dat een toename van het aantal fibroblasten het meest nadelig is voor de contractie van hartspier microweefsels. Vanuit een klinisch perspectief suggereert dit dat bij het behandelen van hartziekten het herstellen van de hartspiercel populatie, bijvoorbeeld via stam cel injectie, het meest veelbelovend lijkt om de functie van het hart te verbeteren. Om een compleet herstel van de pompfunctie te bereiken, is het echter ook belangrijk om de hoeveelheid collageen te verminderen en de anisotrope oriëntatie van cellen en matrix terug te krijgen.

De resultaten van dit proefschrift laten zien dat niet alleen de hartspiercel zelf, maar ook de micro-omgeving, essentieel is voor efficiënte contractie van hartspierweefsel. Daarnaast is aangetoond dat het ontwikkelde weefselmodel geschikt is om systematisch verschillende eigenschappen van de micro-omgeving van het gezonde en zieke hart na te bootsen en te bestuderen. Op deze manier kunnen deze weefselmodellen bijdragen aan het verbeteren van regeneratieve behandelingen of kunnen ze worden ingezet om het effect van nieuwe medicijnen of therapieën te screenen.

Dankwoord

Het is af! Wat een goed gevoel om nu de laatste woorden van mijn proefschrift te typen. En die laatste woorden wil ik dan ook graag wijden aan degenen zonder wie dit boekje nooit tot stand was gekomen.

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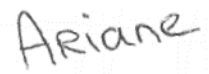

Curriculum Vitae

Ariane van Spreeuwel was born on March 30, 1987 in Eindhoven, the Netherlands. After graduating from the Sondervick College (gymnasium) in Veldhoven in 2005, she studied Biomedical Engineering at the Eindhoven University of Technology. During her studies she performed a research internship at the University of California San Diego in the United States, to investigate the mechanical properties and biocompatibility of nanocomposite hydrogels for tissue engineering purposes. For her graduation project in the group Soft Tissue Biomechanics and Engineering at the Eindhoven University of Technology, she focused on the formation of blood vessels in tissue engineered skeletal muscle. After her graduation in 2010, she continued working on this project as a researcher. Subsequently, she started as a PhD student in the same group, resulting in the research as presented in this thesis.

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