The Effects of Cardiac Fibroblasts on Cardiac Myocyte Structure and Excitation-Contraction Coupling Through Paracrine Mediators

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For those who have made me who I am today,
above all my parents.

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Declaration of originality

The work included in this thesis is my own. All information presented that is not is referenced appropriately.

Copyright declaration

Abstract

Cardiac fibroblasts are the most numerous cells within the heart. Their traditional roles are the maintenance of the extracellular matrix to support the structure and contraction of the heart, and their activation and production of increased extracellular matrix in disease. Over the past 15 years, evidence has grown that shows fibroblasts are capable of modulating myocyte function. This is achieved through paracrine mediators, the release of biologically active soluble substances into the local environment, and through direct cell contact, involving gap junctions and mechanical connections. Fibroblasts can also modulate myocyte function indirectly through modification of the extracellular matrix which can transfer various signals to myocytes. However, research into the interaction between fibroblasts and myocytes has been largely limited to using neonatal cells and fibroblasts that have been maintained in culture.

This thesis sets out to examine the paracrine effects of fibroblasts on myocyte structure and excitation contraction coupling. Adult myocytes and, where possible, fibroblasts before their activation in culture were used. The thesis also aimed to determine whether these effects are different using fibroblasts after pressure overload of the myocardium and potential mediators involved in the effects.

Initial studies looked at the effect of normal rat cardiac fibroblasts isolated from healthy rat hearts, and showed that these fibroblasts reduced myocyte viability, induced myocyte hypertrophy and increased the amplitude of the Ca^{2+} transient. We then compared the effect of fibroblasts from pressure overloaded hearts with control fibroblasts. The pressure overloaded fibroblasts caused a similar reduction in myocyte viability and also induced myocyte hypertrophy. However, the functional effects were different and the Ca^{2+} transient amplitude was reduced in myocytes co-cultured with pressure overload fibroblasts.

TGF-β was elevated in the both pressure overload and control fibroblasts co-cultures and was therefore investigated as the potential mediator of the effects. It was found

that fibroblast derived TGF-β directly causes myocyte hypertrophy, whereas the other effects are due to more complex signalling with the involvement of secondary mediators released from the fibroblasts in response to TGF-β signalling.

Finally, we investigated the paracrine effects of cultured canine fibroblasts on the electrical activity of adult myocardium using canine myocardial slices. These are thin viable slices of the left ventricle and allowed us to investigate the field potential and the conduction velocity of intact adult myocardium. It was found that fibroblasts-derived paracrine mediators altered the conduction velocity of these slices.

This work supports the growing body of evidence that fibroblasts are capable of modulating myocyte structure and function and, specifically, myocyte excitation contraction coupling. We have shown that these effects are evident in adult cells and are altered using fibroblasts from normal and diseased heart. Furthermore, we have shown that TGF-β appears to be central to these effects.

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Abbreviations

3 R's	Replacement, Reduction, Refinement.
α-SMA	alpha smooth muscle actin
AM	acetoxymethyl ester
Ang II	Angiotensin II
BSA	Bovine serum albumin
CICR	Ca^{2+} induced Ca^{2+} release
CM	Cardiomyocyte
CTGF	Connective tissue growth factor
CV	Conduction velocity
DDR2	Discoidin Domain Receptor 2
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin
FB medium	Fibroblast culture medium
FB	Fibroblast
FGF	Fibroblast growth factor
FPD	Field potential duration
FSP1	Fibroblast specific protein 1
g	Gravitational force
HEK 293	Human embryonic kidney 293 cells
Hrs	Hours
HW	Heart weight
ICa,L	L type Ca^{2+} current
IGF-1	Insulin like growth factor 1
Il-6	Interleukin 6

KLF Krüppel like factor

LC Low calcium solution

LTCC L-type Ca^{2+} channel

LV Left ventricle

MEA Multi electrode array

M-mode Motion mode

MMP Matrix metalloproteinase

Myocyte Cardiomyocyte

NCX $Na+/Ca^{2+}$ exchanger

NRVM Neonatal rat ventricular myocyte

NT Normal Tyrodes

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PGF Placental growth factor

PVDF Polyvinylidene fluoride

RyR Ryanodine receptor

SDS Sodium dodecyl sulphate

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SERCA Sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase

SR Sarcoplasmic reticulum

TAC Thoracic aortic constriction

TBS Tris-buffered saline

TGF- β Transforming growth factor beta

TNF-α Tumour necrosis factor α

T-tubule Transverse tubule

CHAPTER 1.

Introduction

The heart is fundamental to life. The supply of blood, with the nutrients it carries and the waste it removes, is essential for every tissue within the body. To achieve this, a mammalian heart beats an average of 730,000,000 times over a lifetime (Levine, 1997).

Heart failure, which is defined as the inability of the heart to meet the metabolic demands of tissue (Jessup and Brozena, 2003), is universally prevalent. It is the leading cause of mortality in Europe and North America, accounting for nearly 50% of deaths, and is increasingly encountered in developing countries where it is already ranked the third most common cause of death (Murray and Lopez, 1997, Mendez and Cowie, 2001). Although care for heart failure is improving, 6 month mortality was 14% in 2004-5 (Mehta et al., 2009) , and heart failure also has a particularly significant impact on quality of life (Hobbs et al., 2002). In addition it presents a substantial burden on health care. Heart failure is one of the top ten diagnoses for hospital bed use and in 2009 was estimated to cost the NHS £9 billion and the UK economy as a whole £19billion.[1]

Heart failure is a complex syndrome that can result from a number of prior conditions, for example genetic disease, such as hypertrophic cardiomyopathy, or after ischemic injury from a myocardial infarction. Heart failure can involve failure of the left, right or both ventricles, and results in symptoms including breathlessness (due to pulmonary oedema), lethargy and reduced exercise capacity. The progression of heart failure is assessed using the New York Heart Association class system[2]. Class I describes patients with no functional restriction but with increasing symptoms patients enter higher classes, with class IV (the highest class) describing patients who cannot perform physical activity without breathlessness or discomfort and show symptoms at rest.

Care for patients with heart failure has improved considerably. Early treatment included positive inotropes such as digitalis, to increase cardiac contractility, and

[1] British Heart Foundation Coronary Heart Disease Statistics 2012 http://www.bhf.org.uk/publications/view-publication.aspx?ps=1002097

[2] The Criteria Committee of the New York Heart Association. Nomenclature and Criteria for Diagnosis of Diseases of the Heart and Blood Vessels. Boston: Little Brown, 1964.

diuretics and rest, to reduce the load placed on the ventricles (Eichhorn and Bristow, 1996). This provided symptomatic relief but in clinical trial was shown to have no benefit on mortality (Cohn et al., 1986, Packer et al., 1991). With the improving understanding of heart failure progression and novel therapies, treatment moved from positive inotropes and rest to negative inotropes, exercise and angiotensin converting enzyme inhibitors. This improvement in treatment is reflected by improved mortality figures with 6-month mortality falling from 26% in 1995-7 to 14% in 2004-5 (Mehta et al., 2009) .

However, the treatment for heart failure is still largely only able to slow the progression of the disease. Therefore further understanding of the processes involved in heart failure initiation and progression are important in providing potential new targets for intervention and further improve patient outcomes.

Despite the clear importance of the heart in health and the burden of heart disease, there are still many unknown elements to cardiac physiology and the processes involved in the pathogenesis of cardiac pathology, and an improved knowledge may improve future therapy. The focus of this thesis is to advance the understanding of the most common of all cells within the heart, cardiac fibroblasts, and their influence on myocyte excitation contraction coupling.

1.1. Makeup of the heart

The heart's ultimate function is to pump blood around the body, which is achieved by the contraction of myocytes within the myocardium. However, to achieve successful cardiac muscle contraction there are many specialist structures and cells that are important in controlling and optimising heart function.

1.1.1. **Conduction system**

The cardiac conduction system is a network of specialised myocytes that initiates and controls the electrical impulses that stimulate each heartbeat. Cells within the sino

atrial node have specialised currents that allow controlled and consistent initiation of the electrical impulse, that is then directed around the heart via the atrioventricular node, bundle of His and Purkinje fibres to produced directed and efficient contraction of the myocardium (Moorman et al., 1998).

1.1.2. **Valves**

The valves control the unidirectional flow of blood essential for the efficient functioning of the heart (Nolan and Muller, 1965). The importance of the valves is clearly demonstrated by the extensive problems that arise when the valves become diseased and normal blood flow is interrupted (Shipton and Wahba, 2001) and surgical intervention to correct valve function accounted for 42% of all adult cardiac surgery in the UK in 2010.[3]

1.1.3. **Coronary vasculature**

The high energy demands of the heart mean that the coronary vasculature receives approximately 5% of total cardiac output at rest (Ramanathan and Skinner, 2005). The control of this system is essential for effective cardiac physiology, and disease of the vasculature is the main cause of cardiovascular pathology (Hansson, 2005).

1.1.4. **Working myocardium**

The working myocardium produces the force to expel blood from the heart and pump it around the body, which is achieved by the contraction of myocytes. However, the myocardium also contains non-myocyte cells.

Fibroblasts are the most numerous cell type within the rat myocardium, accounting for 62% of all cells and, although in adult mice they represent only 27% of the total number of cells, they are still the largest population of non-myocyte cells (Banerjee et al., 2007). The study of fibroblasts has historically been limited, due to a perception

[3] National Adult Cardiac Surgery Audit 2010-2011. National Institute for Cardiovascular Outcomes Research.
http://www.ucl.ac.uk/nicor/audits/Adultcardiacsurgery/publications/pdfs/NACSA_annual_report_FINAL

that the cells were passive bystanders in myocardial function. However, more recently interest in these cells has grown and the role of fibroblasts in the structural, biochemical and mechanical functioning of the myocardium is beginning to be unravelled (Camelliti et al., 2005, Porter and Turner, 2009).

1.2. What are fibroblasts

Fibroblasts are cells of mesenchymal origin that are distributed diffusely throughout the heart as well as being found throughout the body. They are traditionally defined by their expression of extracellular matrix (ECM) molecules. The National Library of Medicine Unified Medical Language System defines them as 'connective tissue cells which secrete an extracellular matrix rich in collagen and other macromolecules'.[4] Cardiac fibroblasts produce collagen types I, III, IV, V and VI as well as other ECM proteins such as elastin, laminin and fibronectin (Bosman and Stamenkovic, 2003).

Despite this definition, ECM synthesis and deposition are not commonly used to identify fibroblasts, but instead they are identified by morphological characteristics and cellular markers. Fibroblasts are flat cells with elongated processes that lead to their characteristic spindle shape. They contain a single oval nucleus and an extensive rough endoplasmic reticulum and Golgi apparatus. In cardiac tissue, fibroblasts are the only cells that do not have a basement membrane (Krenning et al., 2010). The identification of fibroblasts by cellular markers is difficult due to the lack of truly specific markers (discussed in more detail in section 1.3.1). However, in general, fibroblasts can be stained for vimentin (Figure 1.1). This also marks smooth muscle and endothelial cells but these can be discriminated by the different morphology (Camelliti et al., 2005)

[4] Unified medical language system, National Institute of Health (https://uts.nlm.nih.gov/home.html)

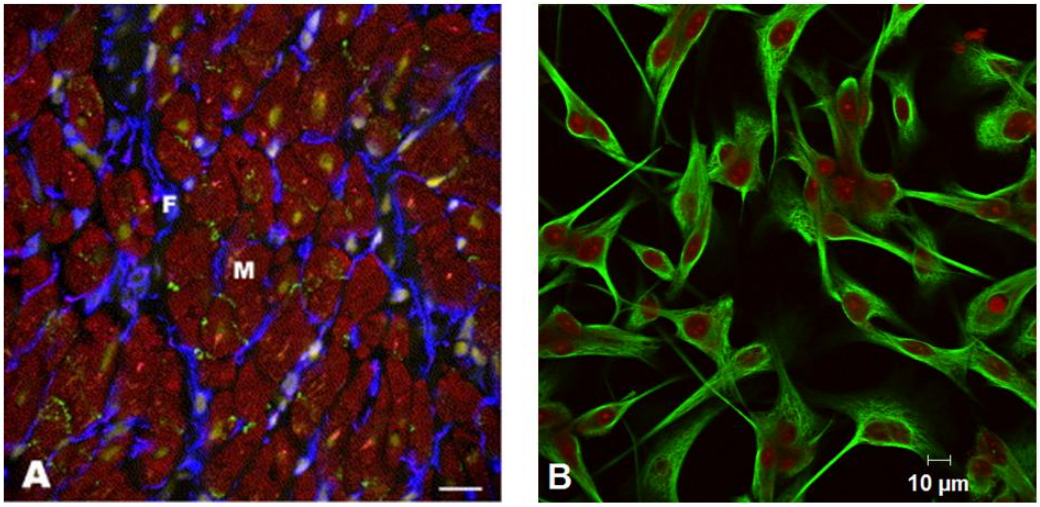


Figure 1.1: Fibroblasts within the myocardium and *in vitro*

A) Transverse myocardial section of sheep left ventricle with myocytes in red (myomesin) and fibroblasts in blue (vimentin) showing the diffuse distribution of fibroblasts throughout the myocardium. Connexin 43 in green and nuclei in pale yellow (DAPI) are also stained. Scale bar: 20µm. (reproduced with permission from (Kohl et al., 2005)) B) Canine cardiac fibroblasts stained for vimentin (green) and nuclei marked with propidium iodide (red) in culture.

1.2.1. **Source of fibroblasts in physiology**

The major source of fibroblasts in the normal myocardium is cells that arise from the proepicardium during embryonic development. Proepicardial cells migrate as an epithelium over the heart to produce the epicardium. The epicardial cells then undergo epithelial to mesenchymal transformation into epicardial derived cells, which invade the myocardium walls during the late embryonic and foetal periods. These epicardial-derived cells then differentiate into fibroblasts (Lie-Venema et al., 2007).

Further to the recruitment of cells during development, the contribution of hematopoietic stem cell derived cells to the fibroblast population in normal myocardium has also recently been demonstrated (Visconti and Markwald, 2006). These may represent a post-natal source of fibroblasts in the normal heart.

Fibroblasts are also found in the cardiac valves which are derived from a different source. They arise from endothelial-to-mesenchymal transformation of cells in the cardiac endothelium which invade the cardiac jelly and mature into fibroblasts (de Lange et al., 2004).

1.2.2. **Source of fibroblasts in pathology**

Increased levels of fibrosis are common to many cardiac pathologies and are associated with an increased number of fibroblasts (Weber, 1989, Vliegen et al., 1991). However, the source of these fibroblasts is not fully established.

Despite the large number of resident fibroblasts in the normal heart and their ability to proliferate, their contribution to the increased fibroblast numbers in pathological conditions may be small. Resident fibroblasts may be activated by mechanical or humoral factors in pathology but their proliferation appears to be very limited (Mandache et al., 1973). Instead, the recruitment of new cells accounts for most of the increase in fibroblasts observed in disease (Zeisberg et al., 2007).

The source of these recruited fibroblasts has been investigated by transplanting fluorescent marked cells into recipient mice. Using this technique, bone marrow derived cells were identified as a major source of fibroblasts in cardiac pathology. After pressure overload, 21% of fibroblasts in the heart were derived from bone marrow cells (Zeisberg et al., 2007). van Amerongen *et al* (2008) found that 24% of fibroblasts in the heart were bone marrow-derived 7 days after myocardial infarction and although the number then reduced, some cells were still present at 28 days. The transfer of fluorescent monocytes into recipient mice showed that they may also contribute to the fibroblast population after myocardial infarction (Fujita et al., 2007). In addition, Zeisberg *et al* (2007) showed that some cardiac fibroblasts in disease were derived from endothelial-to-mesenchymal transformation, the process that gives rise to valve fibroblasts during development.

Evidently, various sources of fibroblasts exist in cardiac disease. It is possible that they produce distinct end products with different roles in the disease process. For example, it has been shown that fibroblasts within the scar and remote regions are dominated by fibroblasts from different origins (Crawford et al., 2012). This is an interesting proposition that may allow more targeted approaches to intervening in cardiac fibrosis.

1.3. Difficulties associated with investigating fibroblasts

Before discussing the role of fibroblasts in the myocardium, the difficulties associated with studying them need to be examined.

1.3.1. Molecular Identification of fibroblasts

One difficulty when working with fibroblasts is the identification of the cells. To date, a truly specific marker has not been identified. As mesenchymal cells fibroblasts stain positive for vimentin, which has been used to identify cardiac fibroblasts in tissue sections (Camelliti et al., 2004a). However, vimentin also marks endothelial cells, smooth muscle cells and other mesenchymal cell types and therefore for accurate identification these cells need to be excluded (e.g. (Chang et al., 2002)).

Early targets for the development of specific antibodies for fibroblast identification were based around the ECM proteins produced by the cells. For example, an antibody was produced against the cleaved carboxy terminus of pro-collagen (McDonald et al., 1986). However, this was found to identify active, dividing fibroblasts only and did not identify quiescent cells. ECM markers are still used to identify fibroblasts. Commercially available fibroblasts are selected for by the expression of fibronectin[5], although fibronectin is also expressed by endothelial and other cells (Jaffe and Mosher, 1978).

[5] ScienCell research laboratories, USA (http://www.sciencellonline.com/)

More recently, further putative specific fibroblast markers have been suggested. Discoidin domain receptor 2 (DDR2) and fibroblast specific protein 1 (FSP1) have both been reported as being fibroblast-specific within the myocardium.

DDR2 is a collagen receptor tyrosine kinase that was initially reported as a fibroblast specific marker (Goldsmith et al., 2004). It is involved in transferring signals from the ECM to cells, and its absence reduces fibroblast proliferation and collagen production (Labrador et al., 2001, Olaso et al., 2011). However, others have reported DDR2 expression in smooth muscle cells (Ferri et al., 2004). Therefore use of DDR2 as a marker for fibroblasts still requires the exclusion of smooth muscle cells by the absence of desmin (Rohr, 2011).

FSP1 was also initially reported to be fibroblast-specific in the heart (Strutz et al., 1995). However, it appears to only stain a sub-population of fibroblasts (Iwano et al., 2002). Another report questioned its specificity as in the liver it did not mark fibroblasts and instead stained a population of macrophages (Osterreicher et al., 2011).

Other markers have been used for fibroblasts in different tissues. TE-7, an antibody raised against thymus stroma (Haynes et al., 1984), has been reported to specifically mark fibroblasts (Goodpaster et al., 2008). Other markers used include fibroblast surface antigen (Wartiovaara et al., 1974) and cadherin-9 (Thedieck et al., 2007).

Despite these reports, a truly specific and also global marker of cardiac fibroblasts is still not available (Krenning et al., 2010).

1.3.2. ***In vivo*** **fibroblast studies**

The lack of specific markers for fibroblasts presents an even greater problem when trying to study fibroblasts *in vivo*. The production of genetically modified animals has provided a valuable tool for cardiovascular research, and biological research in general (Ramachandran et al., 2008). Further insight can be gained by the production of

inducible genetic modification with cre- lox technology (Nagy, 2000) as well as using cell specific promoters, such as α-myosin heavy chain in cardiomyocytes, to produce cell specific modifications (Heine et al., 2005).

An established fibroblast-specific promoter to allow genetic manipulation would provide a platform for a far greater *in vivo* insight into fibroblast function. There are some reports of fibroblast-specific promoters. FTS-1, part of the FSP-1 promoter has been reported to be a fibroblast-specific promoter (Okada et al., 1998) and has been used to produce fibroblast-specific knockout mice (Liu et al., 2010, Kim et al., 2012). A fibroblast-specific regulatory sequence from the proα2(I)collagen gene has also been used to drive fibroblast-specific expression (Zheng et al., 2002) and has been used to target fibroblasts in the heart (Lother et al., 2011). Periostin has also been used to produce a non-myocyte specific modification in the heart (Takeda et al., 2010).

Further establishment of a promoter will aid fibroblast research. Ultimately not just a fibroblast-specific, but a cardiac fibroblast specific promoter would be most beneficial.

1.3.3. **Changes to fibroblast phenotype in culture**

Due to the difficulty of studying fibroblasts *in situ*, they are mainly studied using isolated cells in culture (Rohr, 2011). Fibroblasts are readily isolated and cultured, although they have a limited proliferative capacity (Hayflick, 1965).The ability to isolate and culture fibroblasts has enabled investigations into their function, and is essential for the research involved in this thesis. Fibroblasts, however, are very dynamic cells and undergo alterations to their phenotype in culture (Rohr, 2011). Therefore the application of information gained from cultured fibroblasts to a specific *in vivo* setting is challenging.

The most obvious and widely reported change in fibroblasts in culture is the development of α-smooth muscle actin (α-SMA) expression (e.g. (Benamer et al., 2009, Thompson et al., 2011, Dawson et al., 2012)). *In vivo,* α-SMA is associated with the differentiation of fibroblasts in disease and these α-SMA positive cells are termed

myofibroblasts (Weber, 2004). Throughout this thesis the term *myofibroblasts* will be avoided, as it is potentially confusing and therefore cells will be defined as fibroblasts with information regarding their source provided.

As well as developing α-SMA expression, fibroblasts expression of ECM collagen and fibronectin are altered by culture (Dawson et al., 2012). Fibroblasts from control and myocardial infarction also alter their expression of connexins *in vitro* (Vasquez et al., 2010). In addition, fibroblast electrophysiology is altered by culture (Benamer et al., 2009).

Whether fibroblasts with α-SMA expression induced *in vitro* are representative of fibroblasts from disease is debated. There are some similarities reported. For example, fibroblasts in culture develop an ATP-sensitive K^+ current that also develops in fibroblasts after myocardial infarction (Benamer et al., 2009). Rohr (2011) argues that these cells are comparable and therefore our knowledge from cultured fibroblasts can be used to understand fibroblasts in disease. However, this would appear to oversimplify the matter. Firstly, fibroblasts in disease come from different sources (as discussed in section 1.2.2) whereas the α-SMA positive cells in culture come only from differentiation and proliferation of resident fibroblasts. Fibroblasts isolated from diseased hearts are themselves changed in culture (Vasquez et al., 2010, Dawson et al., 2012). Also, the phenotypes of fibroblasts from normal and diseased hearts in culture are different. Various reports show these differences are maintained for prolonged periods of time (Flack et al., 2006, Jarvis et al., 2006, Vasquez et al., 2010), although others have found that cells from diseased and normal hearts rapidly lose differences (Dawson et al., 2012). Furthermore, cultured fibroblasts treated with transforming growth factor-β (TGF-β), a potent activator of fibroblasts in disease (Gordon and Blobe, 2008), undergo further changes even after prolonged culture (Adapala et al., 2013). It would therefore appear that fibroblasts in culture are different from both fibroblasts in the normal heart and fibroblasts in cardiac disease.

Various elements of culture systems have been shown to affect fibroblast phenotype. One of the main elements that has been investigated is the stiffness or elastic modulus

of the substrate on which fibroblasts are cultured. Normally fibroblasts are cultured on stiff plastic substrates, but the effects of using more flexible substrates have been investigated. Using human gingival fibroblasts, reducing the stiffness of collagen substrates limited the increase of α-SMA expression in response to TGF-β (Arora et al., 1999). Also, fixing granuloma tissue to increase tension induced α-SMA expression in the fibroblasts (Hinz et al., 2001). In a normal rat heart, the elastic modulus is approximately 18kPa and increases in infarcted areas to around 55kPa (Berry et al., 2006). This information may be useful in directing effort to adapt culture conditions. Culture of rat cardiac fibroblasts on substrates with a elastic modulus of 15kPa reduced the amount of α-SMA compared to stiffer substrates. However, this was only seen after the 4^{th} passage and there was no difference during the first 3 passages. Also, 65% of cells were still α-SMA positive (Castella et al., 2010). Embryonic cardiac fibroblasts cultured on a less rigid substrate of 9.6kPa prevented the development of α-SMA stress fibres compared to more rigid substrates, although there was still α-SMA expression within the cytosol (Goffin et al., 2006). Substrate stiffness therefore has some effect but other factors appear to be involved.

The culture medium used has been shown to affect fibroblast phenotype. Using Endothelial Growth Medium-2 limited the development of α-SMA in cardiac fibroblasts compared to using 45% DMEM, 45% F12 and 10% foetal bovine serum (Rossini et al., 2008). The presence of foetal bovine serum also affected the production of elastin in ligament fibroblasts (Mecham et al., 1981). Taurine (Ren et al., 2008b) and high glucose levels (Neumann et al., 2002) also alter fibroblast proliferation *in vitro*, both of which are included in some culture media.

The level of O_2 in which cultures are kept affects fibroblasts. Supra-physiological levels of O_2 result in increased α-SMA expression and increased contractile capacity (Roy et al., 2003). Hypoxic culture also affects fibroblast activity (Shyu et al., 2012). It is therefore clear that fibroblasts are O_2 sensitive. It must however be noted that the definition of hypoxic, normoxic and supra physiological O_2 requires better understanding. Shyu *et al* (2012) used 21% oxygen as their normoxic control, which

Roy *et al* (2003) defines as their supra-physiological level. Despite this, it is clear that the level of O_2 during culture affects fibroblasts.

ECM proteins signal to fibroblasts and their use in culture appear to alter fibroblast phenotype. Fibronectin and laminin are ECM proteins that are used to promote the adhesion of cells to culture substrates and both proteins have been shown to affect fibroblasts. Fibronectin fragments stimulate apoptosis in lung fibroblasts (Hadden and Henke, 2000), although it has also been shown to promote the proliferative response of alveolar fibroblasts to fibroblast growth factor (Bitterman et al., 1983). Laminin has been shown to affect fibroblast morphology (Lin and Bertics, 1995). The effect of collagen has also been investigated. Dawson *et al* (2012) found that culture on collagen I coated plastic did not alter fibroblasts compared to fibroblasts on bare plastic, although another study into the effect of collagen on fibroblasts found that collagen I and III induced proliferation and collagen IV induced α-SMA expression (Naugle et al., 2006). Furthermore, using dermal fibroblasts, Vozenin *et al* (1998) showed that collagen I prevented α-SMA development. The concept that ECM alters fibroblast phenotype in culture is made more complicated by the fact that fibroblasts are releasing their own ECM, and this in turn is affected by other elements of the cell culture system.

Further confounding factors include the density of fibroblasts in culture and the isolation protocol itself. Confluency leads to quiescence and reduced elastin expression in ligament fibroblasts (Mecham et al., 1981) and the protein expression profile of cardiac fibroblasts is different after using different methods of isolation (Xin et al., 2012).

This variety of contributing factors highlights the difficulty of producing a culture system that would not induce changes in isolated fibroblasts and therefore allow more applicable study of their properties *in vitro*. Alongside the development of fibroblast-specific genetic modification *in vivo,* such a culture system would be highly beneficial for the study of cardiac fibroblasts.

1.4. Fibroblast function: modulation of the extracellular matrix

The traditional function of fibroblasts is the modulation of the ECM. Cardiac fibroblasts are responsible for the production of the ECM, producing interstitial collagens, elastin, fibronectin and other molecules. Fibroblasts also control ECM degradation through balancing the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) (Figure 1.2A) (Porter and Turner, 2009).

In the healthy myocardium fibroblasts are arranged in a network of cells throughout the myocardium, and produce a structural scaffold of ECM. The ECM scaffold is essential for maintaining structural integrity, distributing mechanical force and co-ordinating the contraction of individual myocytes into whole heart contraction (Figure 1.2B). However, it is not a static structure and approximately 5% of the ECM is replenished each day (McAnulty and Laurent, 1987). It is the job of the network of fibroblasts throughout the heart to maintain and moderate the ECM structure in response to chemical and mechanical stimuli (Souders et al., 2009).

In disease states, for example after myocardial infarction, fibroblasts become increasingly active and produce more ECM. This increase in ECM production is essential in the reparative fibrosis that replaces areas of myocyte loss with scar tissue. It also drives negative remodelling, mainly through reactive fibrosis, which produces interstitial fibrosis away from the initial site of injury (Figure 1.2C) (Weber, 1989). The scar tissue remains an active tissue with continual modification by resident fibroblasts (Willems et al., 1994), and the number of fibroblasts in the scar may correlate with improved outcomes. The activity of fibroblasts away from the scar has negative consequences as collagen deposition increases the stiffness of the myocardium and disrupts electrical communication between myocytes (Souders et al., 2009).

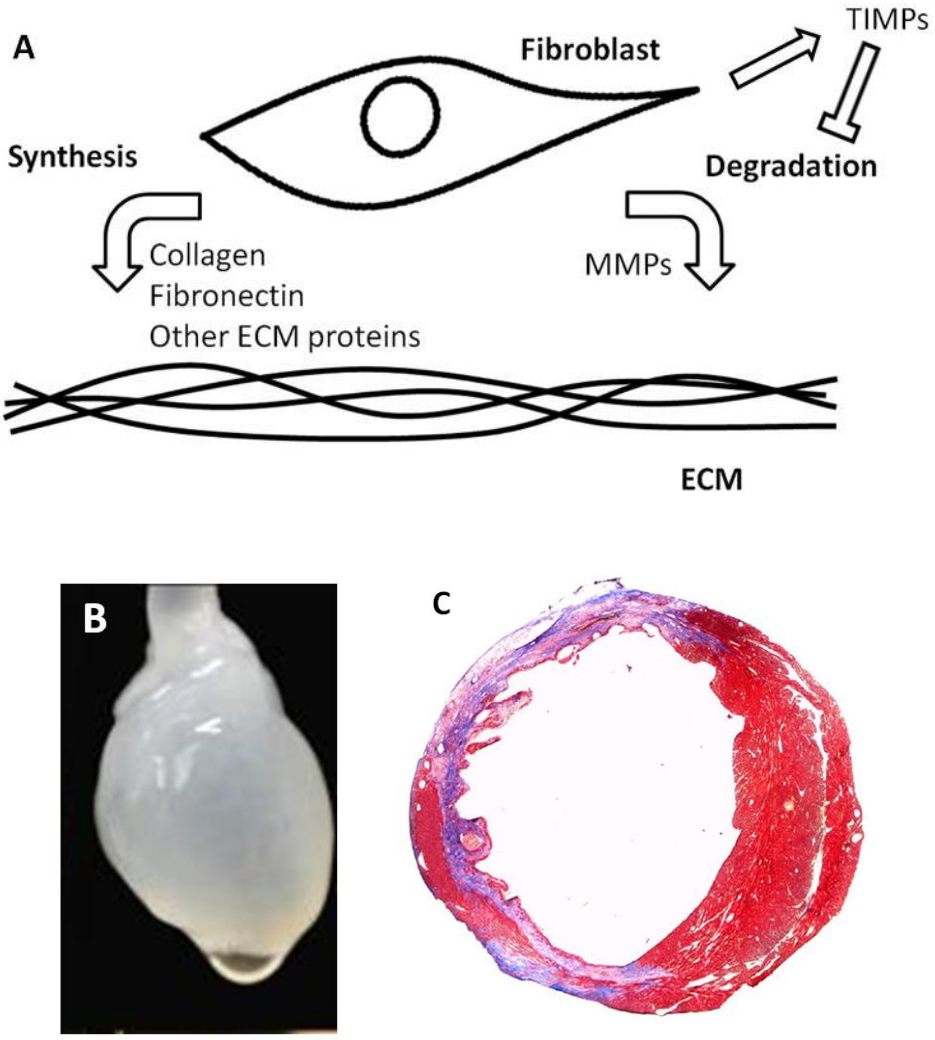


Figure 1.2: Fibroblasts control the ECM in health and disease

A) Schematic showing the elements involved in ECM control. Fibroblasts produce ECM through the synthesis of collagen, fibronectin and other proteins, and degrade the ECM via the release of MMPs. Fibroblasts also release TIMPs as a further control of MMP function. B) A de-cellularised heart. The cells are removed leaving the ECM scaffold, demonstrating the importance of the ECM to normal heart structure (reproduced with permission from (Ott et al., 2008)). C) A cross section of a heart after myocardial infarction. The pale areas show fibrotic replacement of the dead myocytes predominantly produced by fibroblasts (reproduced with permission from (Mollmann et al., 2006)).

Myocardial fibrosis and remodelling have been extensively studied and the pathways involved are increasingly understood. The main signals that activate fibroblasts are mechanical strain and soluble mediators. Fibroblasts have been shown to be exquisitely sensitive to mechanical stress (MacKenna et al., 2000) and respond differently to different strain patterns (Lee et al., 1999). The pathways involved in the response of fibroblasts to mechanical stress are increasingly understood (e.g. (Dalla Costa et al., 2010)). Various mediators also activate fibroblasts, the most important of which appear to be angiotensin II (Ang II) and TGF-β (Eghbali et al., 1991, Hafizi et al., 2004a). These pathways are potential targets for therapeutic intervention, and may account for some of the beneficial effects of current cardiovascular drugs such as angiotensin-converting enzyme inhibitors (Porter and Turner, 2009).

This is a brief introduction into the field of myocardial fibrosis, but excellent reviews are available elsewhere (Porter and Turner, 2009, Souders et al., 2009, van den Borne et al., 2010)

1.5. Fibroblast function: regulating myocyte phenotype

A growing area of interest and the focus of this thesis is how fibroblasts may modulate myocytes through intracellular communication.

1.5.1. **Juxtacrine signalling**

Juxtacrine signalling is the communication between cells by direct contact. Direct cell contact between fibroblasts and myocytes has been shown to affect the myocyte phenotype through gap junctions and adherens junctions.

1.5.1.1. **Heterocellular gap junctions**

Gap junctions are intercellular channels that link the cytoplasm of adjacent cells and allow the rapid passage of electrical impulses and small molecules between connected cells. They are formed by transmembrane protein complexes called connexons, one on each of the connected cells. These form stable bonds with each other to create the

intercellular channel. Each connexon is itself formed of 6 connexins, the basic proteins responsible for the formation of gap junctions (Mese et al., 2007).

Gap junctions are fundamental to cardiac function facilitating the rapid and direct transmission of action potentials between myocytes. They are thus essential in the coordinated and controlled stimulation of each heartbeat (Jansen et al., 2010). Connexin expression has been shown in the fibroblast fraction of both normal and diseased hearts (Vasquez et al., 2010). The expression of connexins along with the intimate physical proximity of myocytes and fibroblasts in the heart makes the presence of heterocellular fibroblast-myocyte gap junctions an interesting proposition.

In vitro, investigating gap junctions between isolated fibroblasts and myocytes has a long history. Electrical coupling between myocytes and fibroblasts was first shown in culture systems as far back as the 1960s (Mark and Strasser, 1966, Hyde et al., 1969). In the late 1980s and early 1990s the conductance between coupled cells was investigated (Rook et al., 1989) and for the first time, the role of connexin 43 in producing the gap junctions was reported (Rook et al., 1992).

There is now a considerable body of research that has investigated the properties of heterocellular fibroblasts-myocyte gap junctions *in vitro* (Figure 1.3), mainly with a focus on the potential arrhythmic effects of this coupling. Using strands of neonatal rat cells, fibroblasts were able to electrically link myocytes over extended distances (Gaudesius et al., 2003). This was associated with a delay in conduction which may be pro-arrhythmic. In unstructured cultures, the presence of increasing numbers of fibroblasts correlated with a depolarization of the resting membrane potential of neonatal myocytes from -78 to -50mV. At low numbers of fibroblasts conduction velocity and action potential upstroke velocity were increased, but as fibroblast number increased these parameters slowed (Gaudesius et al., 2003). These effects were found to be greater with cultured adult rat fibroblasts from a myocardial infarction model compared to the control fibroblasts (Vasquez et al., 2010). Heterocellular coupling between adult fibroblasts and adult myocytes has also been shown, with an intracellular dye spreading between the cell types (Chilton et al., 2007).

However, the action potentials of myocytes were not affected until spingosine-1-phosphate was added which activated a current within the fibroblasts. Connexin 43 appears to mediate heterocellular gap junction coupling as the silencing of its expression in fibroblasts prevented ectopic activity in neonatal cultures (Askar et al., 2012).

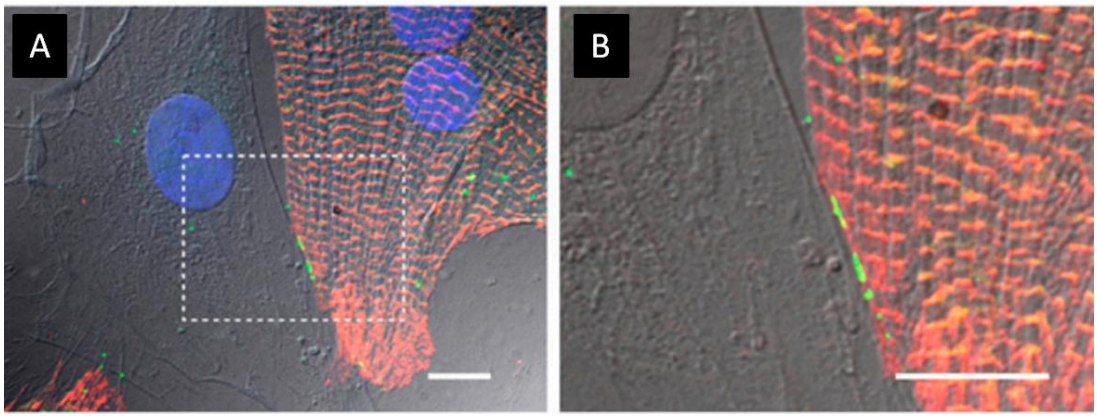


Figure 1.3: Immunofluorescence of heterocellular gap junction *in vitro*

Fibroblasts (unlabelled) and myocytes (red) readily form gap junctions (green) *in vitro*. Red- α-actinin, Green- connexin 43, Blue- nuclear staining. Scale bar 10µm. (reproduced with permission from (Zlochiver et al., 2008)).

However, the existence of gap junctions *in vivo* is still controversial. Using electron microscopy to look for gap junctions in the sino atrial node, homocellular junctions were regularly observed (both myocyte-myocyte and fibroblast-fibroblast) but only one possible small gap junction-like structure was seen between a fibroblast and myocyte (De Maziere et al., 1992). More recently, a search for gap junctions between fibroblasts and myocytes in an infarct border zone after coronary artery occlusion in dogs was conducted. Again, homocellular junctions were regularly seen, but no heterocellular junctions were observed despite extensive membrane appositions. Functionally, the spread of dye between myocytes and fibroblasts was also not seen (Baum et al., 2012). Immunohistochemistry also failed to identify any fibroblast

myocyte gap junctions in sheep ventricular tissue during the progression of remodelling following myocardial infarction (Camelliti et al., 2004a).

Other reports support the presence of gap junctions *in vivo*. Heart transplant recipients sometimes develop synchronisation of host and donor tissue across the graft scar line (Lefroy et al., 1998). One explanation is the conductance of the electrical signal via fibroblasts. Immunolabelling found connexin 45 between fibroblasts and myocytes in the rabbit sino atrial node, and it was suggested that fibroblasts may contribute to dye spreading between myocytes (Camelliti et al., 2004b). Also, double patch clamping of myocytes and fibroblasts in frog atria showed a slight decrease in fibroblast membrane potential, which temporally corresponded with the myocyte action potential (Kamkin et al., 2005).

Conclusive evidence for the existence of fibroblast and myocyte heterocellular gap junctions *in vivo* is still required. However, if they exist, the regional variations in fibroblasts often observed in disease would be pro-arrhythmic, with regional effects on conduction promoting wave front fractionation and re-entry (Kohl et al., 2005). The potential effects of the connections have been subject to computer modelling and highlight the arrhythmic potential of heterocellular gap junctions (Jacquemet and Henriquez, 2007, Sachse et al., 2008, Xie et al., 2009).

1.5.1.2. **Adherens junctions**

Adherens are another type of intercellular junction and produce tight junctions between adjacent cells (Niessen, 2007). They were traditionally seen as structural proteins but have now been attributed a variety of signalling properties (Matter and Balda, 2003).

Adherens junctions are essential for fibroblast function. They allow the coordination of mechanical force between cells (Hinz et al., 2004). They are also important in signalling in fibroblasts; synchronised Ca^{2+} oscillations within contacting fibroblasts were prevented by dissociation of adherens junctions (Follonier et al., 2008).

A role of adherens junctions in the modulation of myocyte electrophysiology by fibroblasts has recently been demonstrated. Thompson *et al* (2011) showed fibroblasts decreased neonatal myocyte conduction velocity and action potential upstroke velocity. However, these effects were decreased when mechano-sensitive channels were blocked. The effects were also evident when using connexin-43 silenced fibroblasts to confirm that this was not a gap junction-induced effect. The authors conclude that fibroblasts are causing the activation of mechano-sensitive channels through mechanical connections with the myocytes, altering their electrophysiology.

1.5.2. **Signalling through the extra cellular matrix**

The ECM provides a framework for the heart, but is also a bioactive material involved in cell signalling (Imanaka-Yoshida et al., 2004). The major receptors involved in ECM signalling to myocytes are the integrins. They were initially identified as adhesion proteins (Hynes, 1987) but the signalling properties of integrins are now widely studied (Brancaccio et al., 2006).

Integrins are transmembrane proteins with a large extracellular portion that binds to the ECM, and a small intracellular tail that is associated with actin in the cytoskeleton (Humphries, 2000). Each integrin is a heterodimer between α and β subunits and in the heart there is a variety of α subunits, which combine with a β1 subunit and have different properties (Ross and Borg, 2001). Bridging between the ECM and the myocyte, integrins signal to myocytes upon binding to an ECM substrate and also act as a sensor for mechanical tension (Ross and Borg, 2001). They are crucial in the development and the disease response of the heart. The proliferation of human foetal myocytes in response to epidermal growth factor was blocked by an antibody against the integrin β1 subunit (Hornberger et al., 2000). Knockout models have also shown the importance of integrins in development, and specifically in the heart. For example, α4 subunit null mice have defects in the development of the epicardium (Yang et al., 1995). In the adult heart, integrin signalling produces a hypertrophic response (Ruwhof and van der Laarse, 2000) and also affects cell function. For example, the binding of

laminin to αβ1 integrin attenuated the response of the L-type Ca^{2+} current to acetylcholine (Wang et al., 2000).

The direct link between fibroblasts, the ECM and myocytes has been neatly highlighted using *in vitro* preparations. Culturing neonatal myocytes on a fibroblast-derived ECM produced more rapid adhesion and spontaneous beating activity (VanWinkle et al., 1996), as well as greater Ca^{2+} uptake and development of organelles (Bick et al., 1998). In similar experiments, neonatal myocytes plated on fibroblast derived ECM had higher glucose usage, ATPase activity and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) expression (Zeng et al., 2013). Fibroblast-derived ECM also promotes the maturation of embryonic stem cell-derived cardiomyocytes improving cell ultrastructure and spontaneous beating properties (Baharvand et al., 2005).

It is therefore clear that as the major cell type involved in ECM regulation, fibroblasts can affect myocyte properties by modulating the ECM.

1.5.3. **Paracrine communication**

Paracrine communication is 'cellular signalling in which a factor secreted by a cell affects other cells in the local environment'.[6] Fibroblasts release a large number of mediators and the paracrine effects of these fibroblast-released factors are increasingly recognised in many fields of research. For example, fibroblasts associated with cancer stroma have paracrine effects that promote cancer progression (e.g. (Ao et al., 2007)). In dermal wound healing and intestinal research, paracrine mediators released from fibroblasts have been shown to affect epithelial cells (Visco et al., 2009, Peura et al., 2012).

In cardiovascular research there has been a growing interest in the paracrine effects of fibroblast-released factors. Research has mainly focused on the effect of fibroblasts on myocyte size, with more limited research into the functional effects. The majority of

[6] http://www.ncbi.nlm.nih.gov/mesh/68019899

this research has used rat neonatal cells *in vitro.* During this discussion it is highlighted when adult cells have been used.

1.5.3.1. **In vitro**

The hypertrophic response of myocytes to fibroblast paracrine mediators was first suggested over 20 years ago. Increasing the number of fibroblasts in myocyte cultures correlated with an increase in myocyte volume (Long et al., 1991). This heterocellular culture is unable to differentiate between paracrine effects and the effects induced by direct cell contact, but Long *et al* (1991) suggest it is paracrine mediated and identified a putative novel paracrine factor 'non-myocyte derived growth factor' released from the fibroblasts.

Since this initial experiment a large number of studies have shown *myocyte hypertrophy* or increased protein expression in response to fibroblast-derived paracrine mediators. A direct hypertrophic response to mediators released from fibroblasts has been observed in multiple studies, in neonatal, dedifferentiated adult mouse and adult rat myocytes. Harada *et al* (1997) showed that the size of neonatal myocytes was increased when myocytes were exposed to medium conditioned with neonatal fibroblasts. Guo *et al* (1998b) also showed an increase in neonatal myocyte size as well as in total protein production in response to neonatal fibroblast conditioned medium. The rise in protein synthesis in neonatal myocytes in response to neonatal fibroblasts conditioned medium was also shown by Booz *et al* (1999) and the increase in cell size was further shown by LaFramboise *et al* (2007). Fredj *et al* (2005b) used adult mouse dedifferentiated myocytes, and found that co-culture with fibroblasts increased the volume of these cells. Finally, work previously carried out in our lab showed that co-culture with fibroblasts increased the volume of adult rat myocytes (Cartledge et al., 2011).[7]

[7] This work was mainly carried out by a previous PhD student, and I finished it off when I joined the lab. I presented this work as an oral presentation at the American Heart Association Scientific Sessions 2011. The abstract is available online.
http://circ.ahajournals.org/cgi/content/meeting_abstract/124/21_MeetingAbstracts/A10255

In other studies the presence of fibroblasts has been shown to be essential for the hypertrophic response of myocytes to classical hypertrophic stimuli. Harada *et al* (1997) found that Ang II and TGF-β did not induce myocyte hypertrophy unless fibroblasts were present in the culture. In pure myocyte cultures these classical hypertrophic stimuli did not induce hypertrophy. Ponicke *et al* (1997), Gray *et al* (1998) and Sano *et al* (2000) also showed that Ang II drives hypertrophy through the stimulation of downstream mediators from fibroblasts rather than through direct effects on myocytes. Bhavsar *et al* (2010) found that fibroblasts were also required for the hypertrophic response of clenbuterol, another hypertrophic agent. The hypertrophic effect of cultured fibroblast-derived mediators is therefore well documented.

The effects of fibroblasts derived factors on the *function of myocytes* are less extensively studied. Guo *et al* (1998b) first showed that fibroblasts could affect myocyte electrical properties through paracrine communication. Fibroblast conditioned medium prolonged the action potential and caused a reduction in the transient outward K^+ current (Guo et al., 1998b, Guo et al., 1999). More recently the effects on electrical properties have been examined further (Pedrotty et al., 2009, Vasquez et al., 2010). Supporting the earlier findings, fibroblast conditioned medium prolonged the action potential. The myocyte resting membrane potential was also raised, the upstroke velocity of the action potential was slowed and conduction velocity in a confluent monolayer of myocytes was reduced. This was underpinned by a reduction in the Na^+ current, the rapid delayed rectifier K^+ current and the transient outward K^+ current (Pedrotty et al., 2009). A further study looked at the effect of adult fibroblasts isolated from a model of myocardial infarction and control hearts (Vasquez et al., 2010). In this study only fibroblasts from the myocardial infarction model slowed the conduction velocity and decreased the duration of the action potential. These differences were observed despite culturing fibroblasts for up to one month. The different effects to the previous reports could be due to adult source of the fibroblasts, or the protocol used for conditioning the medium.

The effect of fibroblast conditioned medium on myocyte contractility has also been the subject of a single study where fibroblast conditioned medium reduced the spontaneous rate of contraction of neonatal myocytes and after 72 hours the intrinsic beating of the myocytes stopped (LaFramboise et al., 2007).

The effects of fibroblasts on adult myocyte are even less studied. Shivakumar *et al* (2008) showed that adult rat fibroblasts exposed to hypoxic conditions reduced the viability of adult rat myocytes in culture. Previous work from our lab has shown that cultured adult rat fibroblasts caused a reduction in the Ca^{2+} transient amplitude and contractility of adult rat myocytes (Cartledge et al., 2011). A recent study has also looked at the effect of adult fibroblast conditioned medium on adult myocyte electrophysiology. Kaur *et al* (2013) showed that fibroblast conditioned medium increased the voltage gated Na^{+} current and reduced the transient outward K^{+} current. These changes are similar to those observed in neonatal myocytes, and show that fibroblasts are capable of affecting adult myocyte electrophysiology.

These studies into the paracrine effects of fibroblast on myocytes *in vitro* have mainly exposed myocytes to fibroblast conditioned medium. However, various paracrine effects of myocytes on fibroblasts have also been demonstrated. Paracrine mediators released from neonatal myocytes or HL-1 cells (an atrial cancer cell line that maintains myocyte features (Claycomb et al., 1998)) increase fibroblast proliferation (Fredj et al., 2005b, Panse et al., 2012, Tsoporis et al., 2012), α-SMA expression (Burstein et al., 2007, Dolmatova et al., 2012) and ECM protein expression (Burstein et al., 2007, Tsai et al., 2011, Zhang et al., 2011, Panse et al., 2012). It is therefore important to consider this whole relationship as a bidirectional, dynamic communication.

1.5.3.2. **In vivo**

The use of genetic modification to examine fibroblasts is still limited (as discussed in section 1.3.2). However, it has started to allow investigations into fibroblast paracrine communication and its importance *in vivo.* These studies, in support of the findings *in vitro,* show that cardiac hypertrophy is limited when the activity of fibroblasts is

suppressed and this appears to be due to paracrine communication (Pellieux et al., 2001, Jaffre et al., 2009, Takeda et al., 2010, Accornero et al., 2011).

Pellieux *et al* (2001) found that fibroblast growth factor-2 knock out (FGF-$2^{-/-}$) mice had a dilated heart which did not hypertrophy in response to hypertension. As fibroblasts are the main source of FGF-2 (although myocytes also release FGF-2 (Jimenez et al., 2004)), and Pellieux *et al* (2001) showed that FGF-2-silenced fibroblasts do not induce myocyte hypertrophy *in vitro,* it was argued that the *in vivo* findings were due to the lack of paracrine release of FGF-2 from fibroblasts.

Jaffre *et al* (2009) examined the role of serotonin receptor 2b in non-myocyte cells by crossing a global knockout with a myocyte specific transgenic mouse line. Expression of serotonin receptor 2b in myocytes was recovered but it was still absent in other cells including fibroblasts. The transgenic animals did not develop cardiac hypertrophy in response to isoproterenol infusion and had decreased cytokine levels. These effects were recapitulated with fibroblasts *in vitro* and therefore it was argued that fibroblasts were involved in the effects observed *in vivo*.

Selective knockout of the transcription factor Krüppel like factor 5 (KLF-5) under the control of the periostin promoter ($Klf5^{fl/fl}$;Postn-Cre) was used to look at the role of KLF-5 in fibroblasts. These animals did not have a hypertrophic response to pressure overload in part due to downstream paracrine signalling (Takeda et al., 2010).

Conversely, the overstimulation of fibroblast paracrine mediator release increases the hypertrophic response *in vivo.* The over expression of placental growth factor (PGF) stimulated the release of cytokines in fibroblasts in response to pressure overload and this correlated with a greater hypertrophic response (Accornero et al., 2011).

It is clear that fibroblasts have the ability to induce myocyte hypertrophy and that this appears to be important in the hypertrophic response of the heart. Although it is not as widely demonstrated, the available data suggest that fibroblast paracrine mediators have a negative effect on myocyte function, with reduced conduction velocity,

contractility and Ca^{2+} transient amplitude, which may also play a role in cardiac pathology. To gain a better understanding of these processes the underlying paracrine mediators need to be understood.

1.6. Paracrine signalling pathways

The investigation and understanding of the mediators and pathways involved in paracrine communication between fibroblasts and myocytes is complex. Firstly, both fibroblasts and myocytes release mediators. Secondly, both fibroblasts and myocytes can respond to these mediators in an autocrine (responding to a signal derived from themselves) or paracrine fashion. Thirdly, the mediators released are altered by the biochemical and physical signals received. Finally, there are complex interactions between different mediators rather than just additive effects (van Nieuwenhoven et al., 2013). It is therefore important to realise that there is a bidirectional, dynamic communication between the two cell types. There are also many potential mediators involved, and they are summarised here. A full overview of all the mediators involved is beyond the capacity of this introduction. Therefore a detailed overview of some of the potentially important mediators is provided, followed by a summary of the direct research into the paracrine effects of other mediators. A particular focus is paid to TGF-β which is the main mediator that has been investigated in this thesis.

1.6.1. **TGF-β**

TGF-β exists as three isoforms TGF-β1, TGF-β2 and TGF-β3 (Leask and Abraham, 2004). Within the cardiovascular system it is viewed mainly in its role as a pro-fibrotic mediator (Leask, 2007). TGF-β is increased in human heart failure tissue (Li et al., 1997, Sivakumar et al., 2008) and in animal models of cardiac disease (Li and Brooks, 1997, Hao et al., 1999, Hao et al., 2000). The role of TGF-β in the progression of heart disease appears to be biphasic. Early after myocardial infarction TGF-β is associated with improved outcomes, but in later stages it promotes progression to heart failure (Lefer et al., 1990, Ikeuchi et al., 2004, Okada et al., 2005).

1.6.1.1. **Release**

TGF-β is produced in a latent form which is then activated by changes in pH or by proteases (Lim and Zhu, 2006). It is released by both fibroblasts (Zhao and Eghbali-Webb, 2001) and myocytes (Thompson et al., 1989, Taimor et al., 1999). The level of TGF-β production is increased by Ang II (Lee et al., 1995, Campbell and Katwa, 1997) and other mediators (Fisher and Absher, 1995, Thaik et al., 1995) and mechanical stretch also induces TGF-β release from fibroblasts (van Wamel et al., 2001).

1.6.1.2. **Effects**

TGF-β has three receptors type I, type II and type III and all three TGF-β receptor subtypes are present on both cardiac fibroblasts (Sigel et al., 1996) and myocytes (Engelmann and Grutkoski, 1994). TGF-β type III receptors are non-signalling and their role is to bind TGF-β and then transfer it to type II receptors. Type II and type I receptors then form hetero-tetramers that transfer the signal across the cell membrane (Lim and Zhu, 2006). Activation of TGF-β receptors phosphorylates Smad 2 and Smad 3 which form a complex with Smad 4. This complex then translocates to the nucleus where they alter gene transcription (Heldin et al., 1997). The genetic targets of this Smad complex include Smad 6 and 7 which form a negative feedback loop, inhibiting the activation of Smad 2 and 3 (Park, 2005) (Figure 1.4). TGF-β receptors also signal through Smad-independent pathways including TGF-β-activated kinase 1, extracellular signal-regulated kinase, c-Jun-N-terminal kinase, and p38 mitogen-activated protein kinase pathways (Derynck and Zhang, 2003).

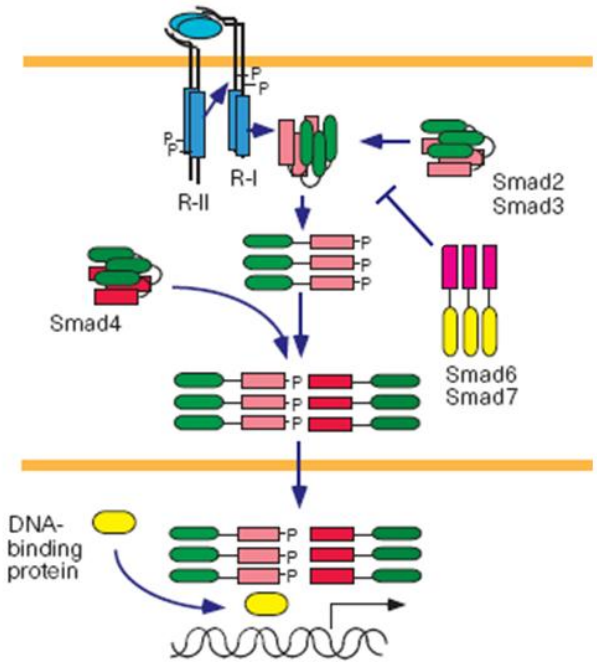


Figure 1.4: Diagram of TGF-β Smad signalling

TGF-β binds to the type II receptor resulting in the formation of a hetero-tetrameric signalling complex of type I and II receptors. This phosphorylates the Smad 2 and Smad 3 complex, which interacts with Smad 4 and translocates to the nucleus where it affects gene transcription. Smad 6 and 7, which are induced by the Smad 2, 3 and 4 complex, block the activation of Smad 2 and 3 in an auto inhibitory feedback loop. (Adapted by permission from Macmillan Publishers Ltd: Nature (Heldin et al., 1997), copyright (1997)).

TGF-β stimulates ECM production in fibroblasts (Eghbali et al., 1991, Heimer et al., 1995, Agocha et al., 1997, van Nieuwenhoven et al., 2013). It also reduces the degradation of the ECM by fibroblasts, by decreasing collagenases and increasing their inhibitors (Chua et al., 1991, Overall et al., 1991, Seeland et al., 2002) although this is not ubiquitously observed (Stawowy et al., 2004). In addition, TGF-β drives the development of α-SMA, which is associated with increased activity in fibroblasts (Lijnen and Petrov, 2002, Adapala et al., 2013, van Nieuwenhoven et al., 2013). Reports of TGF-β effects on migration and proliferation of fibroblasts are conflicting. TGF-β may or may not increase migratory capacity (Stawowy et al., 2004, Brown et al., 2007) and in different studies it has been found to increase (Squires et al., 2005) or

decrease proliferation (Agocha et al., 1997). In another report the effect was dependent on the level of TGF-β. At low levels of TGF-β proliferation was increased slightly, but at higher levels proliferation decreased slightly (Sigel et al., 1996).

In myocytes, TGF-β induces hypertrophy (Gray et al., 1998) and *in vivo* over expression of TGF-β induces a mild cardiac hypertrophy (Rosenkranz et al., 2002). TGF-β also increases and stabilises the beating rate in neonatal myocytes (Roberts et al., 1992). This is possibly achieved through regulation of Ca^{2+} handling properties. TGF-β reduces neonatal atrial myocyte L type Ca^{2+} current (ICa,L) (Avila et al., 2007) and also improves sarcoplasmic reticulum (SR) Ca^{2+} handling, as without TGF-β SR Ca^{2+} oscillations are lost in neonatal myocytes (Neylon et al., 1994). In adult myocytes, TGF-β slows the Ca^{2+} transient and reduces contractility (Li et al., 2008). It also affects myocyte electrophysiology. The reported effects are different in neonatal and adult myocytes. In neonatal myocytes TGF-β reduces the voltage gated Na^{+} current and the inward rectifier and outward sustained K^{+} currents, but not the transient outward K^{+} current (Ramos-Mondragon et al., 2011). In adult myocytes TGF-β prolongs the action potential with an associated increase in the voltage gated Na^{+} current and decrease in the transient outward K^{+} current (Kaur et al., 2013). The effects of TGF-β on myocytes and fibroblasts are summarised in Figure 1.5.

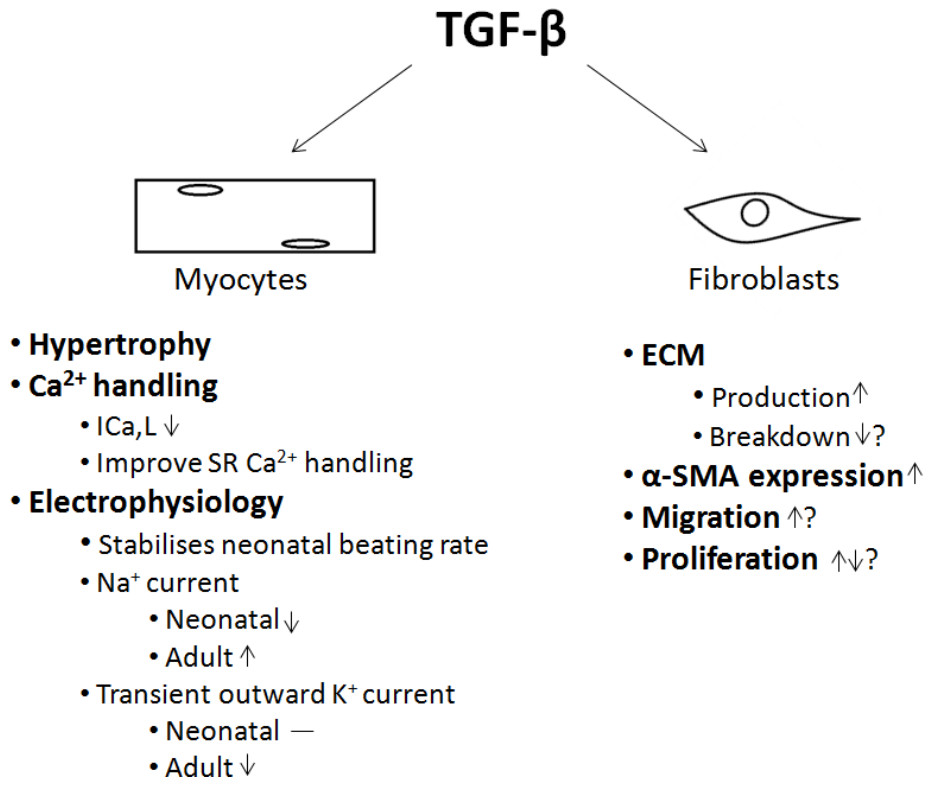


Figure 1.5: Summary of the effects of TGF-β on myocytes and fibroblasts

↑: Increased. ↓: Decreased. —: Unchanged. ?: Conflicting reports.

TGF-β has been directly investigated in the paracrine effects of fibroblasts on myocytes. Gray *et al* (1998) found that neutralising TGF-β in the conditioned medium of fibroblasts stimulated with Ang II prevented the hypertrophic response in myocytes. Neutralising TGF-β in conditioned medium from stretched fibroblasts also prevented hypertrophy (van Wamel et al., 2001). *In vivo,* TGF-β-deficient mice do not develop cardiac hypertrophy in response to a sub-pressor dose of Ang II infusion (Schultz Jel et al., 2002). Although this effect has not been shown to be fibroblast-mediated, in combination with the work carried out by Gray *et al* (1998), it can be hypothesised that TGF-β release from fibroblasts is important in the cardiac hypertrophic response to Ang II. The effects of fibroblast conditioned medium on adult myocyte electrophysiology have also been shown to be due to TGF-β signalling. The increase in the voltage gated Na^+ current and the decrease in the transient outward K^+ current

were blocked when a TGF-β neutralising antibody was added to the conditioned medium (Kaur et al., 2013).

1.6.2. **Angiotensin II**

Ang II is the effector of the renin-angiotensin system that has pleiotropic effects on the cardiovascular system. Initially characterised as a potent vasoconstrictor, it was later shown that Ang II has direct effects on myocardial remodelling, stimulating myocardial hypertrophy and fibrosis (Dostal and Baker, 1992, Brilla et al., 1993). Ang II is increased in cardiovascular disease and levels of Ang II in heart failure patients correlate with mortality (Dzau et al., 1981).

1.6.2.1. **Release**

Ang II can be produced both systemically and locally in the heart. Systemically, renin released from the kidneys converts angiotensinogen in the liver to angiotensin I. This is subsequently converted to Ang II by angiotensin converting enzyme. Within the heart, both fibroblasts and myocytes produce all the components necessary to produce Ang II (Dostal et al., 1992, Katwa et al., 1997).

1.6.2.2. **Effects**

Ang II acts via either AT-1 or AT-2 receptors, both of which are found in the heart (Gullestad et al., 1998). Both receptor subtypes are found on myocytes but in fibroblasts the expression of AT-2 is uncertain. Fibroblast AT-2 expression has been identified in some studies (Tsutsumi et al., 1998) but not in others (Crabos et al., 1994, Hafizi et al., 1998).

In fibroblasts, Ang II causes an increased expression of ECM protein which is blocked by AT-1 receptor antagonists (Crabos et al., 1994, Brilla et al., 1997, Hafizi et al., 1998). Ang II also has a proliferative effect on fibroblasts through an AT-1 receptor mediated pathway (Samuel et al., 2004, Wang et al., 2013) whereas overexpression of the AT-2 receptor did not alter proliferation (Warnecke et al., 2001). However, whether this

increased proliferation is a direct effect of AT-1 stimulation or via the release of secondary autocrine mediators is unclear (Bouzegrhane and Thibault, 2002). Fibroblasts respond to Ang II by secreting an array of mediators including TGF-β (Lee et al., 1995, Campbell and Katwa, 1997), endothelin 1 (ET-1) (Ito et al., 1993, Cheng et al., 2003), interleukin 6 (Il-6) (Sano et al., 2000) and tumour necrosis factor α (TNF-α) (Yokoyama et al., 1999).

Initially Ang II was thought to produce hypertrophy in myocytes (Aceto and Baker, 1990); however, this direct effect was later questioned (Ponicke et al., 1997). The presence of fibroblasts was found to be essential for the hypertrophic effect of Ang II on myocytes (Harada et al., 1997, Ponicke et al., 1997, Gray et al., 1998). Ang II also increases myocyte contractility through increased myofilament sensitivity to Ca^{2+} (Ikenouchi et al., 1994).

Ang II therefore appears to drive myocyte hypertrophy through paracrine mediators released from fibroblasts. ET-1 and TGF-β have been investigated as downstream mediators and are discussed in the relevant sections. Interestingly, myocyte and fibroblast specific aldosterone receptor knockout mice have been produced, which is another downstream mediator of Ang II signalling (Fraccarollo et al., 2011, Lother et al., 2011). Early studies show that the myocyte specific knockout of aldosterone receptors prevent hypertrophy in response to pressure overload, whereas fibroblast-specific knockout does not have an effect (Lother et al., 2011). This suggests that aldosterone induces myocyte hypertrophy directly *in vivo* and, although a role of fibroblasts in this relationship has not been studied, may also represent indirect Ang II signalling through downstream mediators.

1.6.3. **Endothelin 1**

ET-1 was initially identified as a potent long lasting vasoconstrictor released by endothelial cells (Masaki, 2004). The level of ET-1 is raised in heart disease (Parker and Thiessen, 2004) and inversely correlates with patient outcome (Motte et al., 2006). Long term treatment with an antagonist improved outcomes in a rat myocardial

infarction model (Sakai et al., 1996), although clinical trials have produced varied results (Motte et al., 2006).

1.6.3.1. **Release**

Both fibroblasts and myocytes are able to produce ET-1 (Suzuki et al., 1993, Katwa, 2003), and the level of expression is sensitive to external factors including mechanical stimuli (Yamazaki et al., 1996) and biochemical mediators such as Ang II and other ET-1 (Fujisaki et al., 1995).

1.6.3.2. **Effects**

ET-1 acts through two receptors ET_A and ET_B. Fibroblasts and myocytes express both, with a higher level of ET_B in fibroblasts (Katwa et al., 1993) and a higher level of ET_A in myocytes (Fareh et al., 1996).

ET-1 causes an increase in collagen production and decrease in collagenase activity in fibroblasts acting through both ET_A and ET_B receptors (Guarda et al., 1993, Hafizi et al., 2004b). The reported effects of ET-1 on fibroblast proliferation have been conflicting. Although ET-1 has been reported to increase proliferation (Piacentini et al., 2000), other reports show no direct effect (Turner et al., 2004) and others report a reduction in DNA synthesis (Hafizi et al., 2004b).

In myocytes, ET-1 stimulates hypertrophy (Inada et al., 1999) and increases protein synthesis (Guo et al., 1998a). It also increases individual myocyte contractility (Kelly et al., 1990, Kramer et al., 1991), although this effect appears to be reversed in pathology (Suzuki et al., 1998). It also affects myocyte Ca^{2+} handling, resulting in an increased Ca^{2+} transient amplitude in healthy adult myocytes (Kohmoto et al., 1993), although this effect is reversed in heart failure myocytes where ET-1 reduces the Ca^{2+} transient amplitude (Suzuki et al., 1998). In neonatal myocytes ET-1 also results in a smaller and slower Ca^{2+} transient (Kohmoto et al., 1993, Uehara et al., 2012).

There was early interest in ET-1 in the paracrine communication of fibroblasts and myocytes. In heterocellular cultures, the myocyte hypertrophic response induced by Ang II was blocked by an ET_A antagonist (Ponicke et al., 1997). The hypertrophic response to fibroblast conditioned medium was also blocked by ET_A antagonists. This was observed with untreated fibroblasts (Harada et al., 1997, Kuwahara et al., 1999), when fibroblasts were treated with Ang II (Gray et al., 1998), and when fibroblasts were stimulated with cyclic stretch (van Wamel et al., 2001). However, this effect is not ubiquitously observed; another report found that an ET_A antagonist did not block an increase in protein expression resulting from fibroblast conditioned medium (Booz et al., 1999).

1.6.4. **Insulin like growth factor 1**

Insulin like growth factor 1 (IGF-1) is an important mediator of normal growth throughout the body. There are conflicting data regarding IGF-1 in heart failure. Both high and low levels of IGF-1 have been linked with cardiovascular disease (Andreassen et al., 2009, van Bunderen et al., 2010). However, acute administration of IGF-1 improves cardiac function in heart failure patients (Donath et al., 1998) and long term overexpression is protective in animal models (Welch et al., 2002).

1.6.4.1. **Release**

IGF-1 is released in the liver and kidneys in response to growth hormone (Castellano et al., 2009). However, it can also be produced within the heart by fibroblasts (Horio et al., 2005) and myocytes (Reiss et al., 1993). The amount of IGF-1 released from fibroblasts is increased upon mechanical stretch (Hu et al., 2006) and although it has not been shown in fibroblasts, IGF-1 expression is regulated by mediators such as Ang II in smooth muscle cells (Delafontaine et al., 2004).

1.6.4.2. **Effects**

IGF-1 acts via the IGF-1 receptor that is found on fibroblasts and myocytes with increased expression in myocytes in disease (Reiss et al., 1994b, Reiss et al., 1995).

IGF-1 increases ECM production from fibroblasts (Butt et al., 1995, Horio et al., 2005). IGF-1 also appears to promote fibroblast proliferation (Reiss et al., 1995, van Eickels et al., 2000, Samuel et al., 2004), although this is not always detected (Butt et al., 1995). IGF-1 also promotes fibroblast migration (Kanekar et al., 2000) and contraction (Diaz-Araya et al., 2003).

In myocytes, IGF-1 has been shown to be hypertrophic (Guo et al., 1999) and increases general protein synthesis (Guo et al., 1998a, Horio et al., 2005). It increases the contractility of normal myocytes (Freestone et al., 1996, Cittadini et al., 1998) and heart failure myocytes (Kinugawa et al., 1999). Interestingly, it has also been suggested that IGF-1 may promote mitotic division of surviving myocytes after myocardial infarction (Reiss et al., 1994a).

IGF-1 possibly mediates the hypertrophic paracrine effect of fibroblasts. Antibodies against IGF-1 or the IGF-1 receptor prevented, at least partially, the hypertrophic effect of fibroblast conditioned medium (Guo et al., 1999, Horio et al., 2005). IGF-1 antagonists also blocked the hypertrophic effect of clenbuterol. Clenbuterol is a partial β-adrenoceptor agonist that induces myocyte hypertrophy. *In vitro* this was found to be via stimulating the release of IGF-1 from fibroblasts, which in turn exerted paracrine hypertrophic effects on the myocytes (Bhavsar et al., 2010). Furthermore, IGF-1 was identified as a paracrine mediator stimulating myocyte hypertrophy downstream of KLF (Takeda et al., 2010). IGF-1 does not seem to contribute to the paracrine prolongation of myocyte action potentials (Guo et al., 1999).

1.6.5. **Other mediators**

The Il-6 family of cytokines have been implicated in the hypertrophic response of myocytes to fibroblast conditioned medium. Il-6 expression was increased in myocytes in response to fibroblast conditioned medium and induced hypertrophy by autocrine signalling (Fredj et al., 2005a). A neutralising antibody against cardiotropin-1, another member of the Il-6 family, partially prevented the hypertrophic response of myocytes to fibroblast conditioned medium (Kuwahara et al., 1999). Il-6 was also increased in

fibroblasts in response to PGF, which may be linked to an increased hypertrophic response in PGF transgenic mice (Accornero et al., 2011).

FGF-2 deficient fibroblasts do not induce hypertrophy in myocytes although this appears to be mainly through autocrine regulation of a secondary mediator that induces hypertrophy (Pellieux et al., 2001). FGF-2 deficient mice did not have a hypertrophic response to pressure overload, highlighting the potential importance of this pathway in the whole heart.

Fibroblast released Il-1b is putatively involved in down-regulation of myocyte connexin-43 in the border zone after infarction (Baum et al., 2012). It is also increased in fibroblasts by PGF, was decreased in PGF knockout mice and increased in PGF over-expressing mice in response to pressure overload. The level of Il-1b correlated with the level of hypertrophy observed in these animals (Accornero et al., 2011).

Another possible paracrine mediator is TNF-α. TNF-α was identified in the conditioned medium of hypoxic fibroblasts, which caused a reduction in viability of adult myocytes. The addition of TNF-α to normoxic fibroblast conditioned medium mimicked these effects (Shivakumar et al., 2008).

A recent publication suggests a novel role for matrix metalloproteinase-13 (MMP-13) as a paracrine mediator. MMP-13, which is released by fibroblasts, can activate protease activated receptor-1 and blocking MMP-13 or protease activated receptor-1 prevents cardiac dysfunction upon isoproterenol infusion (Jaffre et al., 2012).

Various mediators have also been identified in the signalling from myocytes to fibroblasts. Some of the effects of tachypaced myocyte conditioned medium on fibroblasts were blocked by an AT-1 receptor antagonist. Burstein *et al* (2007) found the induction of α-SMA was blocked, and Tsai *et al* (2011) found that TGF-β and collagen release from fibroblasts were prevented.

Connective tissue growth factor (CTGF) from myocytes also induced fibroblast proliferation and collagen synthesis (Zhang et al., 2011). Another report suggests CTGF is a binding partner with follistatin-like 3 in paracrine effects of myocytes on fibroblasts (Panse et al., 2012).

The release of vascular endothelial growth factor from myocytes has also been reported to drive fibroblast proliferation (Tsoporis et al., 2012). Stressed myocytes have also been found to induce α-SMA expression in fibroblasts through the release of ATP (Dolmatova et al., 2012).

This research into these various paracrine mediators (summarised in Table 1.1) highlights the complexity of these paracrine interactions and also their potential importance.

Mediator	Released by	Receptor expression	Main paracrine effects investigated	References
TGF-β	Both	Both	Myocytes: Hypertrophy Changes in electrophysiology Fibroblast: α-SMA expression ↑ ECM production	Thompson et al., 1989, Eghbali et al., 1991, Engelmann and Grutkoski, 1994, Sigel et al., 1996, Gray et al., 1998, Zhao and Eghbali-Webb, 2001, Lijnen and Petrov, 2002, Kaur et al., 2013.
Ang II	Both	AT-1: Both AT-2: Myocytes Fibroblasts?	Myocytes: ↑ Contractility Direct hypertrophy? Fibroblasts: ↑ ECM production ↑ Proliferation Secretion of mediators	Dostal et al., 1992, Ikenouchi et al., 1994, Lee et al., 1995, Ponicke et al., 1997, Gullestad et al., 1998, Hafizi et al., 1998, Tsutsumi et al., 1998, Samuel et al., 2004.
ET-1	Both	Both	Myocytes: Hypertrophy Fibroblasts: ↑ ECM production ↓ ECM breakdown	Katwa et al., 1993, Suzuki et al., 1993, Fareh et al., 1996, Inada et al., 1999, Katwa, 2003, Hafizi et al., 2004.
IGF-1	Both	Both	Myocytes: Hypertrophy ↑ Contractility Fibroblasts: ↑ ECM production ↑ Migration	Reiss et al., 1993, Reiss et al., 1994, Butt et al., 1995, Reiss et al., 1995, Freestone et al., 1996, Guo et al., 1999, Kanekar et al., 2000, Horio et al., 2005.
Il-6	Both	Both	Myocytes: Hypertrophy Fibroblasts: Proliferation	Fredj et al., 2005.
FGF-2	Both, but mainly fibroblasts	Both	Myocytes: Direct hypertrophy? Fibroblasts: Release of other mediators	Pellieux et al., 2001.

Il-1β	Predominantly fibroblasts	Both	Myocytes: Hypertrophy Reduction in connexin 43 Fibroblasts: ↑ ECM degradation ↓ α-SMA expression	Long, 2001, Baum et al., 2012, Accornero et al., 2011, Turner et al., 2010, van Nieuwenhoven et al., 2013.
TNF-α	Both	Both	Myocytes: Apoptosis Fibroblasts: ↑ Proliferation ↑ MMP expression	Comstock et al., 1998, Shivakumar et al., 2008, Awad et al., 2010, Porter et al., 2004.
MMP-13	Both	Both	Whole heart: ↓ fractional shortening	Steinberg, 2005, Awad et al., 2010, Jaffre et al., 2012.
CTGF	Both	Both	Fibroblasts: ↑ ECM production ↑ Proliferation	Chen et al., 2000, Hayata et al., 2008, Zhang et al., 2011.
VEGF	Both	Both	Fibroblasts: ↑ Proliferation	LaFramboise et al., 2007, Zentilin et al., 2010, Tsoporis et al., 2012.
ATP	Both	Both	Fibroblasts: ↑ α-SMA expression	Saini et al., 2005, Dolmatova et al., 2012, Lu et al., 2012.

Table 1.1: Summary of mediators investigated in the paracrine interaction between fibroblasts and myocytes, and their main effects

This thesis examines the effect of fibroblasts on myocyte function, and specifically myocyte excitation contraction coupling. In addition it looks at myocyte volume and a possible hypertrophic response. An introduction to these aspects of myocyte physiology is provided here.

1.7. Cardiomyocyte function: Excitation contraction coupling

The primary function of the heart is to pump blood around the body. On a cellular level this is achieved by the contraction of individual myocytes acting as a coordinated syncytium. Myocyte contraction is produced out of an electrical stimulus by excitation contraction coupling (expertly reviewed in (Bers, 2002)).

The electrical stimulus is the action potential. This activates the voltage dependent L-type Ca^{2+} channel (LTCC) and Ca^{2+} flows into the myocyte in the form of the ICa,L. This influx of Ca^{2+} triggers the release of further Ca^{2+} from the sarcoplasmic reticulum (SR), which raises the intracellular Ca^{2+} concentration. Ca^{2+} binds to troponin C allowing the cross-bridging of myofilaments, which produces the force for contraction. Ca^{2+} is then rapidly removed to allow relaxation, and is extruded from the cell by the Na^{+}/Ca^{2+} exchanger (NCX) and taken into the SR by SERCA (Figure 1.6).

The elements of excitation-contraction coupling are therefore the action potential, the transient rise in cytoplasmic Ca^{2+} (the Ca^{2+} transient) and the contraction of the myofilaments and the whole cell.

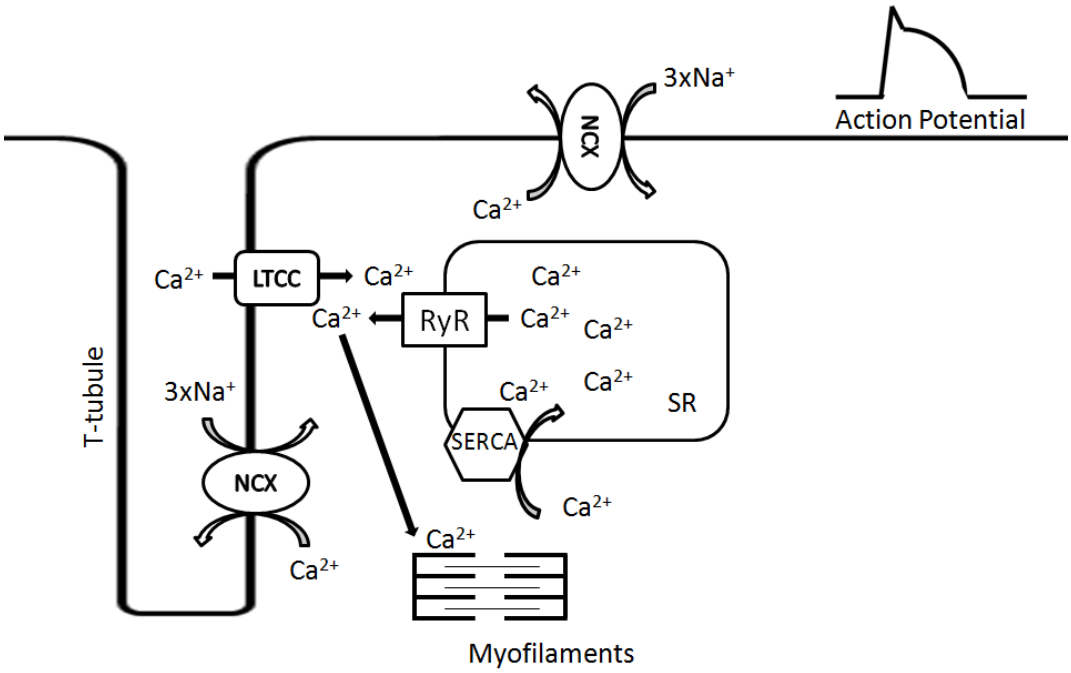


Figure 1.6: Simplified overview of excitation contraction coupling

1.7.1. **The action potential**

First described by Hodgkin and Huxley (1952) in a giant squid axon, the action potential is a voltage waveform produced by multiple currents flowing across the membrane of excitable cells (DiFrancesco and Noble, 1985). The resting membrane potential of a ventricular myocyte is approximately -85 to -90mV and an action potential is triggered when this is depolarised to a threshold. Once this threshold is reached, positive feedback of the voltage gated Na^+ current initiates a full action potential.

Excitation of the heart is initiated in the sino atrial node. Myocytes within the sino atrial node have particular currents that steadily depolarise the membrane potential towards threshold (Satoh, 2003). The action potential then self propagates through the myocardium. This propagation is facilitated throughout the heart by the electrical coupling of cardiomyocytes by gap junctions, producing an electrical syncytium and allowing the coordinated contraction of the tissue.

In ventricular myocytes, the action potential has a rapid upstroke due to the inward Na^+ current, then a characteristic plateau phase due to a balance between the ICa,L and repolarising outward currents such as the delayed rectifier current, before repolarisation when inactivation of ICa,L and increasing activation of repolarising currents then returns the cell to the resting membrane potential (Varro et al., 1993). During this plateau phase, Ca^{2+} enters the cell as ICa,L through the open LTCC.

1.7.2. **The L-type Ca^{2+} Current**

Ringer (1883) first showed the importance of extracellular Ca^{2+} to cardiac contraction and Reuter (1967) first described a long lasting Ca^{2+} current, by which Ca^{2+} enters cardiomyocytes.

ICa,L flows through the LTCC which are voltage sensitive Ca^{2+} channels that maintain a prolonged open state at depolarised potentials. This characteristic distinguishes it from the T-type Ca^{2+} channel which rapidly inactivates (Bean, 1985). The T-type Ca^{2+} channel is at a very low density in ventricular myocytes and is much less important in excitation contraction coupling.

The LTCCs open in response to the depolarisation of the membrane by the action potential at a threshold of -40mV (in physiological conditions) (Rosenberg et al., 1988). It then allows Ca^{2+} entry into the cells before it inactivates. Inactivation of ICa,L depends on the membrane potential, time and the local intracellular Ca^{2+} concentration (Lee et al., 1985). The dependence of inactivation on local Ca^{2+} allows an auto-feedback mechanism to control the amount of Ca^{2+} entry. Replacing extracellular Ca^{2+} with Ba^{2+} prolongs the current (Hofer et al., 1997). Also, buffering intracellular Ca^{2+} does not prevent inactivation suggesting it is the Ca^{2+} that is entering through the channel which causes inactivation (Hofer et al., 1997). In another feedback control, SR Ca^{2+} release can also inactivate ICa,L (Sipido et al., 1995).

Ca^{2+} entry via ICa,L is not sufficient to activate the myofilaments and myocyte contraction but rather acts as the trigger for Ca^{2+} induced Ca^{2+} release (CICR) and amplification of the Ca^{2+} signal.

1.7.3. **Ca^{2+} induced Ca^{2+} release**

CICR was first described by Fabiato (1983). In skinned Purkinje fibres, the application of Ca^{2+} produced a larger release of Ca^{2+} from the SR, the basic principle of CICR (Fabiato, 1985b, Fabiato, 1985a). In cardiac myocytes, Ca^{2+} from ICa,L stimulates the opening of the ryanodine receptor (RyR) on the SR, which is a Ca^{2+} release channel, thus producing amplification of the Ca^{2+} signal in a positive feedback loop. The importance of Ca^{2+} to the opening of the RyR was shown, as no SR Ca^{2+} release occurred when extracellular Ca^{2+} was replaced with Ba^{2+} (Nabauer et al., 1989). Also, when clamping the voltage of the membrane, increases in Ca^{2+} were still able to stimulate SR Ca^{2+} release (Niggli and Lederer, 1990). This shows CICR is in fact Ca^{2+}-induced and not voltage dependent.

Due to the high buffering of Ca^{2+} in the cytoplasm of myocytes, this process is highly dependent on the close spatial interaction of LTCCs and RyRs. This relationship is achieved by the transverse tubule (t-tubule) system and junctional SR complexes. T-tubules are regular invaginations of the cell membrane and LTCC are concentrated to the t-tubules (Ibrahim et al., 2011). Close to the t-tubule the SR is expanded so that there is a close interaction of LTCCs on the t-tubule and RyRs on the SR (Cheng and Lederer, 2008). The t-tubule system is therefore essential for effective excitation contraction coupling.

Although the recorded Ca^{2+} transient is a whole cell event, it is actually a summation of thousands of local events (Cannell et al., 1994). These local events can be visualised in resting myocytes and are termed Ca^{2+} sparks. Ca^{2+} sparks are the release of Ca^{2+} from functional clusters of LTCC and RyRs (Cheng et al., 1993). The identification and study of sparks has provided insight into CICR, as its control is at this microdomain local level.

The process of CICR is a positive feedback loop and therefore requires a specific event to terminate it. Three possibilities have been suggested: stochastic attrition (the concept that at some point, by chance, all LTCCs and RyRs within the cluster will be closed), depletion of SR Ca^{2+} and RyR inactivation (Stern et al., 1997).

Stochastic attrition cannot explain the repeated termination of Ca^{2+} release with each heart beat. In rat myocytes only 55% of SR Ca^{2+} is released and therefore depletion of the SR also does not appear to be the cause (Delbridge et al., 1997). The inactivation and reduced sensitivity to Ca^{2+} of RyRs have both been reported (Gyorke and Fill, 1993, Sham et al., 1998), and therefore appear to play a role in termination of Ca^{2+} release. However, this is still an active area of research and not fully understood.

1.7.4. **Contraction**

The myofilaments are the contractile machinery of the cell. Ca^{2+} facilitates contraction by binding to troponin C. This causes a conformational change to reveal the myosin head binding site on the actin filament and allows cross-bridge cycling, the production of mechanical force and myocyte contraction (Solaro, 1999).

The contractile force is graded to the Ca^{2+} concentration, although this is not linear, and the Ca^{2+} sensitivity can also be altered by factors, such as pH (Fabiato and Fabiato, 1978) and drugs, such as β-adrenergic agonists (Gambassi et al., 1992). Therefore the control of the Ca^{2+} transient is a major, although not the sole, way of regulating myocyte and thus cardiac contraction.

1.7.5. **The removal of Ca^{2+} from the cytoplasm**

To terminate contraction and allow relaxation of the myocytes, and diastole of the whole heart, Ca^{2+} has to be removed from the cytoplasm. The two major pathways are NCX which pumps Ca^{2+} out of the cell and SERCA which pumps Ca^{2+} back into the SR. Two other pathways exist to remove Ca^{2+}, sarcolemmal Ca^{2+}-ATPase and mitochondrial

Ca^{2+} uniport, although, together these account for approximately 1% of Ca^{2+} removal and therefore have minimal involvement in the Ca^{2+} transient (Bers, 2002).

Although SERCA is responsible for the majority of Ca^{2+} removal, the relative contributions of SERCA and NCX vary between species. In rat myocytes SERCA removes over 90% of Ca^{2+} (Bassani et al., 1994), whereas in humans it removes approximately 60% (Pieske et al., 1999). The relative contributions vary depending on other factors such as the resting membrane potential (Bridge et al., 1988) and heart rate (Pieske et al., 1999).

1.7.6. **Excitation contraction coupling in disease**

All aspects of myocyte excitation contraction coupling are altered in cardiovascular disease (Tomaselli and Marban, 1999, Hasenfuss and Pieske, 2002).

The action potential is prolonged due to changes in the current profile of the myocytes, particularly a decrease in the transient outward K^+ current (Beuckelmann et al., 1993, Tomaselli et al., 1994, Santos et al., 1995). There is also increased heterogeneity of action potential duration which is highly pro-arrhythmic (Misier et al., 1995).

Ca^{2+} handling is also deranged in pathology. The ICa,L current has been found to be reduced in some studies (Ouadid et al., 1995, Santos et al., 1995) but unchanged in others (Beuckelmann et al., 1992, Gomez et al., 1997). However, the Ca^{2+} transient amplitude is consistently decreased and the rate of Ca^{2+} removal from the cytoplasm is reduced (Beuckelmann and Erdmann, 1992, Beuckelmann et al., 1992).

This can at least in part be explained by Ca^{2+} cycling in the SR. SERCA activity is decreased in heart failure, a possible explanation for the slowed removal of Ca^{2+} (Schwinger et al., 1995, Lehnart et al., 1998) and for the observation that SR Ca^{2+} content is reduced (Lindner et al., 1998, Pieske et al., 1999). This also explains why Ca^{2+} transients are smaller as less SR Ca^{2+} is available for release. An additional explanation

may be the disruption of the spatial relationship between LTCCs and RyRs which would reduce the efficiency of CICR (Gomez et al., 1997).

Myocyte contractility is reduced in heart failure (Litwin et al., 1991, Davies et al., 1995) which may be a result of the reduced Ca^{2+} transient but also a reduction in myofilament sensitivity to Ca^{2+} (Morgan et al., 1990).

1.8. Myocyte hypertrophy

Myocyte hypertrophy can be simply defined as an increase in myocyte volume. However, it is the complex result of a large array of different signals including pressure overload and numerous soluble mediators (Shah and Mann, 2011). Myocyte hypertrophy is traditionally sub-divided into physiological hypertrophy and pathological hypertrophy (McMullen and Jennings, 2007) but this is not a clear distinction. There is considerable overlap between the signals involved in the physiological hypertrophy associated with pregnancy or exercise, and the pathological hypertrophy associated with cardiovascular disease.

In disease, hypertrophy is associated with detrimental changes in myocyte and whole heart function and co-exists with increased myocardial fibrosis, which increases the functional decline of the heart (Cohn, 1995). However, the initial hypertrophic response of myocytes to pathological stimuli is protective of heart function. If the initial hypertrophic response is prevented then ventricular dilatation and heart failure develop rapidly (Takeda et al., 2010). It is only after cardiovascular disease develops that the hypertrophic response is no longer protective but starts to contribute to disease progression.

The role of myocyte hypertrophy in protection against functional deterioration, and then disease progression, is therefore complicated. Furthermore, whether the effects of fibroblasts on myocyte volume are beneficial or detrimental to cardiovascular health requires careful investigation.

1.9. Hypothesis

In summary, several studies have shown that fibroblasts are capable of affecting properties of myocytes through juxtacrine and paracrine intercellular communication. Fibroblasts can induce myocyte hypertrophy through paracrine mediators, and there is growing evidence that this may be important in the heart's hypertrophic response to pressure overload. The effect of fibroblast-derived paracrine mediators on myocyte function is less extensively studied, but the available evidence suggests fibroblasts may have a detrimental effect on myocyte electrophysiology and contraction.

Among myocyte properties, excitation contraction coupling is crucial in cardiovascular function and disease. However, the factors that regulate excitation contraction coupling and drive its dys-regulation in disease are not fully understood. It is possible that fibroblasts act as a sensor to pathological situations that in turn drive myocyte dysfunction. Therefore, we set out to test the hypothesis that **fibroblasts can modulate excitation contraction coupling via the release of paracrine mediators, and that these effects are altered by pressure overload of the left ventricle.**

Due to the alteration in fibroblast phenotype in culture, we also set out to limit the effects of culture in order to gain a closer representation of the fibroblasts *in vivo* (although this was not possible with the dog work described in chapter 7).

In particular the following specific hypotheses were tested.

1. Normal fibroblasts exert paracrine effects on excitation contraction coupling of myocytes.

This hypothesis is tested in Chapters 4 and 7. To test this hypothesis a co-culture system was used that allows paracrine communication, but prevents direct cell contact.

- In Chapter 4, adult rat cardiac fibroblasts from healthy rat hearts are co-cultured with adult rat myocytes.

- In Chapter 7, cultured dog fibroblasts are co-cultured with dog myocardial slices (multicellular preparations of the adult myocardium).

2. The paracrine effects of fibroblasts on myocyte excitation contraction coupling are altered by chronic overload.

- In Chapter 5, adult rat cardiac fibroblasts from a model of pressure overload and sham operated controls are co-cultured with adult rat myocytes from healthy hearts.

3. **TGF-β released from fibroblasts is responsible for the paracrine effects of fibroblasts on myocytes**.

- In Chapter 6, the TGF-β type 1 receptor antagonist SB 413542 was used to investigate the role of TGF-β in the paracrine effects observed.

CHAPTER 2.

General methods

2.1. Source of cardiovascular tissue

The experiments in this thesis involved the use of rat and dog tissue. The source of tissue was an important consideration due to the pronounced differences in cardiovascular physiology between species. The most evident of these differences is the heart rate. Mice have a heart rate of approximately 600 beats per minute (Mitchell et al., 1998), ten times more than human heart rate. This is underpinned by differences in excitation contraction coupling of myocytes between species (Bassani et al., 1994, Su et al., 2003). The applicability of information obtained to human physiology and disease is therefore a major consideration when choosing a species to use (Hasenfuss, 1998, Yarbrough and Spinale, 2003). However, a second and often conflicting consideration is the availability of the tissue and the ability to manipulate the species to allow for more in depth study.

This thesis used beagle dog hearts (8-12 months of age) that were supplied by GlaxoSmithKline (Ware site, UK). The hearts were collected from control toxicology dogs that were being euthanized. Dog hearts are a good model for human cardiovascular electrophysiology with greater similarity to humans than other species (Lompre et al., 1981, Su et al., 2003).

However, the supply of this tissue was inconsistent. Also, we did not have the capacity to produce a disease model in dogs. This side of cardiovascular research is where small animals, particularly the mouse and rat, are far more useful. Small mammals are comparatively cheap to buy and maintain compared with large animals. It was also possible to produce models of disease using these animals. The lab had extensive experience with rat models of cardiovascular disease and therefore the rat was used for the majority of the experiments.

2.2. Rat model

As a source of fibroblasts from a pathological situation, the investigations involved in this thesis used a rat thoracic aortic constriction (TAC) model. TAC produced a chronically raised afterload of the heart akin to an aortic stenosis in humans.

There are a number of ways to produce models of heart disease, including coronary artery ligation, tachypacing, chemical insult and TAC (Hasenfuss, 1998, Bader, 2010). Both coronary artery ligation and TAC were established in the Terracciano lab (Lee et al., 2009, Ibrahim et al., 2012) and therefore were considered for these experiments. Both models will eventually lead to heart failure although coronary artery ligation produces this faster than TAC (Molina et al., 2009, Navaratnarajah et al., 2013). Coronary artery ligation produces a myocardial infarction, albeit without reperfusion which is normally achieved in human myocardial infarction. Myocardial infarction is a major presentation of cardiovascular disease in humans (Rosamond et al., 1998), and was therefore considered a good source of pathological fibroblasts. However, coronary artery ligation produces different populations of fibroblasts associated with the infarct zone and remote regions (Squires et al., 2005). Fibroblasts from the infarct zone exhibited greater α-SMA expression and proliferation than fibroblasts from the remote zone. TAC produces a homogenous effect on the left ventricle (LV) and has been shown to cause changes in left ventricular fibroblast phenotype. Fibroblasts isolated from TAC hearts were more proliferative and migrated faster than controls (Stewart et al., 2010). To avoid the issue of different populations of fibroblasts from different areas of the LV, TAC was chosen as the source of pathological fibroblasts. A 10-week TAC model was used as it had been previously characterised within the lab (Ibrahim et al., 2012). This model produces myocyte hypertrophy, and also disruption of the t-tubule network and Ca^{2+} handling properties of isolated myocytes. Myocardial fibrosis was not measured in these experiments, although similar models have shown increased fibrosis throughout the LV (Perlini et al., 2005, Ciobotaru et al., 2008, Chen et al., 2011). Considering this, along with the evidence of altered fibroblast function *in vitro*, TAC was chosen as the most suitable model for use in these experiments.

All operations were performed under licence by the UK Home Office, in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Aseptic technique was used throughout.

2.2.1. **Thoracic Aortic Constriction**

All the animals used for surgery were adult male Lewis rats (Harlan Laboratories, UK). This is a syngeneic strain, which allowed for better controls within experiments due to the lack of underlying genetic differences. Rats were kept in normal rodent cages with simple environmental enrichment. The animals had *ad libitum* access to food and water throughout.

The operations were carried out using weight controlled animals of 200-250g. Along with the consistency of the size of the stenosis, the size of the animal was important in maintaining the level of overload placed on the heart, particularly during the initiation of but also throughout the model.

The rat was anaesthetised in 5% isoflurane (Abbott, USA) in pure O_2 and the chest was shaved. To sterilise the operating area the rat was swabbed with 10% Povidone-iodine (Videne®, Adams Healthcare, UK). 10-15mg/kg amoxicillin trihydrate (Clamoxyl LA®, Pfizer, USA) and 10-20µg/kg buprenorphine hydrochloride (Vetergesic®, Reckitt & Colman, UK) was given subcutaneously for antibiotic prophylaxis and pain prevention. The rat was then moved to the operating area and anaesthesia was maintained with isoflurane through a nose cone. Initially 5% isoflurane was used to maintain a deep anaesthesia before the rat was intubated to allow for mechanical ventilation during the operation. A standard paediatric laryngoscope was used to visualise the larynx, and a light was directed at the outside of the neck to allow visualisation of the movement of the vocal cords. An endotracheal tube, a modified 20-gauge cannula (NHS Supply Chain, UK), was placed down the trachea. Correct insertion of the endotracheal tube was confirmed by checking for exhaled condensation using a scalpel blade, before it was connected to a Model 683 Rodent Ventilator (Harvard Apparatus Ltd, UK). Ventilation was provided at approximately 90 breaths per minute with a tidal volume of 1.5ml throughout the operation. After successful intubation, the anaesthesia was reduced to 1.5% isoflurane for the duration of the procedure. Once the animal was sterilely draped it was ready for the operation.

A right lateral thoracotomy was performed to enter the chest cavity. An incision through the skin was made using a scalpel, and the skin was bluntly dissected away from the underlying tissue and retracted to provide access. Next the pectoralis major and latissimus dorsi were dissected and retracted which allowed visualisation of the chest wall. An intercostal space was identified and the mechanical ventilation was paused as a needle was passed through the muscle, tracking the intercostal space and then back out through the same intercostal space. The ventilation was restarted and the muscle along the needle was cut with the scalpel. Needle holders were used to widen the thoracotomy which was then held open using a rodent thoracotomy retractor (World Precision Instruments, UK). Small swabs (approximately $5mm^2$) were placed to protect the delicate structures of the atria and lungs, and then the lobes of the thymus were split to provide a view of the ascending aorta. The surrounding tissue was bluntly dissected away and curved forceps were passed under the aorta to provide a path for a 3-0 silk suture. The suture was loosely tied around the aorta and a needle of 0.9mm external diameter was laid through the loop. The silk was pulled tight and locked around the needle and aorta, and then the needle was removed, leaving the aorta constricted to the size of the needle. An illustration of TAC is shown in Figure 2.1.

The swabs were removed from the chest, increased expiratory pressure was applied to re-inflate the lungs, and the chest wall was closed with a mattress suture using 4-0 prolene (Ethicon®, Johnson and Johnson, UK). The retractors were released and the muscles replaced before the skin was closed with a continuous suture using 2-0 silk. Expiratory pressure was increased again to make sure the lungs were fully inflated and to help alleviate any pulmonary oedema before the isoflurane was removed. The rat was allowed to self extubate as a clear signal of its recovery from the anaesthesia and then left to recover on its own before being returned to its original box. The operated rats were observed closely for 48 hours and wet food and analgesia were provided when necessary to aid recovery. The rats were weighed weekly after surgery to monitor their health with a humane endpoint of 20% weight loss. No rats reached this point.

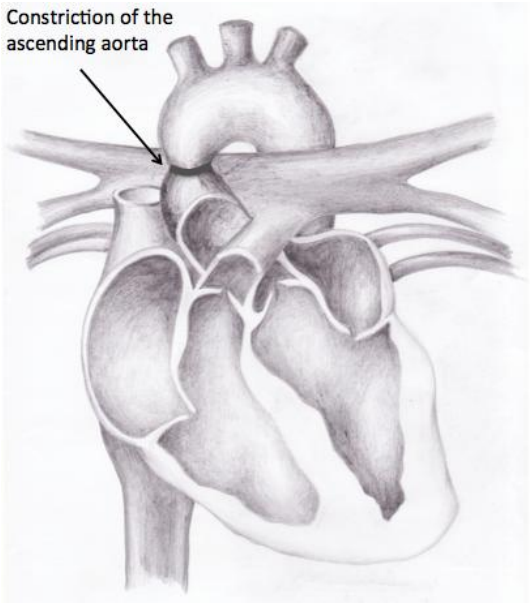


Figure 2.1: Illustration of the placement of thoracic aortic constriction on the ascending aorta
(Courtesy of Dr Mary Curran)

2.2.2. **Controls**

Either sham operated or age matched animals were used as controls. The control used is highlighted within each experiment. Where sham operated animals were used exactly the same procedure was performed as detailed above, excluding the tightening of the suture around the ascending aorta.

2.2.3. **Assessment of operative outcome**

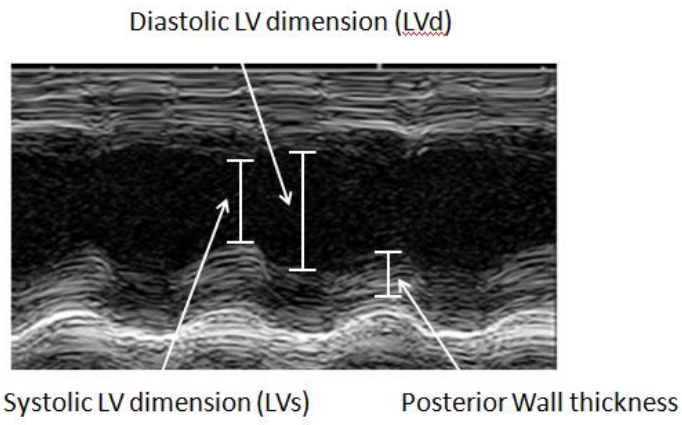
The effect of the 10 week TAC was characterised using echocardiography and heart weight and body weight measurements.

2.2.3.1. **Echocardiography**

At the point of collecting the heart, the rats were lightly anaesthetised with 1.5% isoflurane. The level of anaesthesia was carefully controlled as this can affect cardiac function (Bernard et al., 1990). The chest of the rat was shaved and warm ultrasound transmission gel was applied to the skin (Sonogel®, AnaWiz Ltd, UK). An ACUSON SequoiaTM C256 (Siemens Medical Solutions, USA) echocardiography machine with a

14MHz phased-array transducer (15L8-S, Siemens Medical Solutions, USA) was used to acquire echocardiographic data in motion mode (M-mode).

In order to get comparative data it was essential to assess the same level within each heart. Therefore using the 2 dimensional mode, a long axis view was used to identify the papillary muscles, and switching to a short axis view a level just above these was selected for measurement. M-mode echocardiography involves repeated scanning of a single line over time. Therefore a line was selected across the ventricles and recorded. This image was used to measure LV free wall thickness and ejection fraction. Ejection fraction was calculated by the software from measurements of left ventricular dimensions in systole and diastole using the calculations in Figure 2.2[8]



LV End Diastolic Volume (LVEDV) = $(7.0/(2.4+LVd)) \times LVd^3$

LV End Systolic Volume (LVESV) = $(7.0/(2.4+LVs)) \times LVs^3$

LV EF = $((LVEDV-LVESV)/LVEDV) \times 100$

Figure 2.2: Echocardiography measurements and calculations

Measurements of systolic and diastolic LV dimensions and posterior wall thickness were taken from M-mode images. LV dimensions were used by the software to calculate ejection fraction using the equations shown.

[8] Echo Made Easy. Sam Kaddoura. Churchill Livingstone, Second Edition, 2009.

2.2.3.2. **Biometric measurements**

The body weight of the animals was measured at the point of sacrifice, and before the heart was used for cell isolation the whole heart and LV weights were measured. This allowed the heart weight (HW) to body weight and LV to body weight ratios to be calculated which were used as indices of cardiac hypertrophy.

2.3. Tissue and cell preparation

Three *in vitro* preparations of cardiac cells and tissue are used in this thesis, namely isolated myocytes, isolated fibroblasts and myocardial slices.

Isolated myocytes were prepared from rat hearts and were used to examine the paracrine effects of fibroblasts on myocyte Ca^{2+} handling, contractility, volume and viability. Cell isolation was achieved by enzymatic digestion of cardiac tissue to liberate the cells. The isolation of myocytes was widely established in our laboratory, using a Langendorff set up to perfuse the coronary vascular system via retrograde perfusion of the aorta of small animals with enzymes (Siedlecka et al., 2008). The isolation of intact functional myocytes from the myocardium by retrograde perfusion of the coronary arteries was first described by Berry *et al* (1970). The availability of these cells has been the basis of much of our understanding of the function of myocytes and the effects of exogenous stimuli. The isolation of fibroblasts was less well characterised, and various methods were tested as described in Section 3.3.1, before settling on the technique described below. The isolation of fibroblasts from the heart has also been essential for understanding their properties (Rohr, 2011). The ability to isolate these different cell populations is essential for the work in this thesis.

Myocardial slices were prepared from the dog hearts and were used to examine the paracrine effects of fibroblasts on the electrophysiology of the myocardium at the multicellular level. Unlike isolated cells they allow the measurement of conduction velocity alongside the field potential (an extracellular recording of the voltage changes caused by the action potential). Slices are thin (approximately 300µm) viable sections of living tissue that can be used to study the function of intact tissue from various

organs (Parrish et al., 1995). The methodology for preparing myocardial slices, has been updated such that serial sectioning of a piece of ventricular tissue is now achieved with a high precision vibrating microtome and slices are cut parallel to the epicardial wall so as to run in the direction of the muscle fibres and thus limit damage by cross cutting fibres. These slices can then be used to measure the structure and function of the myocardium (Camelliti et al., 2011).

Slices have advantages over isolated myocytes due to the presence of other elements of the myocardium, including other cells such as fibroblasts and endothelial cells, and an intact ECM. They are therefore considered a more representative model of the intact heart. Other methods used to study intact heart tissue include wedge, trabeculae, and whole heart preparations; slices also have advantages over these (de Boer et al., 2009). Firstly, many slices can be produced from a single heart- approximately 30 from a section of a canine LV. Therefore a greater number of studies can be carried out with fewer animals, an essential aspect of animal research development. Also, wedge preparations measure from the cross cut section of the tissue and are therefore recording from damaged fibres. They also require perfusion through a coronary artery which may be uneven across the tissue, whereas slices remain viable when kept in oxygenated normal Tyrode solution (NT). Trabeculae are columns of muscle that project from the endocardial surface of the LV. They can be detached and studied as whole sections, whereas slices allow the investigation of the main body of the ventricular wall. Whole heart studies are limited to recording from the surface of the heart whereas slices from different depths through the myocardium can be used to generate more information.

2.3.1. **Rat heart collection**

As a result of the surgery, adhesions developed within the chest and around the heart and therefore removing the heart required care. Terminal anaesthesia was induced with 5% isoflurane. The chest and abdomen was shaved along the midline of the animal and the skin was opened from the manubrium down to the mid abdomen with a scalpel. The abdominal wall was opened revealing the xiphisternum and the

diaphragm. Using scissors, the diaphragm was punctured and a median sternotomy was rapidly performed. The heart was quickly excised and placed in NT containing 100 units of heparin (Monaparin™, CD Pharmaceuticals Ltd, UK) per ml (NT+H) at 37°C to prevent blood clotting. It was allowed to beat for approximately 10 seconds to remove the majority of the blood from within the heart and then moved into NT+H at 4°C which led to rapid cessation of beating to preserve the tissue. Unoperated and control rat hearts were collected in the same fashion for consistency.

2.3.2. **Rat myocyte isolation**

While in the cold NT with heparin, the heart was cleaned of excess tissue (e.g. lung, thymus and excess fat) in order to expose the aorta for cannulation. The aorta was cannulated with a 16-gauge cannula on a Langendorff perfusion set up with a constant flow pump (MasterFlex® L/STM Economy Drive, Cole-Parmer Instrument Company Ltd., UK). A flow rate of approximately 12ml per minute was used throughout. The heart was retrogradely perfused with oxygenated NT at 37°C until the heart was clear of blood and contracting well. Perfusion was switched to oxygenated low Ca^{2+} solution (LC) at 37°C for 5 minutes. It was then switched to oxygenated Buffer A containing 0.6mg/ml collagenase type II (Worthington Biochemical Corporation, USA; Cat. No. 4176) and 0.4mg/ml hyaluronidase type I-S (Sigma Aldrich, UK; Cat. No. H-3506) for 8 minutes. In total 35ml of Buffer A with collagenase and hyaluronidase was used.

The heart was removed from the Langendorff and the LV was then removed from the rest of the heart and cut into small pieces of approximately $2mm^3$. These pieces were shaken in 10ml of oxygenated re-circulated collagenase/hyaluronidase containing Buffer A for 5 minutes at 37°C. The solution was collected by filtering through a 400-µm nylon mesh to remove the remaining tissue, which was shaken again in oxygenated fresh collagenase/hyaluronidase containing Buffer A at 37°C for another 5 minutes. At the end of this digestion the tissue was agitated using a Pasteur pipette to encourage the separation of single myocytes from the tissue and the solution was collected again. The remaining tissue was shaken in oxygenated Buffer A at 37°C for a further 5 minutes before agitating the tissue and collecting the solution a final time.

To acquire myocytes, the collected solution which contained the cells in suspension was centrifuged at 500rpm for 1 minute and the supernatant was removed. The pellet of cells was re-suspended in 8ml of Buffer A and re-centrifuged to wash off any remaining enzyme. The supernatant was removed and the cells were finally re-suspended in fresh Buffer A ready for use.

2.3.3. **Rat fibroblast isolation**

To isolate rat fibroblasts, the heart was hung on the Langendorff set up and perfused with oxygenated LC at 37°C for 10 minutes. The heart was then removed from the Langendorff and the LV was dissected from the rest of the heart and cut into small pieces of approximately $2mm^3$. These pieces were shaken in 10ml of oxygenated Buffer A containing 0.36mg/ml proteinase type XXIV (Sigma Aldrich, UK; Cat. No. P8038) for 40 minutes at 37°C. The solution was collected by filtering it through a 400-µm nylon mesh and the remaining tissue was shaken in 10ml of oxygenated Buffer A containing 1mg/ml collagenase type V (Sigma Aldrich, UK; Cat. No. C9263) for 45 minutes at 37°C. The tissue was then vigorously agitated with a Pasteur pipette to help liberate the cells from the tissue, and then the remaining tissue was removed by filtration. Fibroblasts were collected at both time points by centrifuging at 620*g* for 5 minutes and removing the supernatant. The pellet of cells and debris was re-suspended in sterile phosphate buffered saline (PBS) and re-centrifuged to wash the cells. Finally it was re-suspended in fibroblast culture medium (FB medium (82% DMEM, 15% foetal bovine serum, 100U/ml penicillin, 100µmol/l streptomycin, 4mM L-alanyl-L-glutamine)) and placed in culture in $25cm^2$ culture flasks (Corning, USA) in humidified air at 37°C, 5% CO_2. After two hours the medium was changed to remove any debris leaving the fibroblasts attached to the culture dish. This medium change is also important in preventing contamination of the fibroblast population. Fibroblasts attach preferentially compared to endothelial and smooth muscle cells and therefore these potential contaminating cells are washed away by changing the medium (Gustafsson and Brunton, 2000).

2.3.4. **Dog fibroblast isolation**

Dogs were euthanized with intravenous pentobarbital at GlaxoSmithKline. The whole heart was rapidly removed and immediately submerged in ice cold cardioplegia (Plegivex®, Ivex pharmaceuticals, UK). Hearts were couriered to the lab on ice and arrived between 60-90 minutes after they were removed. The sharing and utilisation of this precious tissue, which would have been otherwise wasted, was in line with the 3 R's (Replace, reduce and refine) of animal research.[9]

To isolate fibroblasts, 0.5g of LV tissue was cut into small pieces of approximately $2mm^3$. These pieces of tissue were shaken in 10ml of oxygenated LC at 37°C for 3 minutes. The LC was removed and fresh LC was added and the tissue was shaken for a further 3 minutes. This was done a total of 4 times. It was then treated the same way as the rat tissue by digesting it with proteinase XXIV and collagenase type V and collecting the cells by centrifuging at 620*g*.

2.3.5. **Dog myocardial slices preparation**

The preparation of dog slices was established within the lab (Camelliti et al., 2011) based on the protocol for the preparation of slices from rodent hearts described by Bussek *et al* (2009). The LV free wall was removed from the rest of the heart and a $10mm^2$ transmural block was cut free from the rest of the tissue using clean cuts with a sharp razor blade. The block was mounted on an agar coated specimen holder of a high precision vibrating microtome (7000smz, Campden Instruments Ltd., UK) using surgical glue (Histoacryl®, Braun, Germany) in cold oxygenated slicing solution. This solution contained the excitation-contraction un-coupler 2,3-butanedione monoxime which is used to prevent the tissue contracting while being cut which would damage the tissue.

Slices were cut using a stainless steel blade (Campden Instruments Ltd., UK), set to vibrate at 75 Hz with amplitude of 2mm and deflection in the Z plane of less than 1µm. The advancing speed of the blade was set to 0.04mm/second and slice thickness to

[9] http://www.understandinganimalresearch.org.uk/how/the-three-Rs

300µm. These settings were used to limit tissue damage as previously optimised by Camelliti et al. (2011). Care was taken to keep the tissue covered in solution at all times, and the solution was kept at 4°C to help preserve the tissue and prevent damage while slicing. Once cut, the slices were incubated in oxygenated slicing solution at room temperature for at least 1 hour before being used.

2.3.6. **Fibroblast cell culture**

Fibroblasts remain viable and proliferate in culture over prolonged periods of time, although they have a limited proliferative capacity (Hayflick, 1965). Although the majority of the studies included in this thesis used fibroblast within 48 hours of culture, for some experiments long term culture was required. During maintenance of fibroblast cultures, they were cultured in FB medium. They were split 1:2 when reaching confluency, which normally happened after about 5 days in culture and then approximately twice a week. To achieve this, the medium was removed, and the culture flask was washed with sterile PBS and 2ml of 0.25% Trypsin-EDTA (Gibco®, Life Technologies, USA) was added to detach the cells from the flask. This was left in the incubator for 5 minutes. 5ml of FB medium was added which inactivated the trypsin. The medium and trypsin contained the cells in suspension and was collected and centrifuged at 620*g* for 5 minutes. The supernatant was then discarded and the pellet re-suspended in FB medium. Half the medium was then put back into the flask to achieve a 1:2 split. The other half was either discarded or put in a new flask if more fibroblasts were needed.

2.4. Rat cell studies

The culture of fibroblasts and myocytes was essential to complete the experiments in this thesis. This has inherent problems as both cell types are altered by culture. Fibroblast begin to express α-SMA, which *in vivo* is associated with pathological myofibroblasts, and also undergo other changes in protein expression and cell characteristics (Rohr, 2011). These changes were tracked over time in culture (described in Section 3.3.3.) and the final experimental design was produced so that all

experiments were completed within 48 hours of fibroblast isolation to limit and control for any changes. Adult myocytes also de-differentiate in culture (Mitcheson et al., 1998) and therefore neonatal myocytes are often used in experiments that require *in vitro* myocytes (Chlopcikova et al., 2001). However, these cells have an inherent immature phenotype (Yin et al., 2004). For these experiments, it was therefore necessary to use adult cells where the machinery for Ca^{2+} handling and contractility are fully developed.

The focus of this work was the paracrine interaction of fibroblasts and myocytes. However, it has been widely shown that direct cell contact *in vitro* between fibroblasts and myocytes result in alterations to the myocyte phenotype (e.g. (Miragoli et al., 2006) and discussed in detail in Section 1.5.1.). Therefore a culture system that allowed paracrine communication but prevented direct cell contact was required. There are two ways in which this could be done, either by using conditioned medium or by physically separating the cells within the same culture medium. Conditioned medium involves exposing medium to one cell type and then transferring it onto the target cell type (Harada et al., 1997, Pedrotty et al., 2009, Vasquez et al., 2010). However, this removes the bidirectional aspect of paracrine communication, and therefore a system to physically separate the two cell types within the same medium was used. This system made use of Transwells® (Corning Incorporated ,USA).

Transwells are inserts with a semi permeable membrane (with 0.4µm pores) that allow co-culture of cells in a common media while maintaining physical separation. This allows dynamic bidirectional paracrine communication between the fibroblasts and myocytes. A diagrammatic representation and photos of the set up are shown in Figure 2.2.

All cultures were kept in humidified air at 37°C in 5% CO_2 using a Galaxy S incubator (Wolf laboratories, York, UK) and cell culture work was performed under sterile conditions in a Class II safety cabinet (NuAire, USA).

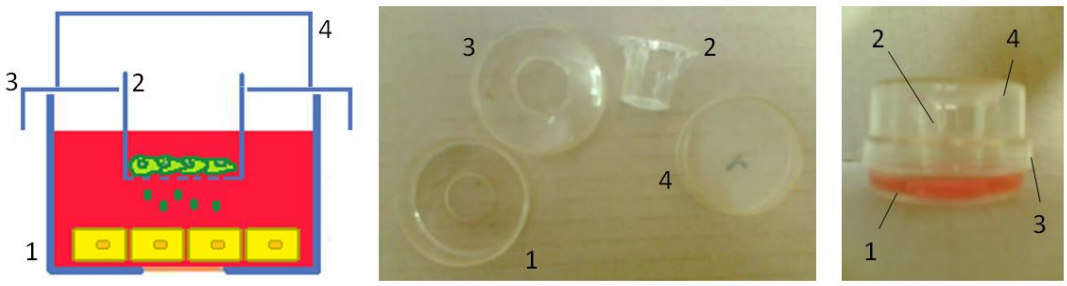


Figure 2.3: Illustration and photos of the co-culture set up.

1. A glass bottom dish, on which the myocytes were plated. 2. The Transwell on which the fibroblasts were plated, which is suspended into the medium of the myocytes. 3. Support for the Transwell to prevent it hitting the base of the glass bottom plate. These were made by cutting a hole into the top of a normal glass bottom dish lid. 4. A lid to produce a fully enclosed environment was fashioned out of a normal 35mm dish.

2.4.1. **Co-culture set up**

The identification of a suitable medium for co-culture was essential for this work and is described in detail in Section 3.3.4. Ultimately, ITS medium was used (M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 1:1,000 dilution ITS supplement (PAA, Austria; Cat. No. F01-015).

Fibroblasts were collected from culture after 20 hours using 0.25% Trypsin-EDTA and counted using a Neubauer Improved Haemocytometer. They were then plated in 6.5mm Transwells with 0.4µm pores, at a density of 10,000 cells/Transwell, in 200µl of ITS medium. These were left for 4 hours to settle and recover from the trypsin exposure before use.

Freshly isolated myocytes were plated at a density of 5,000 cells/dish on laminin (Sigma-Aldrich, UK) coated glass bottom dishes (MatTek, USA). The myocytes were left to attach for 5 minutes and then 2ml of ITS medium was added. 30 minutes later this was replaced with 2ml of fresh ITS medium before they were used. Glass bottom dishes were used as they allowed the use of an inverted microscope to study the

myocytes. They had a thin glass base, so the focal distance between the objective and the cells was smaller. Therefore a more powerful oil immersion objective could be used and a more detailed image recorded.

The Transwells containing the fibroblasts were suspended over the myocytes in the glass bottom dish, so that the base of the Transwell sat within the culture medium on the myocytes but did not touch the base of the dish. This was important to prevent nanotube projections from fibroblasts from protruding through the membrane and contacting with the myocytes (He et al., 2011). As a control 5,000 myocytes were plated in Transwells above the 5,000 myocytes in the glass bottom dishes. This was used to control for the possibility that the extra cells were depleting nutrients from the medium, which could affect the cell phenotype.

The co-culture set ups were incubated for 24 hours before experimental analysis. To characterise the effects of paracrine communication on the myocyte phenotype cell viability, cell volume, the transverse tubule (t-tubule) network, Ca^{2+} transients and contractility were analysed.

Cell viability, volume, the t-tubule network and Ca^{2+} transients were all measured using confocal microscopy. An Axiovert 200M microscope with a LSM 510 confocal laser attachment (Carl Zeiss AG, Germany) was used throughout. Myocyte contractility was recorded using an IonOptix system (IonOptix Corp, USA) to track stimulated sarcomere shortening.

2.4.2. **Cell viability**

Healthy myocytes have a characteristic rod shape and thus a simple way to assess myocyte viability was to classify cells as either rod shaped (viable) or rounded up (non-viable). To achieve this, 3-5 normal light images per co-culture set up were taken of the cells at 20 times magnification. The number of rod and rounded up cells were counted and the percentage of viable rod cells per set up was calculated. The culture of myocytes itself causes changes in cell shape with some rounding of the normal

sharp rod shape. Examples of freshly isolated, viable cultured, dying and dead cells are shown in Table 2.1. Cells that fell into the dying or dead cell categories were considered non-viable.

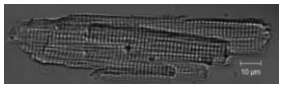
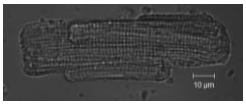
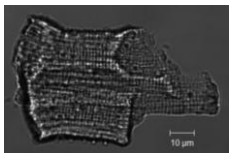
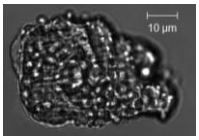
Viable		Non-Viable	
Freshly Isolated	1 day cultured	Dying	Dead
			
Rod shaped with sharp edges and corners, clear sarcomeric structure	Maintained rod shape, with softening of the edges, retains sarcomeric structure	Formation of characteristic 'cauliflower' ends or blebbing of membrane	Rounded up, no sarcomeric structure

Table 2.1: The characteristics of viable and non-viable myocytes when visualised microscopically

These were important in determining cell viability but also when determining which cells to select for data acquisition.

2.4.3. **Cell volume and t-tubule network**

Changes in cell size can be quantified by looking at a cross sectional area (Lee et al., 2009). However, this is limited to measuring any changes in 2 dimensions. Using a membrane binding dye and confocal microscopy, it is possible to reconstruct the volume of the cell in 3 dimensions allowing a more accurate measurement. Staining the membrane also marks out the t-tubule membrane invaginations, and it is therefore possible to characterise this system which is important in Ca^{2+} handling in myocytes (Ibrahim et al., 2011).

Di-8-ANEPPS (Molecular Probes, Life Technologies, USA), a membrane binding voltage sensitive dye, was used to visualise the membrane for these experiments. It was excited at 488nm using an argon laser set at 5% transmission and emission was collected through a 505nm long pass filter. Data was acquired using unidirectional

scanning at 12 bit depth. All handling and recording of di-8-ANEPPS was carried out in the dark to prevent photo-bleaching of the fluorescent molecules.

After co-culture, the myocytes were stained *in situ* on the glass bottom dishes. The ITS medium was removed and 1ml of Buffer A was added to the dish. This was subsequently removed and 10µM di-8-ANEPPS in 500µl of Buffer A was added for 10 minutes at room temperature. The remaining dye was then removed and fresh Buffer A solution added.

The cells were visualised using a 40 times oil-immersion objective using the confocal microscope, and viable cells were systematically selected and imaged. Non-viable cells as defined in Table 2.1 were not imaged, nor were cells that were in contact with another cell or debris due to the difficulty in defining the cell limits upon analysis. Once a cell was selected the microscope was put into laser scanning mode and a single image of the cell was taken. The area of interest was cropped in the x and y axes around the cell. Using the 'mark first and last' z stack function it was also cropped in the z axis to the upper and lower limits of the cell. The area of interest was therefore limited to the area occupied by the cell. A z stack of this area was then recorded with an image interval of 2µm. The images were recorded at 4 image averaging for improved quality (Figure 2.3A).

The di-8-ANEPPS stained area was then reconstructed using ImageJ software (U.S. National Institutes of Health) and cell volume and t-tubule density were analysed using custom written software (provided by Dr Mark Stagg). The images were converted to 8-bit and using auto-threshold were converted into binary images (Figure 2.3B). For t-tubule density, the macro measured the percentage of di-8-ANEPPS staining in the middle 80% of each slice (the 10% at each end were ignored to exclude the cell end membranes). This was done for each slice of the z stack and an average was generated and used to represent t-tubule density.

For cell volume, a second macro was used. The total area within the images that was enclosed by staining was marked positive and measured (Figure 2.3C). The depth of the slice is known and therefore the total volume of the cell can be calculated.

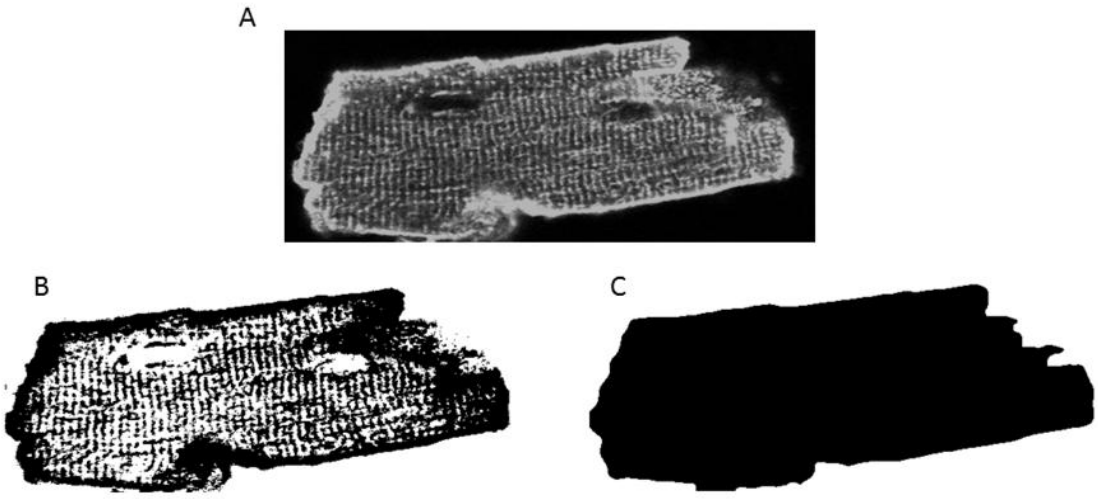


Figure 2.4: Example of the process of recording cell volume and t-tubule density

A) Original recording of di-8-anepps staining showing 1 slice of a z-stack. B) A binary image after auto threshold was applied. The % area stained in the middle 80% of the image is used as a measure of t-tubule density. C) Any area enclosed by staining was marked positive. The cell can therefore be reconstructed in 3 dimensions and the volume calculated.

2.4.4. Ca^{2+} transients

Ca^{2+} transients were measured using the Ca^{2+} sensitive dye Fluo-4 and the line scanning mode of the confocal microscope. Fluo-4 fluorescence is altered by binding of Ca^{2+} and therefore can be used to record changes in the cytoplasmic [Ca^{2+}]. The myocytes were loaded with Fluo-4-acetoxymethyl ester (AM) (Molecular Probes, Life Technologies, Oregon, USA) which easily crosses the cell membrane whereupon it is de-esterified to Fluo-4. This is membrane-impermeable and therefore remains within the cell and reports the cytoplasmic [Ca^{2+}]. Fluo-4 was excited at 488nm using an argon laser set at 5% transmission and emission was collected through a 505nm long pass filter. Data

were acquired using bidirectional scanning for increased time resolution and at 12 bit depth. As Fluo-4 is a non-ratiometric dye it does not report the absolute Ca^{2+} level and therefore it is important that the offset and gain of the channel is kept as constant as possible. Detector gain was set between 750 and 850 in all experiments, amplifier offset was set between 0.05 and 0.08 and amplifier gain was 1. All handling and recording of Fluo-4 was carried out in the dark to prevent photo-bleaching of the fluorescent molecules.

The myocytes were loaded *in situ* on the glass bottom dish after co-culture. The culture medium was removed and 1ml of Buffer A was added and removed to wash off any remaining medium. The cells were then incubated in 500µl of Buffer A containing 5µM Fluo-4-(AM) for 5 minutes at room temperature. After the incubation, this was replaced with fresh Buffer A and left for 20 minutes to allow for de-esterification before being mounted on the confocal microscope.

During these experiments the cells were perfused with NT at 37°C. NT was delivered to the dish by gravity and removed by a suction system. An insert for 35mm dishes was modified with platinum wires attached to a bipolar stimulus generator to field stimulate the cells with 20V at 1Hz.

Once mounted on the confocal, the cells were perfused and stimulated for 5 minutes before recording to allow them to equilibrate with the conditions. Viable contracting cells were then selected and their Ca^{2+} transients were measured in line scan mode. A line was selected along the longitudinal axis of a cell and scanned repetitively. This recorded the Ca^{2+} levels over the passage of time for a specific point of the cell (illustrated in Figure 2.4).

The time taken to scan a single line and the spacing sample (the relationship between a pixel and real distance) needed to be recorded as they were required for analysis. To analyse the transients, the raw images were cropped to within the cell boundaries using AimImageBrowser (Carl Zeiss AG, Germany) and the green signal was isolated from the red and blue using ImageJ. Custom-written Matlab® R2006b (The

MathWorks, Inc., USA) protocols (provided by Dr Mark Stagg) were used to analyse this image and provide the time to peak, the time to 50% and 90% decline and the amplitude of the Ca^{2+} change.

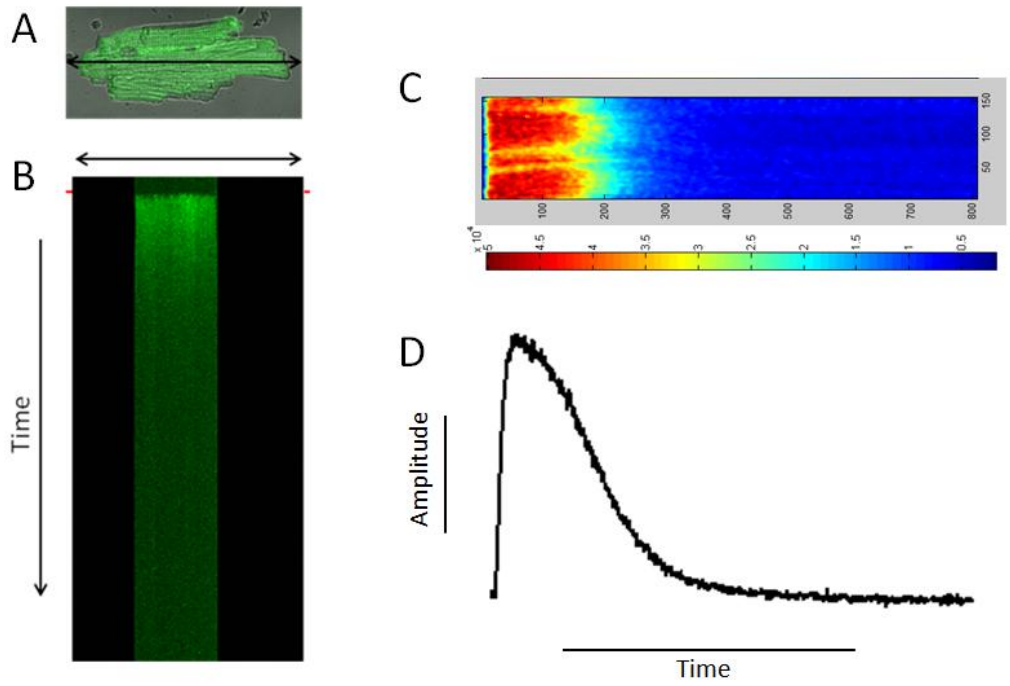


Figure 2.5: Illustration of a line scan output of a stimulated Ca^{2+} transient from a Fluo-4 loaded myocyte

A) A cell loaded with fluo-4. A line is drawn through a myocyte along its longitudinal axis. This is repetitively scanned over time, tracking changes in the cytoplasmic Ca^{2+} levels. B) An original output of a Ca^{2+} transient in a line scan. The red marks show the stimulation point. C) Output of the analysis of the signal. This allows analysis of the parameters of the Ca^{2+} transient. D) A 2 dimensional trace of the Ca^{2+} transient.

2.4.5. **Ca^{2+} sparks**

Ca^{2+} sparks are another element of myocyte Ca^{2+} handling that can be investigated using Fluo-4. Ca^{2+} sparks are spontaneous focal releases of Ca^{2+} from the SR (Cheng et al., 1993) and can be measured by scanning a non-stimulated, quiescent cell. Cells were loaded with Fluo-4 and the dishes were set up on the confocal in the same fashion as for the Ca^{2+} transient recordings. A cell and a line through the cell were

selected. After selecting a line, stimulation was stopped for 30 seconds and then a line scan was recorded for 15,000 lines. This recorded changes in the cytoplasmic Ca^{2+} in a quiescent cell.

Analysis was carried out using a custom written Matlab protocol (provided by Mark Stagg) which reports spark frequency, amplitude, duration and width.

2.4.6. **Contractility**

Myocyte contractility was measured by tracking stimulated sarcomere shortening. Average sarcomere length was calculated by measuring the peak of the power spectrum output of a fast Fourier transform of myocyte striations within a specified area in real time. This could then be tracked over time while the cell was stimulated and contracting, and provided an indication of myocyte contractility.

After co-culture, the glass bottom dishes were placed on an Olympus IX-71™ inverted microscope and viewed through a 63-time magnification oil immersion objective. They were perfused with NT at 37°C throughout the experiment and field stimulated with a 20V pulse using a bipolar stimulus generator. Sarcomere length was tracked in real time using IonOptix IonWizard™ data acquisition software (Version 5.0. IonOptix Corp.). Viable, contracting cells were randomly selected and a section of the cell was identified for input into the fast Fourier transform. By rotating the camera, care was taken that the z lines were lying vertically within this section for accurate assessment. Sarcomere shortening was recorded while stimulating the cells at 1, 2 and 3Hz. Approximately 30 seconds of data was recorded at each frequency before switching to the next to allow the contractions to reach a steady state.

Analysis was conducted using IonWizard™ software. About 10 contractile cycles were averaged for each cell at each frequency, and amplitude, time-to-90% peak, time-to-50% relaxation and time-to-90% relaxation were calculated using the inbuilt analysis (Figure 2.5).

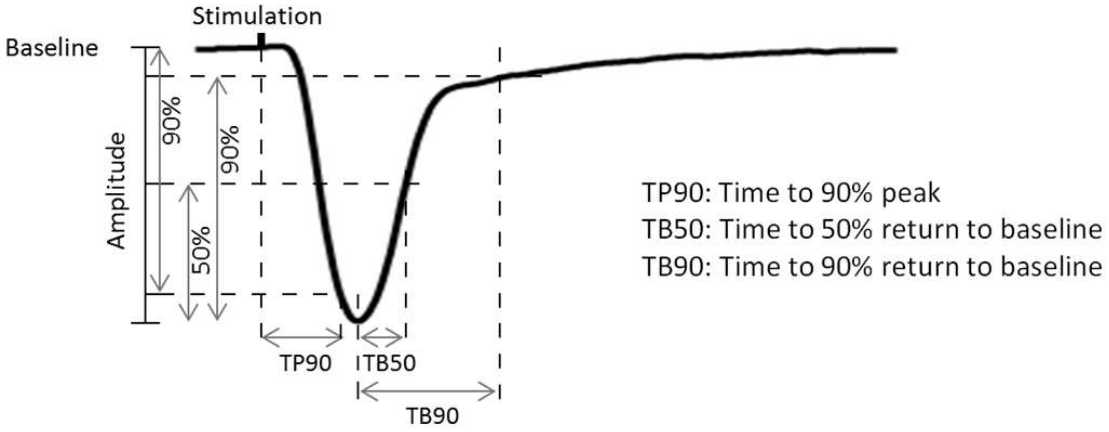


Figure 2.6: Example trace of sarcomere shortening and the parameters measured

2.5. Dog slice studies

Dog myocardial slices were assessed using a 60 electrode multi electrode array (MEA) system (Multi Channel Systems, Germany) to record the field potential of each slice. These are plates that contain a regular 8 by 8 array of electrodes (missing the 4 corners) that can be individually used to stimulate and record electrical activity. Each electrode has a 100µm diameter and they are spaced at 700µm. Each electrode records the field potential of the point of tissue surrounding the electrode. A field potential is the electrical activity recorded from the extracellular solution of a number of cells and is used to represent the action potential. The minimum peak of the field potential correlates with the upstroke of the action potential, and the peak of the second wave form (akin to the T wave of an electrocardiogram) is used to mark the repolarisation of the cells. This can then be used to calculate the conduction velocity of the slice and the field potential duration (FPD). An example of the output of the MEA is shown in Figure 2.6.

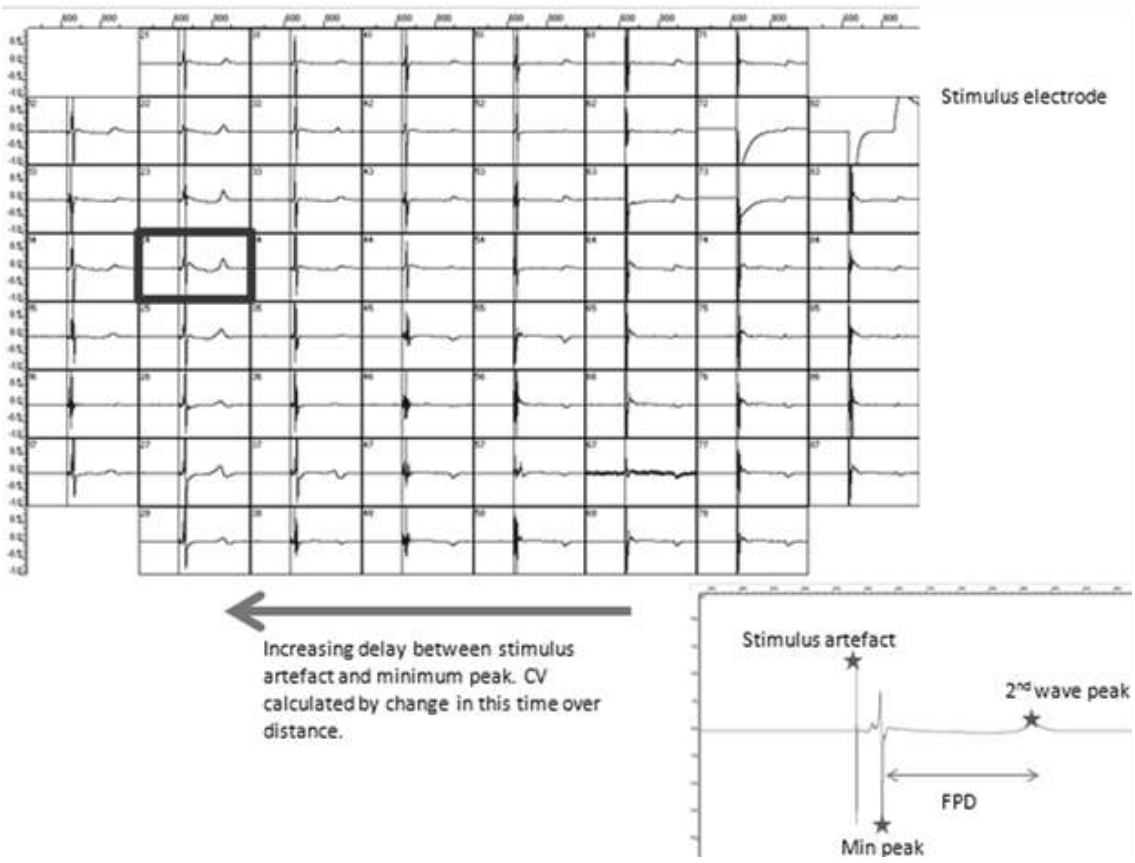


Figure 2.7: Example of the output from an MEA

The 60 electrode recordings are shown along with a zoomed in image of electrode 24 (boxed in red). This clearly shows the points recorded to enable the measurement of FPD and conduction velocity.

2.5.1. **Multi electrode array measurements**

Glass MEA plates, a MEA 1060 amplifier and TC01/02 temperature controller were used throughout these experiments (Multi Channel Systems, Germany). MC_Stimulus (Multi Channel Systems) was used to control stimulation, which was provided as a bipolar pulse of 500µs duration, 1-4 V and 1 Hz. The minimum voltage needed to stimulate the slice was used and normally this was between 1 and 2 V. This is important to minimise the stimulation artefact and also to ensure point stimulation rather than field stimulation of the area surrounding the stimulation electrode. The stimulation electrode was selected using MEA_Select and data was recorded using MC_Rack (both are software from Multi Channel Systems).

The slice was placed in a MEA dish and held down by a slice holder to maintain contact with the electrodes. Throughout the experiment the dish was perfused with oxygenated recording solution at 37°C. The slice was point-stimulated from any electrode and left to equilibrate for 10 minutes before any recordings were made. Due to the anisotropy of cardiac tissue, the slice was stimulated from 4 electrodes such that the slice was stimulated in 4 different directions (Figure 2.7). This allowed identification of the maximum conduction velocity (longitudinal conduction velocity, which runs in the direction of the muscle fibres), and the minimum conduction velocity (transverse conduction velocity, which represents conduction in the direction perpendicular to the muscle fibre orientation). This was essential for the accurate comparison between slices. The stimulation electrodes were always selected at the edge of the array to allow correct analysis of conduction velocity. Between each recording the slice was stimulated for 5 minutes to allow equilibration to the point of stimulation. About 20 seconds of data was recorded for each point.

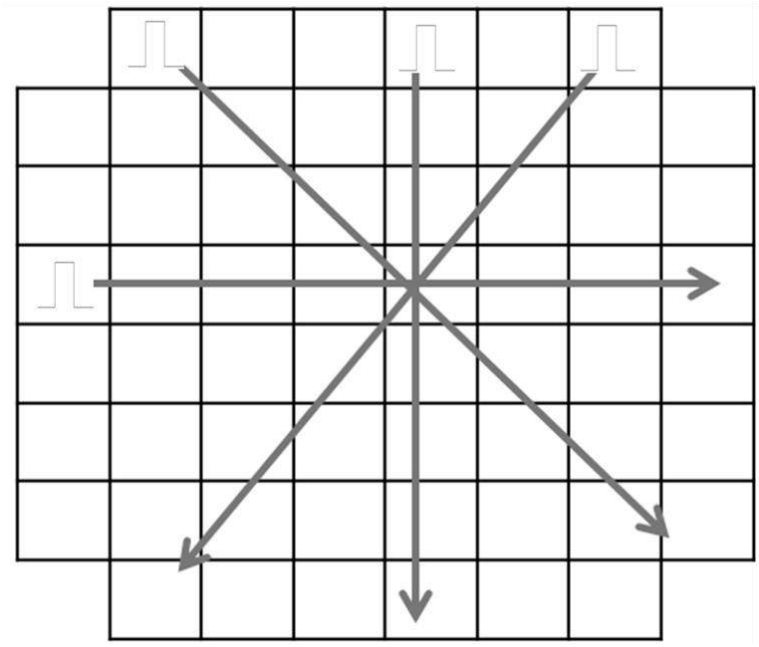


Figure 2.8: The directions of stimulation recorded for each slice

2.5.2. **Slice co-culture**

Various set ups for the culture of slices were tested (described in Section 3.3.6.). Finally, slices were cultured at the interface of culture medium and air as previously reported (Brandenburger et al., 2012). Therefore the co-culture system was reversed compared to the cellular co-culture, with fibroblasts plated on the base of the plate and the slice kept in the transwell (Figure 2.8). Canine fibroblasts were plated at a density of 40,000 cells per well in a 6 well plate and left to attach for 4 hours in 2ml of H.ITS medium (M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 10µg/ml insulin (Sigma Aldrich, UK), 5.5µg/ml apo-transferrin (Sigma Aldrich, UK), 0.005µg/ml Na^+ selenite (Sigma Aldrich, UK)). The slices were then suspended above the cells within the medium with 24mm Transwells with 0.4µm pores (Corning Incorporated, USA). It was important that the slices were laid flat on the Transwells and not folded up, and were also wet but not covered in medium; therefore in addition to the 2ml of medium in the wells, 200µl was added to the slices in the Transwells.

The control used for these experiments was to culture the slices either alone or with 40,000 human embryonic kidney 293 cells (HEK 293) (provided by Dr Nicola Hellen). These cells have very limited intrinsic activity (Thomas and Smart, 2005) and therefore were ideal to use to control for the presence of extra cells, without the release of potential paracrine effectors.

The co-culture was incubated in humidified air at 37°C in 5% CO_2 for 24 hours before MEA recordings were repeated as described above.

Figure 2.9: Slice co-culture set up

The fibroblasts were seeded on the base of a plate and the slice was suspended in a Transwell so that the slice is at the interface of the culture medium and air.

2.5.3. **Analysis**

The data was analysed using MC_Rack, MatLab with MEATools (University of Freiburg, Germany), MC_DataTool (Multi Channel Systems), Clampfit (Molecular Devices, USA) and Microsoft Excel (Microsoft, USA) as described below.

Using MC_Rack a trigger was added to each trace. This aligned each field potential according to the stimulation artefact, which allowed the averaging of traces from the same electrode over the 20 seconds of recording.

2.5.3.1. **Conduction velocity**

The data was opened using MEATools within Matlab, and Extract LFP Features was used to determine the time from the stimulation artefact to the minimum peak of the field potential for each electrode recording. This was then visually checked to ensure accurate acquisition, and the values were put into an Microsoft Excel matrix which arranged them back into the 60 electrode configuration. The local conduction velocity, the velocity at which the field potential travels between electrodes 2 electrodes apart for each electrode, was calculated using an Excel template (provided by Dr Cesare Terracciano). This figure was calculated for each of the four stimulation points for each slice, and the maximum value was considered as the longitudinal conduction velocity and the minimum value was taken as the transverse conduction velocity.

2.5.3.2. **Field potential duration**

Using MC_DataTool, recordings from 16 electrodes were converted into an .abf file for use with Clampfit (Molecular Devices, USA). The recording that related to the longitudinal conduction velocity was used for each slice, and the 16 electrodes used were predetermined and constant as shown in Figure 2.9.

The .abf file produced was then opened in Clampfit and the recorded field potentials for each electrode were averaged. Then the time point of the minimum peak of the field potential and the maximal peak (either positive or negative) of the repolarisation wave were measured, and the time between the two was calculated. The recordings of the 16 points were averaged to give the FPD of the slice, and the standard deviation of the 16 points was used as a representation of FPD heterogeneity.

		1					
	2				3		
			4			5	
		6		7	8		
9	10		11				
				12		13	14
		15					
		16					

Figure 2.10: The predetermined 16 electrodes used for the measurement of field potential duration

2.6. Immunofluorescence

Immunofluorescence was used to characterise and quantify cells and was conducted using both isolated cells and tissue sections.

2.6.1. **Cells**

To stain cells, they were plated on multispot glass slides (Hendley Essex , UK) and left for at least 2 hours to allow them to attach. The culture medium was then washed off with PBS and the cells were fixed and permeabilised with 1ml of ice cold methanol for 5 minutes. The methanol was thoroughly washed off with PBS (at least 5 times over 15 minutes) and the cells blocked with 250µl 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. During this and subsequent incubation, the slide was kept in a humidified chamber to prevent the cells from drying out. Care was taken during washing the slide to this same end. After 1 hour in 1% BSA, the primary antibody was then put on the cells in 100µl of 0.5% BSA in PBS for either 24 hours at 4°C, 4 hours at room temperature or 1 hour at 37°C. Cells were then washed with PBS (at least 5 times over 15 minutes) to remove any unbound primary antibody before the secondary antibody was added in 100µl of 0.5% BSA in PBS at room temperature for 1 hour. The primary and secondary antibodies and dilutions used are shown in Table 2.2. The secondary antibody was linked to a fluorescent probe (alexa 488) and therefore all work was carried out in low light conditions to prevent degradation. The cells were washed thoroughly again with PBS (at least 5 times over 15 minutes) to remove any unbound secondary antibody, and then any excess PBS was removed before the cells were mounted with a propidium iodide-containing mounting medium (Vectashield®, Vector, USA). Propidium iodide stains the nucleus allowing clear identification of cells. A cover slip was then placed carefully over the cells, excess solution was blotted away and the cover slip was fixed in place using nail varnish. The staining was imaged at high resolution with 16 image averaging on the confocal microscope. The alexa-488 fluorescent probe associated with the secondary antibody was excited at 488 nm using an argon laser and emission was collected through a 500-550 nm band-pass filter. The propidium iodide was excited at 453 nm using a helium neon laser and emission was collected through a 565-615 nm band-pass filter. A negative control was used for each secondary antibody. This was achieved by incubating the cells with the secondary but not the primary antibody.

Target	Primary Antibody	Dilution	Secondary antibody	Dilution
α-SMA	Monoclonal Mouse Anti Human (Dako, Denmark; Cat. No. M0851)	1:200	Goat anti mouse A488 (Molecular Probes®, Life technologies; Cat. No. A21121)	1:500
Vimentin	Monoclonal Mouse (Sigma, USA; Cat. No. 6630)	1:1000	Goat anti mouse A488	1:500
Desmin	Monoclonal Mouse Anti Human (Dako, Denmark; Cat. No. M0760)	1:50	Goat anti mouse A488	1:500
CD-31	Monoclonal Mouse Anti Human (Dako, Denmark; Cat. No. M0823)	1:40	Goat anti mouse A488	1:500
DDR-2	Polyclonal Goat Anti Human (R and D systems, USA; Cat. No. AF2538)	1:50	Donkey anti goat A488 (Molecular Probes, Life technologies; Cat. No. A-11001)	1:500

Table 2.2: Antibodies used for immunofluorescence.

2.6.2. **Tissue**

To stain sections of tissue, it was embedded in OCT and frozen. Cryosections of 15µm thickness were cut and placed on glass slides. These were washed with PBS (at least 5 times over 15 minutes). 1ml ice cold acetone was then added for 5 minutes to fix the tissue. This was washed off with PBS (at least 5 times over 15 minutes) and then the tissue was incubated with 1% BSA and 0.1% Triton X-100 in PBS for 1 hour at room temperature. After this step the cryosections were stained using the same method as with cells (except that 0.1% Triton X-100 was present in all solutions) i.e. incubation with the primary antibody, washing, incubation with the secondary antibody, washing and then mounting with propidium iodide.

The stained section was imaged using the confocal microscope. A z stack of images was recorded to allow a 3-dimensional reconstruction of the staining.

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to measure the level of mediators within the culture medium. ELISAs involve the recognition of specific antigens by an immobilised antibody (Engvall and Perlmann, 1971). A solid surface is pre coated with an antibody specific for an antigen on the target molecule, which binds and immobilises the target molecule. A second specific antibody, linked to an enzyme detection system, is then added and binds to the target. Finally a substrate is added. The enzyme reaction produces a signal, normally a colour change, and the level of signal can then be measured and is proportional to the concentration of the target molecule. A standard curve is produced using a number of known concentrations and therefore the concentration within the unknown can be determined. ELISAs have advantages over other techniques such as cytokine arrays because they give an absolute value for the concentration of mediators.

The co-culture medium was collected, centrifuged at 620*g* to remove any cellular debris, and frozen immediately at -20°C. The medium from each experimental condition was collated each day and frozen in 2 portions. When required for the ELISA it was thawed slowly on ice to prevent degradation and undiluted aliquots were used following the manufacturer's instructions. The protocol is outlined below:

- As well as preparation of the samples to be measured, standards were prepared by serial dilution of the standard provided in the kits. The positive control supplied with the ELISA kit was also prepared. For measurement of TGF-β the samples required activation with HCl.

- The standards, samples and positive control were added to the 96 well capture antibody coated plate for 2 hours. Standards were run in duplicates and samples in triplicates.

- After the wells were washed using the supplied wash buffer, the supplied horseradish peroxidase conjugated detection antibody was added to the wells and incubated for 2 hours.

- After another wash, the substrate mixture was added to the wells and incubated in the dark for 30 minutes. The horseradish peroxidase catalyses the oxidation of the substrate changing it from colourless to yellow.

- The reaction was stopped by adding HCl and the optical density of each well was measured with a µQuantTM micro-plate reader (Bio-Tek Instruments Inc., USA) at 450 nm with wavelength correction set to 570 nm. A standard curve was produced and the concentrations in the test samples were calculated from this.

2.8. SDS PAGE and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting allows for quantification of the level of specific proteins. SDS is an anionic detergent that binds to protein at a specific ratio of 1.4g of SDS per 1g of protein and therefore produces a structure that has a large negative charge proportional to the mass of the protein. The size of the electrical charge can then be used to separate the proteins, according to their size, using PAGE. Western blotting then allows the quantity of a specific protein within the sample to be detected by the appropriate selective antibodies.

2.8.1. **Sample preparation**

Tissue was snap frozen in liquid nitrogen and then stored at -80°C. The tissue was then ground into a powder in liquid nitrogen and lysed in solubilisation buffer (SB_{20}) at a ratio of 10µl per mg of tissue. The tissue lysates were vortexed and then sonicated (LSL sonicator, Heat systems-Ultrasonics, Inc) at high power for approximately 10 seconds. The solution was then centrifuged at 15000rpm for 30 minutes to remove any remaining tissue particles. The supernatant was removed from the pellet and was ready for use.

2.8.2. **Calculation of the sample protein concentration**

The concentration of protein within the samples was measured using the Pierce™ BCA protein assay kit (Thermo Scientific, USA). The kit is based on the reduction of Cu^{2+} to Cu^{+} by proteins and a colourimetric detection of Cu^{+} (Smith et al., 1985). A standard curve of known protein concentrations is produced and then the concentration of protein in unknown samples can be calculated.

The samples were diluted 1:20 with Milli-Q water. The supplied standard was serially diluted to produce a standard curve from 20-2000µg/ml as well as a blank of 0µg/ml. 25µl of sample or standard was added to a 96 well plate and 200µl of the supplied working solution was added to each well. The solutions were mixed thoroughly and then incubated at 37°C for 30 minutes after which the plate was allowed to cool to room temperature and the absorbance of each well was measured at 560nm. The reading for the blank was subtracted from all the values and then a standard curve was produced. The concentration of each sample was calculated from the standard curve.

2.8.3. **Sample preparation for SDS-PAGE**

The samples were prepared in loading buffer. This has three functions. SDS binds the protein and adds a negative charge proportional to the protein size, 2-mercaptoethanol breaks the disulphide bridges and bromophenol blue loading dye allows protein migration through the gel to be visualised. The samples were diluted in loading buffer to the desired concentration and incubated at 37°C for 30 minutes to allow the 2-mercaptoethanol to fully denature the proteins.

2.8.4. **SDS PAGE**

A two layered gel was used as first described by Laemmli (1970). The top layer of the gel is a stacking layer which concentrates the protein into a tight space so that their separation in the lower level, the separating gel, has improved resolution.

Gels were prepared using a Hoefer Mini Gel system. For the separating gel, a 12.5% acrylamide gel was prepared and poured into the Hoefer mini gel system leaving approximately 3.5cm at the top for the later addition of the stacking gel. 1ml of propan-2-ol was added to the top of the gel to produce a level top. Once the gel had polymerised the propan-2-ol was washed off with Milli-Q water and the stacking gel was poured on top with an 18-well combs inserted. The stacking gel was a 4.5% acrylamide gel. After the stacking gel had polymerised the gels was ready for use.

The gels were transferred to a Hoefer gel running apparatus (Mighty Small II SE250/SE260) which was filled with electrophoresis running buffer. 10µl of each sample was added to a well, as well as a molecular weight ladder diluted in 10µl loading buffer (Precision Plus Protein Kaleidoscope standards, Bio-Rad). The remaining wells were filled with 10µl loading buffer. Electrophoresis was run at 60V until the bromophenol blue dye reached the separating gel at which point the voltage was increased to 180V. Electrophoresis was stopped when the dye was 0.5cm from the end of the gel.

2.8.5. **Protein transfer**

To blot for proteins, they needed to be electrophoretically transferred from the polyacrylamide gel onto polyvinylidene fluoride (PVDF) membranes (Millipore) using a wet transfer system. The PVDF membrane were cut to match the size of the gel and equilibrated in transfer buffer for 1 hour before use. The gel and membrane were sandwiched between blotting paper and nylon scouring pads in between two electrode plates (illustrated in Figure 2.10). This was placed in a transfer tank containing pre-cooled transfer buffer with a cooling block. The protein transfer was powered at 6V/cm for 1.5 hours at 4°C.

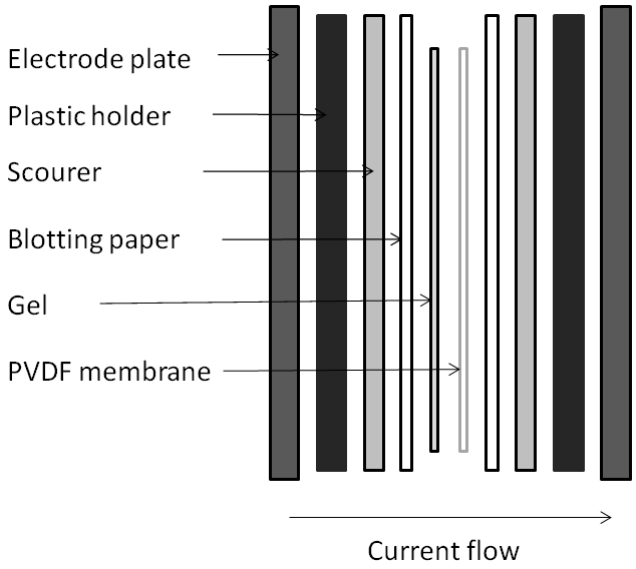


Figure 2.11: Illustration of the set up for protein transfer

2.8.6. **Protein blotting**

The PVDF membrane was removed from the transfer set up, rinsed in Milli-Q water and then incubated for 1 hour at room temperature in blocking solution. The membrane was then incubated overnight at 4°C with the primary antibody diluted in blocking solution. The membrane was then washed for 5 minutes in tris-buffered saline (TBS)-Tween buffer to remove any unbound primary antibody. This was repeated 4 times before the secondary antibody (Pierce, USA), diluted in blocking solution, was added to the membranes and incubated at room temperature for 1 hour. The membrane was washed again with TBS-Tween 4 times for 5 minutes each and then TBS (20mM tris, 150mM sodium chloride; pH 7.6) 4 times for 5 minutes.

Alkaline phosphatase buffer containing 165µg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Promega, USA) and 330µg/ml nitro-blue-tetrazolium (Promega, USA) was used to develop the membrane. The membrane was covered in the solution for 30 minutes at room temperature. This was then washed off with Milli Q water and the membrane was left to dry before it was imaged using a GENE Genius imager and Genesnap software (Syngene, UK). The optical density of the bands was then measured using inbuilt analysis routines of ImageJ software.

2.9. Statistical analysis

Data are represented as mean ± standard error of the mean. Statistical analysis was carried out using GraphPad Prism 4 software (GraphPad Software Inc., USA). Analysis of two data sets was performed by unpaired t-tests, and for before and after measurements paired t-tests were used. When comparing multiple groups of data a Kruskall-Wallis test with Dunn's post test analysis was performed.

$P<0.05$ was taken as significant and on graphical representation of data, one star indicates $p<0.05$, two stars indicates $p<0.01$ and three stars indicates $p<0.001$.

2.10. Solutions

The following solutions were used throughout this work. They are described in mM unless stated otherwise. All chemicals were provided by VWR international or Sigma Aldrich. The solutions were made up in Milli-Q water except low Ca^{2+} solution and enzyme solution which used AnalaR water. Fresh solution was made when required and used for a maximum of 2 weeks.

Normal Tyrode (NT): 140 NaCl, 4.5 KCl, 10 glucose, 10 HEPES (free acid), 1 $MgCl_2$, 1 $CaCl_2$; pH 7.4

Low Ca^{2+} solution (LC): 120 NaCl, 5.4 KCl, 5 $MgSO_4$, 5 Na^+ pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid), 5 nitrilotriacetic acid, 0.04 $CaCl_2$; pH 6.96

Buffer A: 120 NaCl, 5.4 KCl, 5 $MgSO_4$, 5 Na^+ pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid), and 0.2 $CaCl_2$; pH 7.4

Slicing solution: 140 NaCl, 6 KCl, 10 glucose, 10 HEPES (free acid), 1 $MgCl_2$, 1.8 $CaCl_2$, 10 2,3-butanedione monoxime; pH 7.4

Recording solution: 140 NaCl, 4.5 KCl, 10 glucose, 10 HEPES (free acid), 1 $MgCl_2$, 1.8 $CaCl_2$; pH 7.4

Cardioplegia (Plegivex, Ivex Pharmaceuticals, UK): 147 NaCl, 16 KCl, 16 $MgCl_2$, 1.2 $CaCl_2$, 1 Procaine hydrochloride; pH 7.8

Solubilisation buffer (SB_{20}): 20% SDS w/v, 0.1M Tris, 10mM ethylenediaminetetraacetic acid (EDTA); pH 6.8

Loading buffer: 96.5% SB_{20}, 2.5% 2-mercaptoethanol, 1% bromophenol blue

Electrophoresis running buffer: 0.192mM glycine, 25mM trizma base, 0.1% SDS.

Transfer buffer: 192mM glycine, 25mM trizma base, 10% methanol, 0.01% SDS

Blocking solution: 20mM tris, 150mM sodium chloride, 0.1% tween20, 4% non fat dried milk; pH 7.6

Tris-buffered saline Tween (TBS-Tween): 20mM tris, 150mM sodium chloride, 0.1% tween20; pH 7.6

Tris-buffered saline (TBS): 20mM tris, 150mM sodium chloride; pH 7.6

Alkaline phosphatase buffer: 100mM tris, 100mM sodium chloride, 5mM magnesium chloride; pH 9.5

Below is the make up of the culture medium used throughout these experiments. All products were supplied by Gibco® (Life technologies, USA) or Sigma Aldrich. Fresh culture medium was made in 50ml batches when required.

Fibroblast culture medium (FB medium): 82% DMEM, 15% foetal bovine serum, 100U/ml penicillin, 100µmol/l streptomycin, 4mM L-alanyl-L-glutamine

Cell co-culture medium (ITS medium): M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 1:1,000 dilution ITS supplement (PAA, Austria; Cat. No. F01-015)

Slice co-culture medium (H.ITS medium): M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 10µg/ml insulin, 5.5µg/ml apo-transferrin, 0.005µg/ml Na^+ selenite.

CHAPTER 3.

Optimisation of culture

3.1. Introduction

The experiments conducted in this thesis involved the preparation and culture of three separate preparations, namely isolated fibroblasts, isolated myocytes and myocardial slices, and it was necessary to optimise these processes.

3.1.1. **Fibroblasts**

It was necessary to optimise the isolation of fibroblasts to provide the largest immediate yield, and to avoid contamination with other cell types. It was also important to track the development of α-SMA in fibroblasts in culture.

A reliable way of producing cardiac fibroblasts is by using an outgrowth method (Etzion et al., 2002, Messina et al., 2004). This involves plating small pieces of tissue (approximately $2mm^2$) in culture, and after a few days fibroblasts start to grow out of the tissue chunks. However this takes a number of days to provide usable fibroblasts. Alternatively, fibroblasts can be isolated by enzymatically digesting the tissue and collecting the cells from the solution by centrifugation to provide an immediate yield of cells. Enzymatic digestion of cardiac tissue can be achieved in many different ways, for example using trypsin and/or collagenase type II (Hafizi et al., 1998, Vasquez et al., 2010). Our lab had experience using enzymatic digestion of cardiac tissue for the isolation of myocytes using a Langendorff set up (Siedlecka et al., 2008) and also from human tissue using a protocol that shakes the tissue with protease and collagenase (Terracciano et al., 2003). It is then possible to collect fibroblasts by centrifuging the supernatant following these protocols.

Once isolated, it was necessary to check the purity of the cell population. This was essential in order to attribute any observed effects of co-culture to the fibroblasts, rather than as a result of contaminating cells. Also, although fibroblasts can be readily cultured, culture is known to alter their characteristics (as discussed in Section 1.3.3.). α-SMA is used as a marker for these changes and therefore it was necessary to track the time frame of these changes by characterising the development of α-SMA in order to direct the time course of future experiments.

3.1.2. **Myocytes**

The isolation of myocytes is well established within our lab (Siedlecka et al., 2008), and although the culture of myocytes has been regularly conducted (Lee et al., 2008) various considerations were necessary for this work. Previously, a culture medium containing foetal bovine serum had been used. However, serum contains high and inconsistent levels of various biologically active molecules (Honn et al., 1975) and some foetal bovine serums contain TGF-β (Zheng et al., 2006). The presence of TGF-β in the culture medium may mask the release of TGF-β from fibroblasts. Optimisation studies on culture medium for adult myocytes have identified possible alternatives that allow better control over the contents of the culture medium which help to maintain the myocyte phenotype (Volz et al., 1991, Viero et al., 2008). It was therefore necessary to identify an ideal medium, based on these previous reports, for the co-culture experiments.

3.1.3. **Myocardial slices**

The preparation of thin slices of viable tissue has been widely used in brain research (Gahwiler et al., 1997). The use of the technique to study the heart has developed more slowly, but the preparation of viable myocardial slices has now been described in detail (Bussek et al., 2009). The preparation of slices is also well established within our lab (Camelliti et al., 2011). However, the culture of slices was not established and therefore needed developing.

3.1.4. **Chapter aims**

- Fibroblasts:
  - Identify an optimal technique for isolation
  - Characterise the cell type
  - Characterise the time course of α-SMA development in culture
- Myocytes:
  - Identify a suitable serum free medium
  - Characterise the effects of culture on the myocytes phenotype

- Myocardial slices:
  - Identify a culture set up for slices
  - Characterise the effects of culture on the electrical activity of myocardial slices

3.2. Methods

3.2.1. **Fibroblast isolation and cell counting**

Three protocols for the isolation of fibroblasts were tested to identify a method to provide the maximal yield of fibroblasts. 0.5g of rat ventricular tissue was used for each isolation procedure.

1. Isolation from the supernatant after myocyte isolation using the Langendorff set up (the isolation of myocytes is described in Section 2.3.2.). Once the heart was removed from the Langendorff, the ventricular tissue was weighed and 0.5g was used for the rest of the protocol.
The supernatant removed from the myocyte pellet was re-centrifuged at 620*g* for 5 minutes to collect the fibroblasts, which were washed in sterile PBS and then put into culture with FB medium.

2. Isolation using digestion with protease type XXIV and collagenase type V as described under fibroblast isolation in Section 2.3.3.

3. Isolation using collagenase type II.
The tissue was prepared by perfusing the heart with LC for 10 minutes and then cutting the LV into 2mm^3 pieces as in Protocol 2. These pieces were then digested with 500units/ml collagenase type II in 10ml of Buffer A for two 1-hour periods. Fibroblasts were collected from the solution as described for Protocol 1.

To assess the efficacy of each method, the cells were counted after 48 hours in culture using an Improved Neubauer Haemocytometer. The culture medium was removed,

and the culture flask was washed with PBS. 2ml of 0.25% Trypsin-EDTA was added and the flask was left at 37°C for 5 minutes. 5ml of FB medium was then added to inactivate the trypsin and the solution was centrifuged at 620*g*. The supernatant was carefully removed and the cell pellet was re-suspended in 3ml of FB medium. 80µl of the cell suspension was taken and mixed with 20µl 0.4% trypan blue solution. This marked the dead cells and therefore allowed identification of the living cells. 10µl of this mixture was added to the Haemocytometer and viewed using 20 times magnification for counting. The number of cells per ml was calculated. Three cell counts were made for each isolation and the average was recorded as the number of cells per ml from the isolation. As the cells were re-suspended in 3ml this was multiplied by three to give the total number of cells from the isolation.

3.2.2. **Immunofluorescent characterisation of fibroblasts**

To examine the purity of the isolated cell population, the cells were stained on Day 3 after isolation for vimentin, DDR2 and desmin. Vimentin is used to mark cells of a mesenchymal origin. Most vimentin-positive cells within the heart are fibroblasts, although endothelial and smooth muscle cells also express vimentin (Camelliti et al., 2005). DDR2 is a putative fibroblast specific marker, although it may stain smooth muscle cells and therefore desmin was also used to exclude the presence of smooth muscle cells (Rohr, 2011).

To characterise the effect of culture on the rat fibroblasts, they were stained on Day 1, 3, 5 and 7 after isolation for α-SMA. The final experimental protocol involved 24 hours in FB medium and then 24 hours in ITS medium, and therefore fibroblasts were also stained for α-SMA after exposure to these conditions.

Cells from three isolations were stained for each marker. The protocol for immunofluorescence is described in Section 2.6.1.

3.2.3. **Testing different myocyte culture media**

To test the suitability of different culture media for the co-culture set up, Ca^{2+} transients were recorded from freshly isolated myocytes and from myocytes cultured in different media for 24 hours.

The culture media tested were:

1. Neonatal Rat Ventricular Myocyte medium (NRVM): 67% DMEM, 16% medium-199, 10% horse serum, 4% FBS, 2% 1 M HEPES and 1% penicillin/streptomycin.

2. ITS medium: M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 1:1,000 dilution ITS supplement (PAA, Austria; Cat. No. F01-015).

3. Volz medium: M199 (without l-glutamine), 0.2% (weight/volume) bovine serum albumin, 100mM ascorbate, 5mM creatine, 5mM taurine, 2mM carnitine, 0.1µM insulin, 100U/ml penicillin, 100µmol/l streptomycin.

To record Ca^{2+} transients in freshly isolated myocytes they were loaded with Fluo-4 in solution and then plated on laminin coated glass bottom dishes for recording. Myocytes were suspended in 1ml of Buffer A containing 10mM Fluo-4-AM for 10 minutes. After 10 minutes the myocytes had settled on the base of the test tube and the solution was removed and replaced with 1ml of Buffer A. The cells were left for 20 minutes to allow the de-esterification of the Fluo-4-AM before a couple of drops were placed on a laminin coated glass bottom dish. The cells were left for 5 minutes to adhere to the dish and then visualised and recorded in the same fashion as for cultured cells.

To record Ca^{2+} transients after 24 hours in culture, freshly isolated myocytes were plated at 5,000 cells per dish on laminin-coated glass bottom dishes and 2ml of culture medium was added. After 24 hours in culture, the Ca^{2+} transients were recorded as described in Section 2.4.4.

3.2.4. **Effects of culture on myocyte volume**

Three-dimensional reconstruction of di-8-ANEPPS staining of freshly isolated myocytes and myocytes after 24 hours in culture was used to assess the effects of culture on cell volume.

To stain freshly isolated myocytes, the cells were suspended in 10µM di-8-ANEPPS in 1ml of Buffer A for 10 minutes, which was then replaced with fresh Buffer A to remove the dye. Two drops of cells were then put on laminin coated glass bottom dishes and left to attach for 5 minutes before use. The cells were visualised and recorded in the same fashion as for cultured cells as described in Section 2.4.3.

3.2.5. **Myocardial slice culture set up**

The culture of myocardial slices was attempted in 4 different configurations using either ITS medium or H.ITS medium (formulations below) (Table 3.1). After 24 hours in culture, the ability to record an electrical signal from the slice using the MEA set up was used as a marker for viability.

ITS medium: M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 1:1,000 dilution ITS supplement.

H.ITS medium: M-199 (without l-glutamine), 100U/ml penicillin, 100µmol/l streptomycin, 10µg/ml insulin, 5.5µg/ml apo-transferrin, 0.005µg/ml Na^+ selenite.

3.2.6. **The effect of culture on myocardial slice electrophysiology**

The electrical activity of slices was recorded in freshly prepared slices and then after 24 hours in culture using the MEA system. The MEA recording allowed analysis of the longitudinal and transverse conduction velocity, the field potential duration and the variation in the field potential duration.

3.3. Results

3.3.1. **Isolation with protease and collagenase provide the greatest yield of cells**

To optimise the number of cells collected from each isolation, three protocols were tested (Figure 3.1). The number of cells produced by digestion with protease XXIV and collagenase type V was significantly greater than the number of cells produced when using collagenase type II alone.

The isolation of cells using the Langendorff set up protocol did not produce a significantly different number of cells than using protease and collagenase, but appeared to produce a more variable yield of cells. Although two of the isolations produced a similar yield to using protease and collagenase, the other two isolations produced considerably fewer cells.

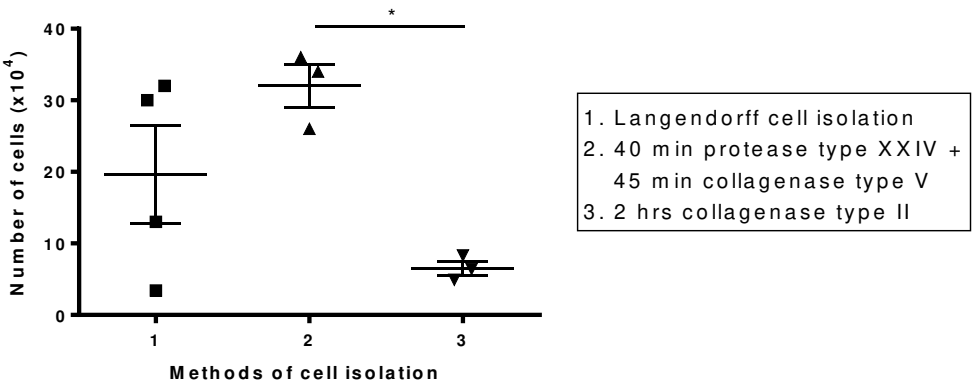


Figure 3.1: The yield of cells produced by different isolation procedures

The number of cells 48 hours after isolation. Protocol 1 n=4; protocols 2 and 3 n=3;

3.3.2. **The isolated cells were fibroblasts**

Immunofluorescence of isolated cells three days after isolation showed that all the cells were vimentin positive (111/111 cells) and DDR2 positive (132/132) (Figure 3.2). The cells were also stained for desmin due to a concern that DDR2 might also stain smooth muscle cells. All cells were negative for desmin (197/197 cells) supporting the identification of the cells as fibroblasts.

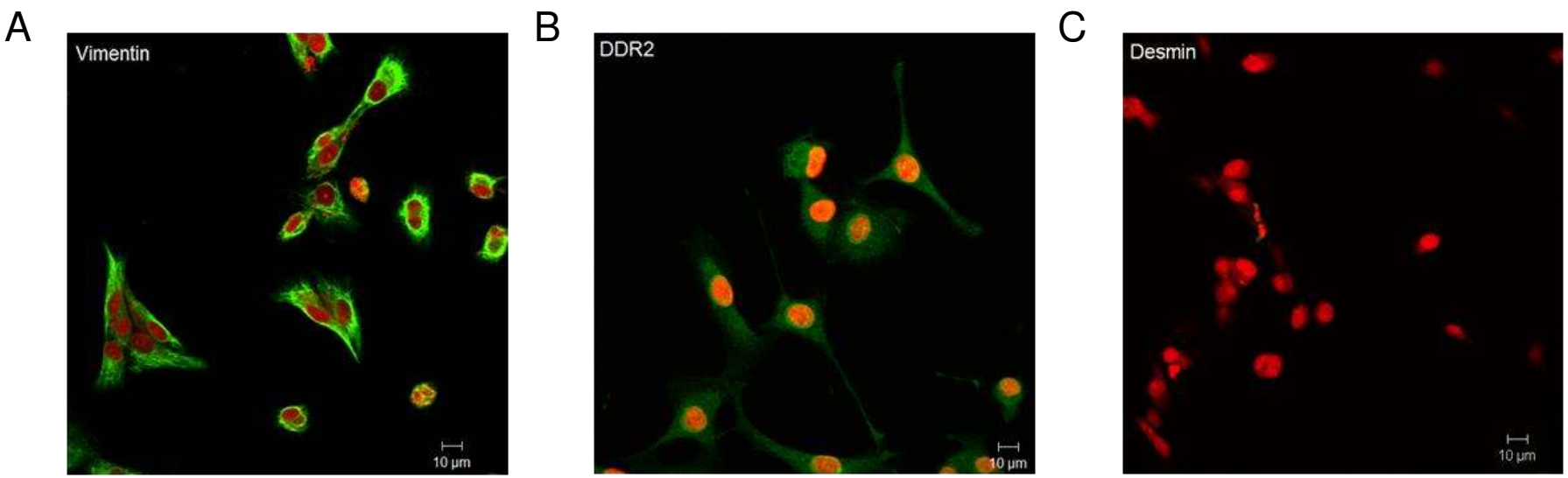


Figure 3.2: Immunofluorescence of isolated rat cells

A) Isolated cells stained positive for vimentin, a marker of mesenchymal cells. B) The cells were positive for discoidin domain receptor 2. C) Staining for desmin was negative. This is a characteristic staining pattern of fibroblasts. Green: Target protein. Red: Propidium iodide nuclear marker.

3.3.3. **α smooth muscle actin developed in culture**

To characterise the time frame of changes in fibroblast phenotype in culture, the isolated cells were stained for α-SMA at different time points of culture (Figure 3.3). At Day 1 the cells were α-SMA negative (0/47 cells). At Day 3 a minority of cells showed a low level of α-SMA expression (12/144 cells). At Day 5 α-SMA was expressed in the majority of cells (177/197 cells). However, the incorporation of α-SMA into the stress fibres was not evident in all the cells at Day 5. By Day 7, almost all the cells imaged were α-SMA positive and showed a highly organised expression with clear stress fibre staining of α-SMA (122/124 cells). The final experimental protocol involved keeping fibroblasts for 24 hours in FB medium and then 24 hours in ITS medium. At this time point the fibroblasts were α-SMA negative (0/95 cells).

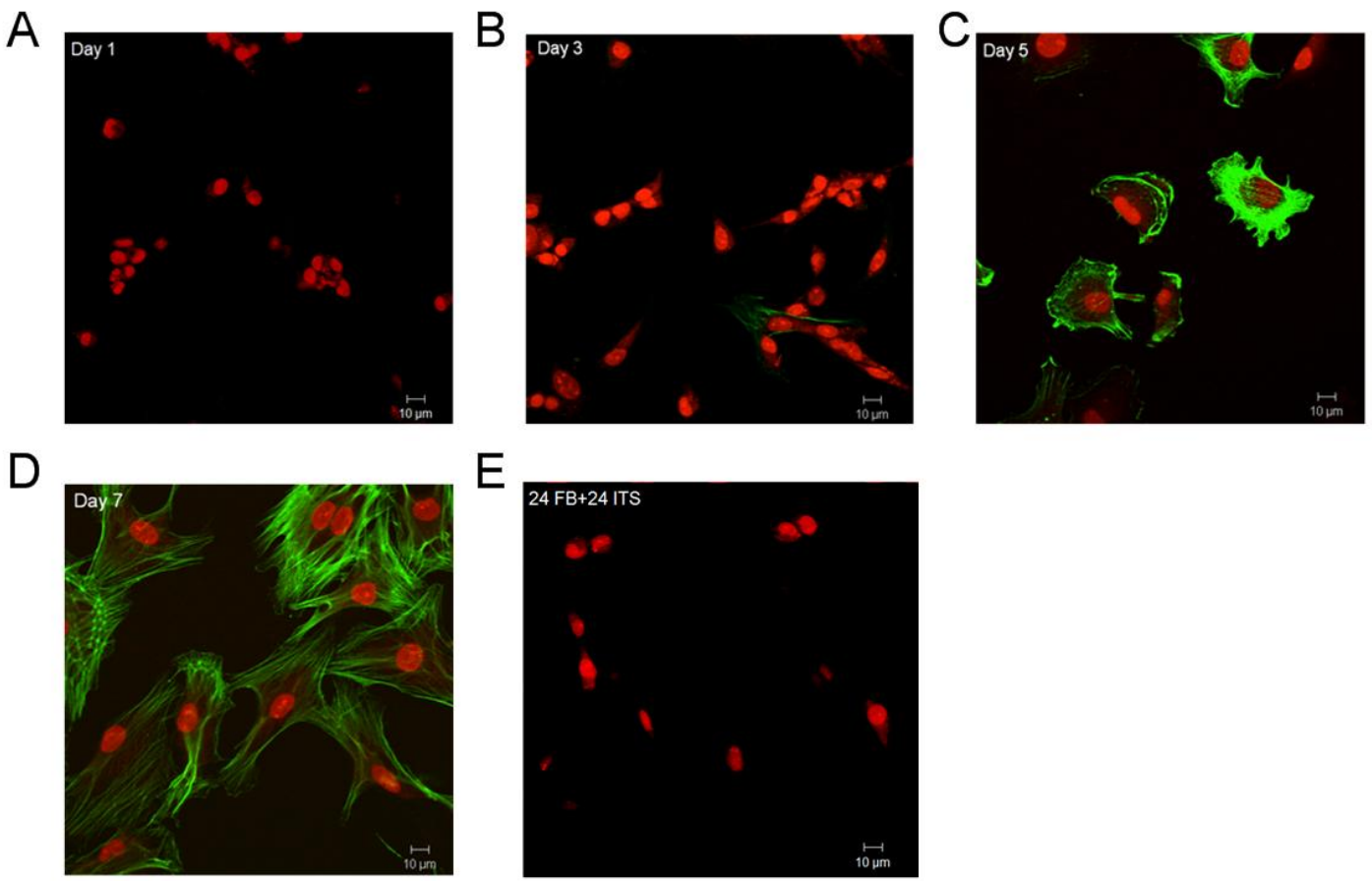


Figure 3.3: The development of α-SMA in culture

The development of α-SMA was tracked over time in culture in FB medium. A) At Day 1 α-SMA was not present in any cells. B) By Day 3, α-SMA was present in 8% of cells. C) At Day 5, α-SMA was seen in 90% of cells imaged and D) by Day 7 it was well developed into stress fibres and present in 98% of cells. E) When exposed to the experimental protocol of 24 hours in FB medium and 24 hours in ITS medium the cells were α-SMA negative. Green: α-SMA. Red: Propidium iodide nuclear marker.

3.3.4. **ITS medium provides a serum free medium**

It was important to identify a serum free medium to reduce myocyte de-differentiation and allow insight into possible paracrine mediators. Previously NRVM medium was used in the lab which is serum supplemented. Ca^{2+} transients were examined after culture in different culture media and compared to parameters measured in fresh cells (Figure 3.4). Transient amplitude was unchanged by culture or the culture medium used. Time to peak was also not significantly changed although it appeared to be more variable after culture. The interquartile range for Day 0 was 12.46 whereas for NRVM, ITS and Volz it was 16.46, 23.61 and 25.58, respectively. The decay of the Ca^{2+} transient was affected by the culture medium used. Time to 50% decay was faster with NRVM or ITS medium but unaffected by culture in Volz medium compared to Day 0. Time to 90% decay was prolonged with Volz medium but unaffected by NRVM or ITS medium.

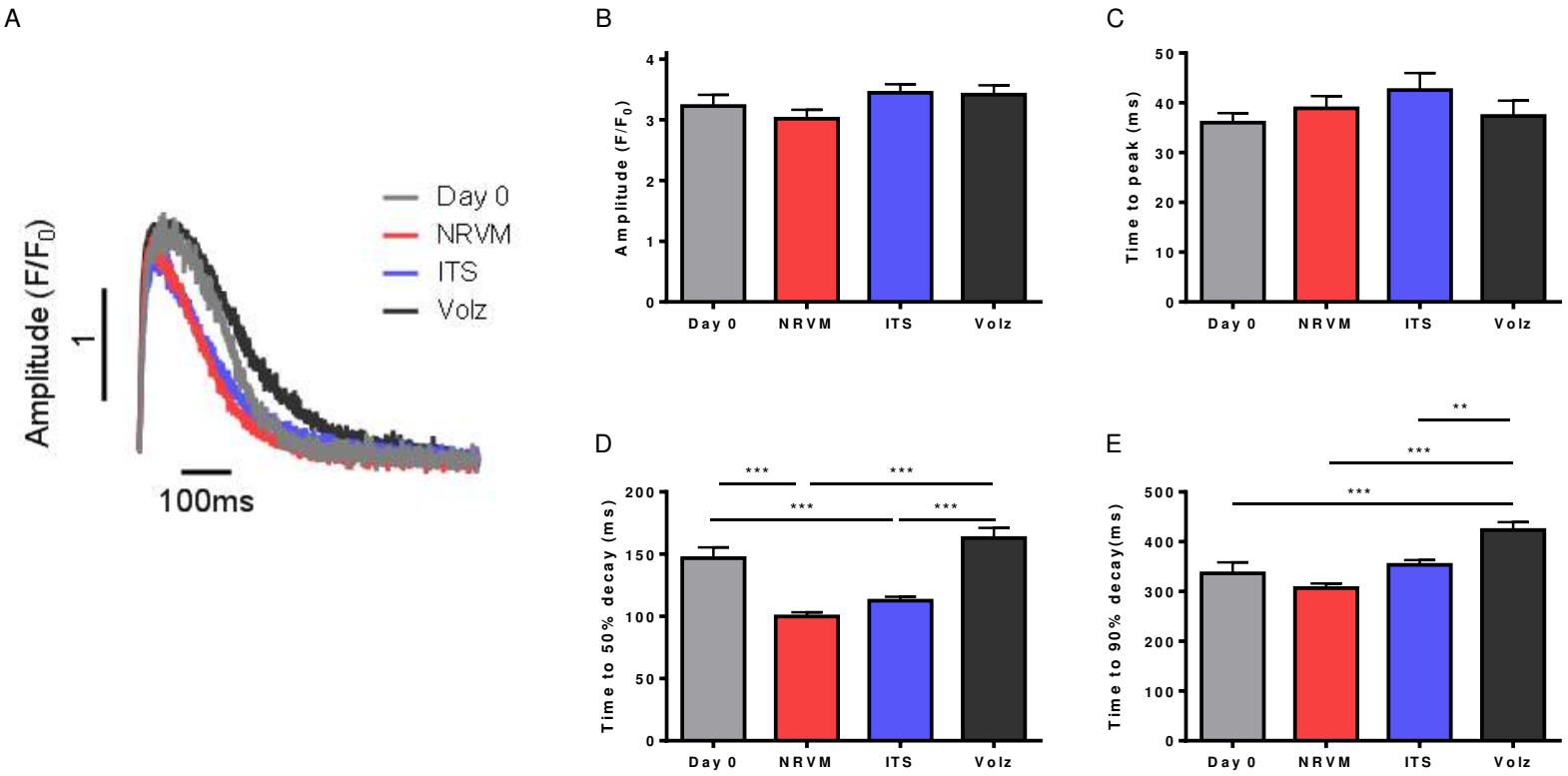


Figure 3.4: Ca^{2+} transient properties of fresh myocytes and after 24hour culture in different culture medium

A) Representative traces of recorded Ca^{2+} transients. B) Ca^{2+} transient amplitude and C) Time to peak were not affected by the different culture media. D) Time to 50% decay was faster in NRVM and ITS medium compared to Day 0 and Volz medium. E) Time to 90% decay was prolonged with Volz medium but not changed with NRVM or ITS medium compared to Day 0. Day 0 n=27; NRVM n=30; ITS n=28; Volz n=25.

3.3.5. **Myocyte volume is unchanged but Ca^{2+} handling is altered after 24 hour culture**

To understand the effects of culture on myocytes, cell volume, Ca^{2+} transients and Ca^{2+} sparks were compared between fresh cells and cells cultured for 24 hours in ITS medium. Culture of myocytes did not affect volume, which was unchanged between freshly isolated cells and cells cultured for 24 hours (Figure 3.5).

Ca^{2+} handling was affected by culture. Figure 3.4 shows that the time course of the Ca^{2+} transient was affected. Time to 50% decay of the transient was shortened in myocytes cultured in ITS medium. Also, Ca^{2+} sparks were altered by culture, and only seen in cultured cells very infrequently. Only 6 out of 50 cells recorded showed any Ca^{2+} sparks (12%) whereas, using historical data from our lab (courtesy of Dr Michael Ibrahim), sparks were seen in 42% of freshly isolated cells (100/173).

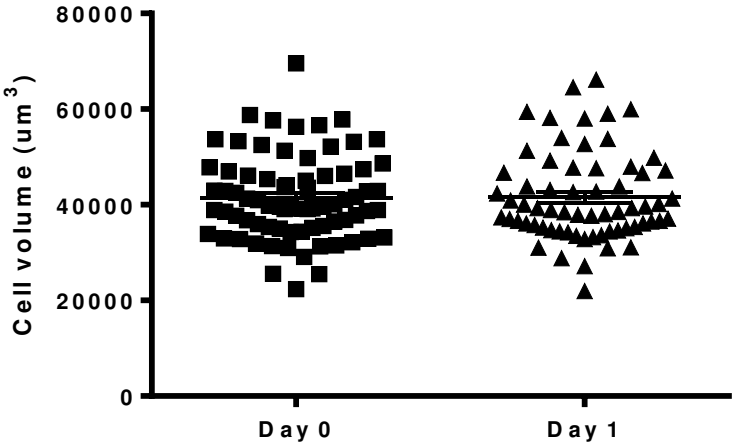


Figure 3.5: Culture for 24 hours did not affect myocyte volume

Myocyte volume was not different between freshly isolated cells (Day 0) and cells cultured in ITS medium for 24 hours (Day 1). Day 0 n=75; Day 1 n=65.

3.3.6. **Myocardial slice culture**

In order to identify a methodology to culture myocardial slices, four different set ups were tested and in two of the set ups two different media were also tested (Table 3.1).

Initially slices were cultured free floating in medium. In ITS medium these slices did not survive. In H.ITS medium 50% of slices had recordable electrical activity. On visual inspection the slices had curled up and looked hyper-contracted. Therefore two methods were tested to maintain the structure of the slices. The slices were held in place on an upturned cell strainer either using surgical glue or a mesh. The perceived advantage of these techniques where the slice was fully submerged was that it would allow continual field stimulation of the slices in culture in future experiments, which has previously been shown to help preserve myocytes in culture (Berger et al., 1994). However the slices were not viable after culture in these set ups with H.ITS medium. The final set up tested was culturing the slices at the interface of the culture medium and air, as used in a previous study (Brandenburger et al., 2012). This was the most successful set up with 50% viability using ITS medium and 100% viability using H.ITS medium.

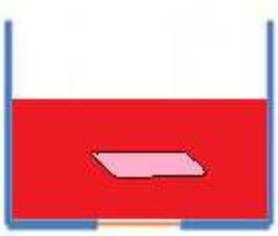
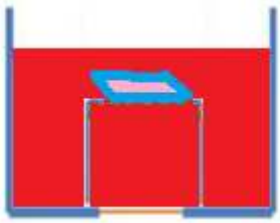
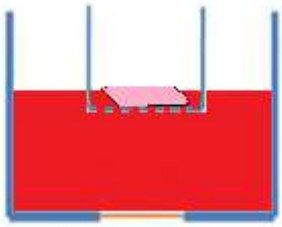
		Survival	
Set up	Diagram	ITS medium	H.ITS medium
Free floating in medium		0/6	3/6
Glued to upturned cell strainer		-	0/3
Held down on upturned cell strainer with a mesh		-	0/3
At the culture medium/air interface		2/4	8/8

Table 3.1: Culture set ups tested for myocardial slices and relative viability of the slices at 24 hours. Viability was quantified as the ability to record an electrical signal from the slice using a MEA and is shown as viable number/total number.

3.3.7. **Myocardial slice electrophysiology is affected by culture**

The effect of culturing slices for 24 hours at the interface of H.ITS medium and air was assessed using the MEA. The field potential of slices cultured for 24 hours was longer compared to freshly prepared slices (Figure 3.6). This effect appeared to be homogeneous as the variation in the length of the field potential within each slice was unchanged by culture. The longitudinal conduction velocity was increased after culture, but there was no effect on the transverse conduction velocity (Figure 3.7).

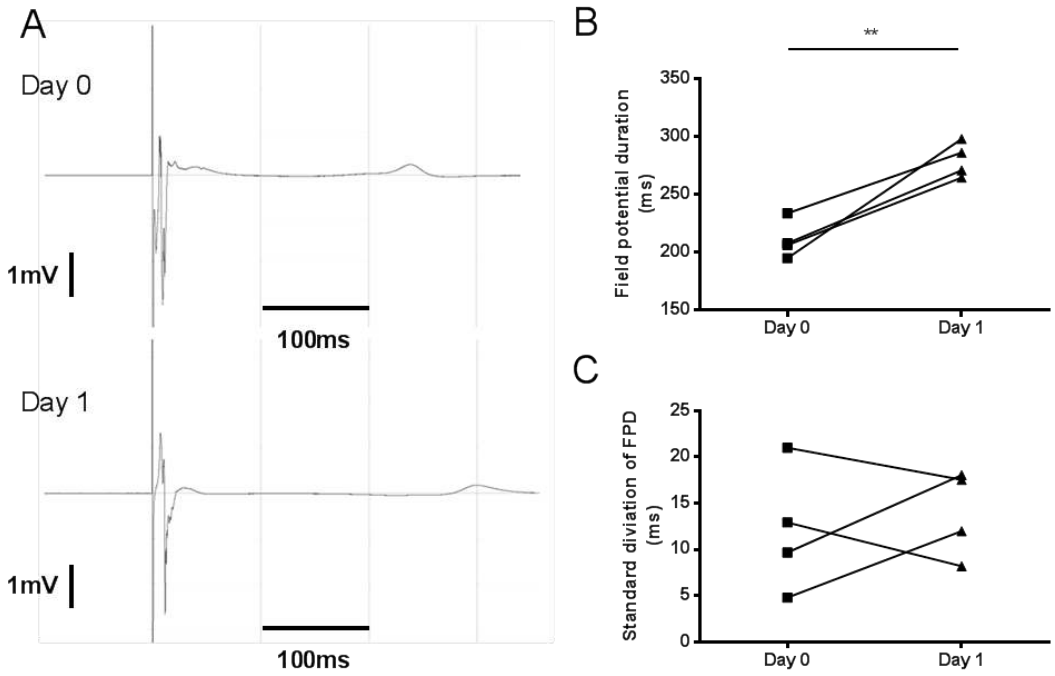


Figure 3.6: The field potential duration of myocardial slices was longer after 24 hours culture

A) Representative recordings of the field potential at a single electrode of the MEA. B) Field potential duration was longer after culture, but C) the standard deviation of the field potential duration between the 16 analysed electrodes from each slice was not altered. n=4.

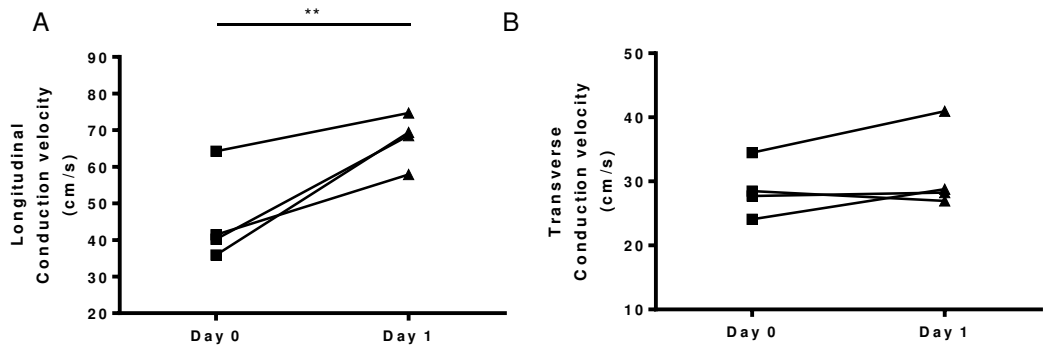


Figure 3.7: Longitudinal conduction velocity was increased after 24 hours in culture

A) The longitudinal conduction velocity of myocardial slices was increased after 24 hours in culture, but B) the transverse conduction velocity was not changed. n=4.

3.4. Discussion

The results presented in this chapter are essential to the planning and understanding of the work presented in the rest of this thesis.

3.4.1. **Fibroblasts**

These results show that a pure population of fibroblasts can be reliably isolated from ventricular tissue of the rat. The protocol used for the isolation of cardiac fibroblasts varies between published studies and therefore different protocols were tested. The identification of a reliable high yield method for the isolation of cells was important in allowing the use of fibroblasts at an early time point of culture. The rat co-culture set ups used for the following experiments required 10,000 fibroblasts per set up and therefore the availability of approximately 300,000 fibroblasts at 48 hours post isolation showed that it would be possible to produce multiple co-culture set ups from each isolation at an early time point.

The identification of the purity of the cells once isolated and put into culture was also important. The cells stained positive for vimentin and DDR2 but were negative for desmin. This staining profile supports the conclusion that these cells were fibroblasts.

Previously, a similar protocol has also been reported to produce a population of fibroblasts with only very limited potential contamination (Gustafsson and Brunton, 2000). Fibroblasts are the most numerous cells within the rat heart and also attach to the culture flask more rapidly than endothelial or smooth muscle cells. Therefore, changing the medium after plating the cells reduces potential contamination (Gustafsson and Brunton, 2000). Importantly, only a section of the total isolated cells were stained and imaged and therefore it cannot be definitively concluded that minor numbers of contaminating cells were not present. The purity of the cells was important for this work as endothelial and smooth muscle cells are also capable of releasing paracrine mediators (Pintavorn and Ballermann, 1997, Coutts et al., 2001, Justewicz et al., 2012, Yin et al., 2013). Thus, it was important that smooth muscle and endothelial cell contamination was minimal to associate any observed effects with fibroblasts.

The characterisation of α-SMA development in culture was also important in directing the design of these experiments. In the culture conditions used here, α-SMA developed in most cells by Day 5. Although α-SMA is only one marker of fibroblast differentiation *in vitro*, there is evidence that other properties of fibroblasts track these changes, making it a good marker to use. Changes in ECM protein expression occur in the same time frame as α-SMA development (Dawson et al., 2012). The development of an ATP-sensitive K^+ current also develops over the same time course as α-SMA in culture (Benamer et al., 2009). Furthermore, a study of α-SMA negative and positive cells from skin, subcutaneous tissue and lung found distinct phenotypes in fibroblasts depending on their α-SMA expression irrespective of the source of the cells (Dugina et al., 1998).

Previous reports have found differences in the time course of α-SMA development. α-SMA development in mouse ventricular fibroblasts has been reported to evolve between 5 and 9 days in culture (Benamer et al., 2009). Adapala *et al* (2013) used rat ventricular fibroblasts at passage 2-3, stating that these had not undergone culture-induced differentiation (no exact time point was given, although in our experience this takes approximately 8-12 days). In canine atrial fibroblasts, α-SMA was evident after 48 hours in culture (Dawson et al., 2012). The same group reported that rat atrial

fibroblasts developed α-SMA within 3 days of isolation (Harada et al., 2012). These differences may be explained by differences between species, which has been reported previously (Dawson et al., 2012), or differences between atrial and ventricular cells. Burstein *et al.* (2008) reported that atrial fibroblasts were more reactive than ventricular fibroblasts. The time frame identified in our set up generally fits with these previous reports.

This rapid development of α-SMA meant that the planned time frame of the experiments was kept to a minimum to limit the possible culture-induced changes in fibroblast phenotype. Due to the number of cells produced by each isolation, it was possible to complete the experiments with rat fibroblasts within 48 hours of isolation. During this time the fibroblasts were kept in FB medium for 24 hours and in ITS medium for 24 hours. At this time point α-SMA expression had not developed in the fibroblasts.

In the literature to date the amount of time fibroblasts were cultured for before use in paracrine studies is very variable. In some experiments it is reported in number of days. Fredj *et al.* (2005) used fibroblasts up to 12 days after isolation. Harada *et al.* (1997) used cells up to 5 days after isolation. Others report the number of passages providing limited information about the actual amount of time spent in culture. Shivakumar *et al.* (2008) used fibroblasts from passage 2 or 3 and Kaur et al (2013) used fibroblasts from passages 3-5. Pedrotty *et al.* (2009) allowed fibroblasts to reach confluency, then collected them with trypsin and used the cells at passage 1. LaFramboise *et al.* (2007) also allowed fibroblasts to reach confluency and then collected the medium to use as conditioned medium. Although the process of passaging fibroblasts may in itself have effects on their function, the lack of reported specific time points makes it difficult to compare different studies. Further to these variations in the reporting of the length of culture, none of these studies report whether there is expression of α-SMA in the fibroblasts at the point of use. The experiments included in this thesis may be the first to use fibroblasts at a time point before the induction of α-SMA expression by culture.

3.4.2. **Myocytes**

All aspects of myocyte excitation contraction coupling have been shown to be altered over time in culture. Action potentials progressively become more variable in culture with a loss of the notch and plateau phase (Mitcheson et al., 1996). The Ca^{2+} handling of adult myocytes reverts to that of neonatal myocytes over 7 days in culture with a shift in reliance from SR Ca^{2+} to extracellular and nuclear Ca^{2+} as the source for Ca^{2+} transients (Poindexter et al., 2001). Other parameters including cell volume, the t-tubule network and contractility also decrease progressively over 5 days in culture (Banyasz et al., 2008).

The Volz and ITS media were designed to preserve myocyte function in culture (Volz et al., 1991, Viero et al., 2008). Despite using these media there were changes in Ca^{2+} handling of cells in culture. The decline of the Ca^{2+} transient was altered by culture. In Volz medium it was prolonged whereas with ITS medium it was shortened. It was reasoned that although these were different effects they were of similar importance, and ultimately, ITS medium was selected for use because it did not contain taurine. Taurine is used in the formulation of Volz medium and is known to affect fibroblast function (Ren et al., 2008b).

Further experiments showed that Ca^{2+} sparks were also markedly reduced in cultured myocytes. Therefore, Ca^{2+} sparks were not recorded in the co-culture experiments.

3.4.3. **Slices**

The results reported in this chapter show that canine myocardial slices can be successfully cultured for the 24 hours necessary for the completion of the co-culture experiments. The most successful culture of slices was achieved by culturing the slices at the interface of air and culture medium. This corresponds with previously published reports of cardiac and brain slices culture (Stoppini et al., 1991, Brandenburger et al., 2012). However, even in this set up the electrophysiology is altered with culture with changes in the longitudinal conduction velocity and the field potential duration.

Brandenburger *et al* (2012) maintained human myocardial slices in culture for 28 days, but also found that there was rapid remodelling. 70% of cells lost their typical striations. The authors also found that only 67% of the cultured slices still showed cardiomyocytes-like action potentials, and contractility was substantially reduced. Much of the loss of contractility was seen after 1 day. The electrophysiology was more stable with changes to the upstroke velocity and amplitude of the action potential, but no change in the action potential duration, unlike the prolongation seen in our experiments. Although after 28 days the conduction velocity had fallen, the conduction velocity at an earlier time point was not reported which meant we were unable to compare this with our work. Despite the changes observed by Brandenburger *et al* (2012) and in the results reported in this chapter, the ability to maintain viable slices of adult myocardial tissue in culture provides an exciting platform for cardiovascular research.

3.4.4. **Summary**

The results included in this chapter show that a pure population of fibroblasts can be produced from the rat LV and that it is possible to use these fibroblasts before they begin to express α-SMA in culture. Also, an optimal culture medium that is serum free and produces limited changes in myocytes in culture was identified. Finally, the culture of myocardial slices has been developed, although there are changes in the electrophysiology after 24 hours in culture. The optimisation of these processes was essential for the following work investigating the paracrine effects of fibroblasts on myocyte properties.

CHAPTER 4.

The paracrine effects of fibroblasts from normal hearts on myocyte volume and Ca^{2+} transients.

4.1. Introduction

Fibroblasts within the un-diseased myocardium have been viewed as passive bystanders. However, they are constantly active cells, responsible for the maintenance and turnover of the ECM. The on-going activity of normal fibroblasts is highlighted by the fact that 5% of the cardiac extracellular matrix is replaced every day (McAnulty and Laurent, 1987). Whether fibroblasts have a role in modulating myocyte structure and function in the normal myocardium is unclear.

The baseline data of *in vivo* studies can provide an insight into the potential role of fibroblast in the normal myocardium. Fibroblasts-specific knock down of KLF-5 prevented the adaptation of the heart to pressure overload in mice, but there were no apparent differences at baseline (Takeda et al., 2010). KLF-5 expression is upregulated in pathological stimulation of fibroblasts and therefore may only be important in pathology (Manabe et al., 2002). PGF overexpression or knock out, which affected hypertrophy in response to pressure overload through fibroblast paracrine mediators, also did not affect the baseline phenotype of the heart (Accornero et al., 2011). In mice expressing the 5-HT_{2B} serotonin receptor in myocytes only, the mice appear to have slightly lower LV weights and reduced function as assessed by echo (although this was not directly analysed or discussed in the paper). Paracrine signalling from non-myocytes was important in the heart's hypertrophic response to isoproterenol and therefore it is possible it may also be involved in these potential changes in the baseline characteristics (Jaffre et al., 2009). Also, global FGF-2 deficient mice had a dilated heart at baseline (Pellieux et al., 2001). Fibroblasts are the main source of FGF-2 and these effects are possibly due to changes in ECM maintenance by fibroblasts but may also be partly due to paracrine signalling from fibroblasts to myocytes. These studies do not investigate any potential effects on individual myocytes and their function.

The *in vitro* study of fibroblasts from normal hearts is flawed by the changes induced in culture (discussed in detail in Section 1.3.3.) (Rohr, 2011). All the studies to date looking at paracrine communication *in vitro* have used fibroblasts from normal hearts

(e.g. (Harada et al., 1997, LaFramboise et al., 2007, Shivakumar et al., 2008)). However, they have all been kept in culture for prolonged periods of time. Although the changes associated with the culture of fibroblasts are not fully understood, it is clear that cultured fibroblasts have different properties from fibroblasts in the normal myocardium (Dawson et al., 2012). Therefore the results of these studies do not aid in our understanding of the role of fibroblasts under physiological conditions. The methodology used in this thesis requires the co-culture of fibroblast and myocytes for 24 hours and therefore the culture of fibroblasts could not be eliminated completely. However, the optimisation of techniques described in Chapter 3 shows that it is possible to use fibroblasts before they begin to express α-SMA, a marker of culture-induced differentiation, to investigate paracrine communication.

4.1.1. **Chapter aims**

The work described in this chapter aims to investigate the effects of isolated fibroblasts from normal hearts on isolated adult myocyte volume and Ca^{2+} handling. Fibroblasts were used within 24 hours and the experiments were completed within 48 hours of isolation to avoid culture-induced changes.

4.2. Methods

A schematic of the experimental protocol is shown in Figure 4.1.

4.2.1. **Cell isolation and co-culture set up**

Cardiac fibroblasts were isolated from the LV of adult male Lewis rats weighing 250-300g. They were kept in culture in FB medium for 20 hours before being plated at 10,000 cells per well in Transwells in 200µl of ITS medium ready for co-culture.

Myocytes were isolated from adult male Lewis rats weighing 175-225g and plated immediately on laminin-coated glass bottom dishes for co-culture at 5,000 cells per dish in 2ml of ITS medium and co-culture of fibroblasts and myocytes was set up as described in Section 2.4.1.

Fibroblasts and myocytes were each isolated from 3 hearts. Each parameter was recorded from multiple co-culture set ups per round of cell isolation.

4.2.2. **Assessment of myocyte phenotype**

The paracrine effects of the fibroblasts on the myocytes were assessed by measuring the following parameters after 24 hours of co-culture:

- Cell viability
- Cell volume
- Ca^{2+} transients
- T-tubule density

The detailed methodology for measuring these parameters is described in Section 2.4.

4.2.3. **Statistical analysis**

The data was analysed by unpaired t-test. N numbers represent the number of cells measured unless stated otherwise.

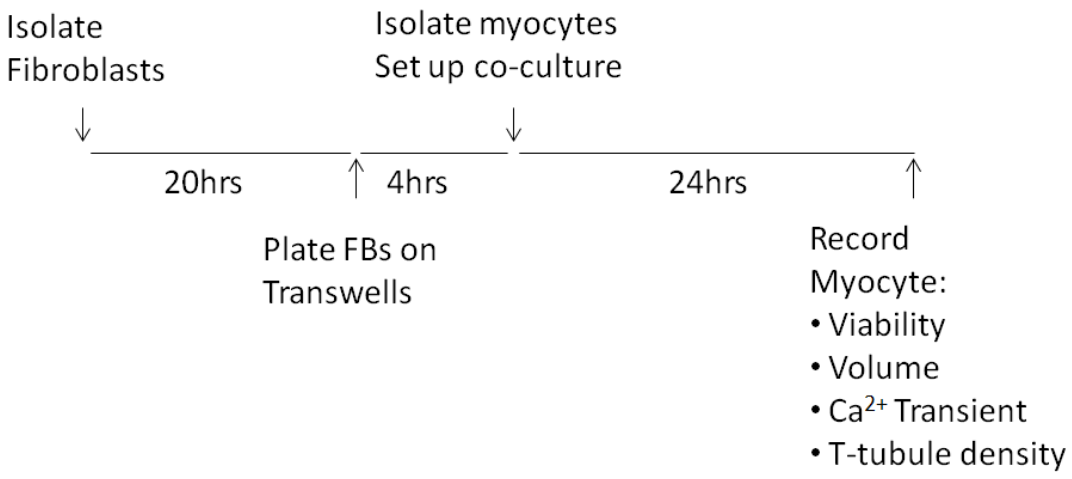


Figure 4.1: Schematic of the experimental protocol

4.3. Results

4.3.1. **Fibroblasts reduced myocyte viability**

The viability of myocytes was assessed by calculating the percentage of myocytes that retained their classic rod shape structure. Myocyte viability was reduced after co-culture with normal fibroblasts (Figure 4.2). The percentage of rod shaped cells was reduced from 64% in control to 53% in co-culture with fibroblast. There is a loss of cell viability in both groups with culture, as the percentage of viable cells at the start of culture was 84.7 ± 1.28% (n=7 set ups).

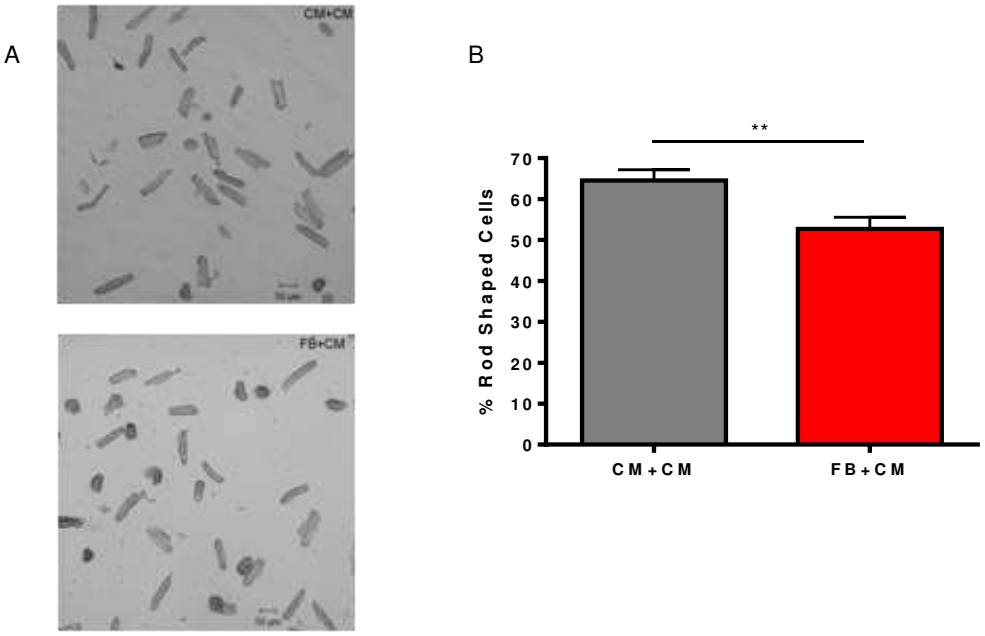


Figure 4.2: Co-culture with fibroblast reduced myocyte viability

A) Representative images used for assessing viability. B) The percentage of myocytes retaining their rod shape was reduced by co-culture with fibroblasts compared to control. CM+CM n= 8 set ups; FB+CM n=10 set ups.

4.3.2. **Fibroblasts induced myocyte hypertrophy**

Myocyte cell volume was measured using 3-dimensional reconstruction of di-8-ANEPPS staining. The volume of myocytes co-cultured with fibroblasts was increased by 8.5%

compared to control (Figure 4.3). Myocyte volume did not change in culture (as demonstrated in Section 3.3.5.). Thus the increase in cell volume induced by co-culture with fibroblasts is a marker of myocyte hypertrophy and not an effect on culture-induced volume remodelling.

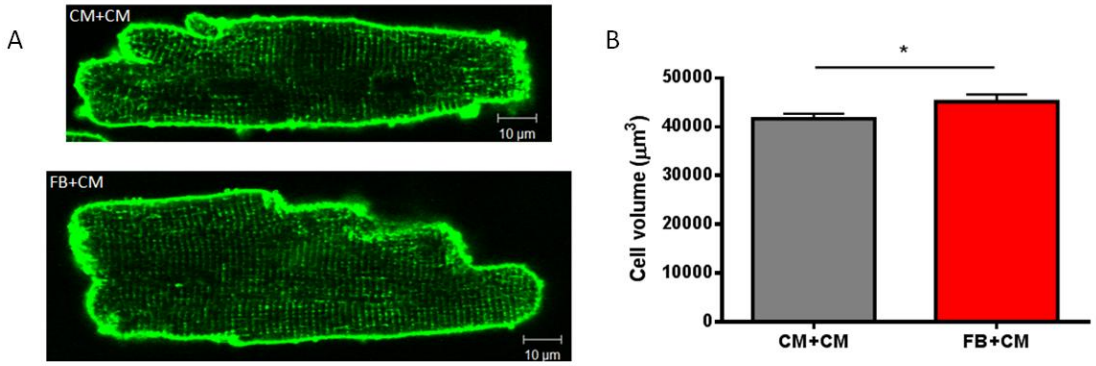


Figure 4.3: Co-culture with fibroblast led to myocyte hypertrophy

A) Representative recordings of di-8-ANEPPS staining. B) Myocyte volume was significantly increased after co-culture with fibroblasts compared to control. CM+CM n=73; FB+CM n=46.

4.3.3. **Fibroblasts increased the amplitude of the Ca^{2+} transient**

Stimulated Ca^{2+} transients were recorded from the myocytes using the Ca^{2+} sensitive dye Fluo-4. The peak amplitude of the Ca^{2+} transient was increased in myocytes co-cultured with normal fibroblasts compared to control (Figure 4.4). The time course parameters of the transient were unchanged by the presence of fibroblasts in co-culture. Time to peak, time to 50% decay and time to 90% decay were not significantly different in control myocytes and myocytes co-cultured with fibroblasts.

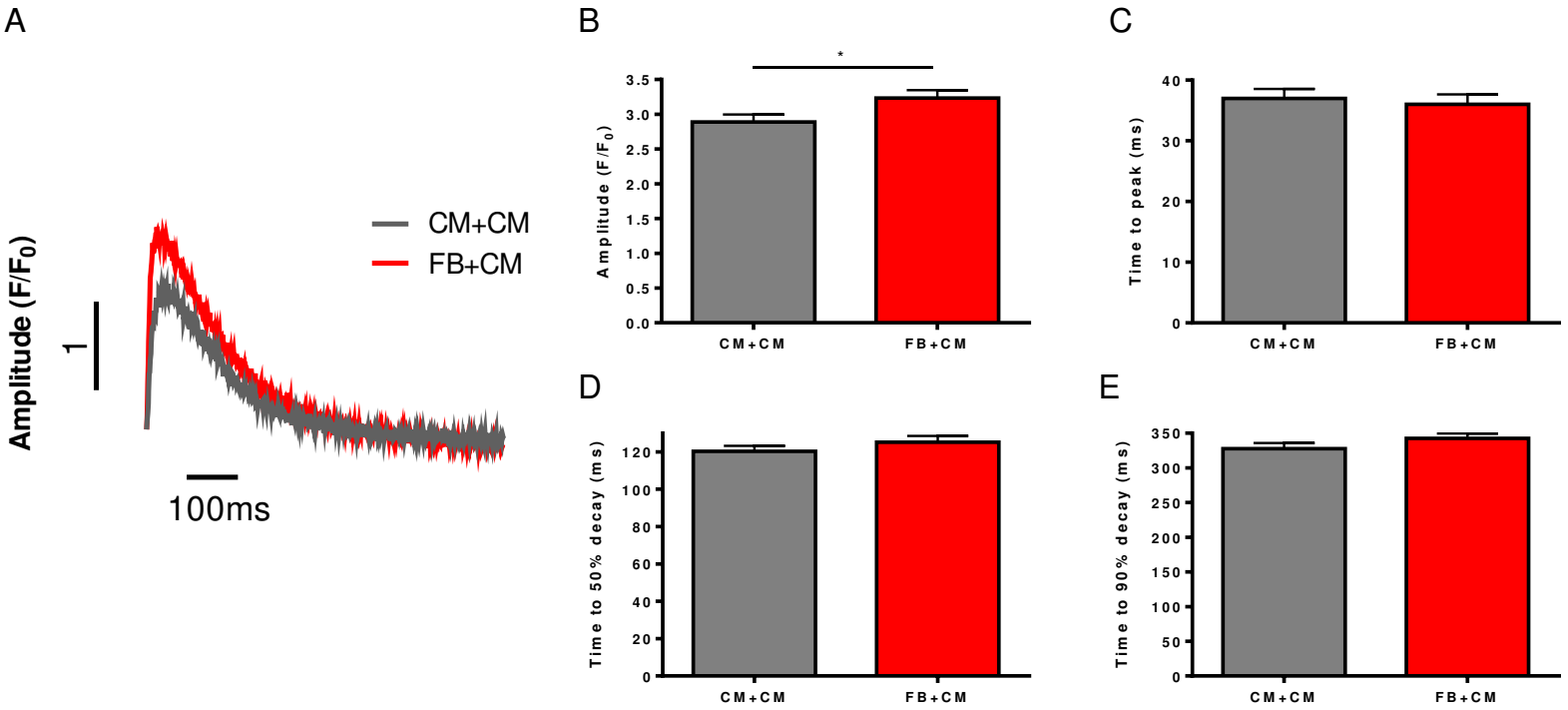


Figure 4.4: Fibroblasts increased the Ca^{2+} transient amplitude

A) Representative Ca^{2+} transients. B) Myocyte Ca^{2+} transient amplitude was increased after co-culture with normal fibroblasts compared to control. C) Time to peak, D) time to 50% decay and E) time to 90% decay were not affected by co-culture with fibroblasts. CM+CM n=39; FB+CM n=61.

4.3.4. **Fibroblasts did not affect the t-tubule density**

Di-8-ANEPPS staining also allowed the study of the t-tubule network, which is an important system in the control of Ca^{2+} handling in ventricular myocytes (Ibrahim et al., 2011). The density of the t-tubule network was represented as the % of the cellular area stained positive for di-8-ANEPPS. T-tubule density was unaffected by co-culture with fibroblasts compared to control (Figure 4.5).

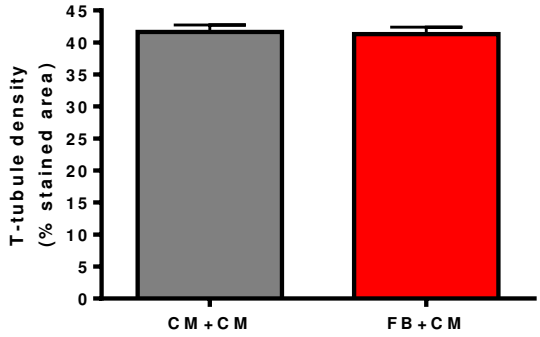


Figure 4.5: Fibroblasts did not affect the t-tubule density

T-tubule density was unaffected by co-culture with fibroblasts. CM+CM n=73; FB+CM n=46.

4.3.5. **The variance of data was unaffected by the reduced viability seen in co-culture**

The decrease in the number of viable myocytes after co-culture with fibroblasts raised the question of whether there was a selection pressure towards larger cells or cells with larger Ca^{2+} transient amplitude. That is to say, are the fibroblasts causing the selective loss of smaller cells, or cells with smaller Ca^{2+} transient amplitudes, that would result in an apparent increase in these parameters? To look into this potential confounding factor, the variance of the data was examined (Table 4.1). If the reduced viability of the myocytes was due to the selective death of smaller cells or cells with smaller Ca^{2+} transient amplitudes then a lower variance would be expected. The variance of the recordings from each day was therefore calculated to allow a statistical comparison between the two groups.

The variance of the cell volume was unchanged and the variance of the Ca^{2+} transient amplitude was increased in myocytes co-cultured with fibroblasts compared to control. This supports the conclusion that fibroblasts are inducing these changes and they are not just an effect of selective survival.

	CM+CM	FB+CM
Cell volume ($x10^7$)	7.73 ± 1.38	7.34 ± 2.05
Ca^{2+} transient amplitude	0.252 ± 0.03	0.709 ± 0.10*

Table 4.1: Variance of cell volume and Ca^{2+} transient amplitude

The variance of cell volume is unchanged and the variance of the Ca^{2+} transient amplitude is increased after co-culture with fibroblasts. n=3. * $p<0.05$ vs. control.

4.4. Discussion

The results reported in this chapter show that fibroblasts isolated from normal hearts, used before they are activated to express α-SMA by culture, exert paracrine effects on adult myocytes. Co-culture with fibroblasts reduced myocyte viability, increased myocyte volume and increased the amplitude of the Ca^{2+} transient.

4.4.1. **Viability**

The percentage of myocytes that maintained their rod shape after culture was used as a marker for viability. This was reduced after co-culture with fibroblasts compared to control. The loss of viability is conceivably due to apoptotic or necrotic cell death or uncontrolled hyper-contraction of the cells.

A previous study to look at the effects of fibroblasts on adult myocytes was focused on myocyte survival. Shivakumar *et al* (2008) exposed adult rat myocytes to conditioned medium from hypoxic and normoxic fibroblasts and after 5 hours incubation the number of dead cells was increased in the hypoxic fibroblast group. Addition of TNF-α

to the normoxic fibroblast conditioned medium mimicked this effect. This increased myocyte death was linked to an increased sensitivity to reactive oxygen species which may explain the loss of cell viability due to myocyte apoptosis. Although Shivakumar *et al.* (2008) report that changes in viability were only seen with hypoxic fibroblasts, the effect was seen in our experiments with normal fibroblasts. Fibroblasts were kept in a normal 5% CO_2 incubator without any specific O_2 control. Therefore, the culture conditions were not hypoxic and O_2 levels were close to atmospheric levels of 21%. However, in these experiments co-culture of fibroblasts and myocytes was used, whereas Shivakumar *et al* (2008) used fibroblast conditioned medium. Also we kept the co-cultures for 24 hours whereas Shivakumar *et al* (2008) only kept the myocytes in conditioned medium for 5 hours. These differences in the protocols used may explain the differences observed.

The possibility of myocyte hyper-contraction or necrosis has not been investigated. Hyper-contraction of myocytes occurs when there is loss of control of the intracellular Ca^{2+} concentration. This can be due to spontaneous entry of Ca^{2+} through ICa,L or release from the SR, or due to the reversal of NCX to pump Ca^{2+} into the cell. Alternatively, disruption of the cell surface membrane would allow uncontrolled entry of Ca^{2+}. Increased sensitivity of the myofilaments to Ca^{2+}, for example due to changes in intracellular pH, could also result in hyper-contraction (Piper and Garcia-Dorado, 1999). Paracrine mediators released from fibroblasts can depolarise the resting membrane potential which could result in opening of the ICa,L allowing Ca^{2+} entry and stimulating hyper-contraction of the cells (Vasquez et al., 2010). Necrotic cell death involves the uncontrolled rupture of the cell membrane (Piper and Garcia-Dorado, 1999). Whether this is involved in these results is not clear.

4.4.2. **Volume**

Co-culture with fibroblasts increased the cell volume of myocytes. Fibroblasts have previously been shown to induce hypertrophy by paracrine mediators in neonatal rat myocytes (Harada et al., 1999, Fredj et al., 2005a, LaFramboise et al., 2007) and also in de-differentiated adult mouse cells (Fredj et al., 2005a). Work carried out in our lab

has previously reported that cultured fibroblasts induce hypertrophy in adult rat myocytes (Cartledge et al., 2011). Osmotic swelling could also explain the increased cell size observed. However, although not studied here, the link between increased cell size and hypertrophy is supported by raised levels of atrial natriuretic peptide and brain natriuretic peptide in myocytes exposed to fibroblast conditioned medium (Harada et al., 1999).

4.4.3. **Ca^{2+} transients**

Myocyte Ca^{2+} transient amplitude was increased after co-culture with fibroblasts. The paracrine effects of fibroblasts on myocyte function are less studied than the effects on cell volume. In neonatal myocytes fibroblast conditioned medium reduced contractility (LaFramboise et al., 2007). Work carried out in our lab showed cultured fibroblasts could affect adult myocyte function in co-culture, depressing the Ca^{2+} transient and contractility (Cartledge et al., 2011). Interestingly the opposite effect on the Ca^{2+} transient was observed here. This difference may be explained by changes in fibroblast activity in culture, which has been demonstrated here by the development of α-SMA but also affects the secretory profile of fibroblasts (Dawson et al., 2012).

The basis of excitation contraction coupling has been discussed in Section 1.7. The Ca^{2+} transient is predominantly made up of Ca^{2+} release from the SR through RyRs. Therefore, the increase in the Ca^{2+} transient is likely to be due to an increase in SR Ca^{2+} release. This could be due to an increase in ICa,L which is the trigger for RyR opening and Ca^{2+} release, or due to a higher level of Ca^{2+} within the SR available for release (Hussain and Orchard, 1997, Eisner et al., 2012). Although the effect of fibroblasts on the L-type Ca^{2+} current has not been determined, it has previously been reported that fibroblast paracrine mediators prolong the action potential (Guo et al., 1999, Pedrotty et al., 2009). Prolongation of the action potential is associated with an increase in the ICa,L and therefore this may contribute to the increase Ca^{2+} transient amplitude (Wickenden et al., 1998, Hirano and Hiraoka, 2003). However, the prolongation of the action potential would also be expected to prolong the Ca^{2+} transient and this was not evident here. The level of SR Ca^{2+} is determined by the activity of SERCA which

removes Ca^{2+} from the cytoplasm into the SR, and therefore increased SERCA activity could result in increased SR Ca^{2+} content and a larger Ca^{2+} transient amplitude (Meyer and Dillmann, 1998, Miyamoto et al., 2000). However, an increase in the activity of SERCA would be expected to increase the speed of the decline of the Ca^{2+} transient. In these experiments there was no change in the decline of the Ca^{2+} transient. Further work is required to define what underlying mechanisms are responsible for the changes in the Ca^{2+} transient.

Theoretically it is challenging that myocytes isolated from a normal heart, where they are surrounded by normal fibroblasts, should be altered when cultured with normal fibroblasts. It is therefore necessary to consider the possibility that some changes have been induced in the fibroblasts. More subtle changes in fibroblasts can lead to the formation of cells termed 'protomyofibroblasts' that are somewhere between fibroblasts and myofibroblasts (Rohr, 2011, Turner and Porter, 2013). Although the cells used here are α-SMA negative it cannot be excluded that other subtle changes to the fibroblast phenotype have occurred. Also, as well as fibroblasts affecting myocytes by paracrine communication, myocytes affect fibroblasts (Fredj et al., 2005a, Dolmatova et al., 2012, Tsoporis et al., 2012) and there may be a signal from the myocytes that is inducing changes in the fibroblasts. This concept is explored further in Chapter 6.

4.4.4. **Summary**

The results presented in this chapter show for the first time that fibroblasts isolated from normal hearts and used before α-SMA induction by culture affect adult myocytes via paracrine mediators. Fibroblasts reduced myocyte viability, increased myocyte volume and increased myocyte Ca^{2+} transient amplitude.

This is the first time the paracrine effects of α-SMA-negative fibroblasts on myocytes have been explored, and raises the possibility that fibroblasts in the normal heart are actively regulating the activity of myocytes. A closer examination of fibroblast-targeted

knockout and transgenic mice under physiological conditions will allow further insight into this exciting possibility.

Having shown these effects of fibroblasts from normal hearts, the next chapter investigates whether the effects are altered when fibroblasts isolated from a pathological situation are used.

CHAPTER 5.

The paracrine effects of fibroblasts from pressure overloaded hearts on myocyte structure and function

5.1. Introduction

There are only very scarce data regarding the effects of pathology on the paracrine interaction between fibroblasts and myocytes. Vasquez *et al* (2010) showed that cultured fibroblasts from a rat model of myocardial infarction had a greater effect on neonatal myocyte electrophysiology compared to controls, causing a significant shortening of the action potential and slowing of conduction. Other studies have used fibroblasts from normal hearts that have differentiated in culture and it has been argued that these studies can provide insight into the role of fibroblasts in disease (Rohr, 2011). However, it would appear that fibroblasts activated in culture are a different phenotype to those activated by disease (as discussed in detail in Section 1.3.3.).

The best insight into the role of fibroblast paracrine mediators in a disease setting comes from transgenic mice. Depressing fibroblast paracrine properties prevents the cardiac hypertrophic response to pressure overload (Takeda et al., 2010, Accornero et al., 2011) or to infusion of Ang II or isoproterenol (Pellieux et al., 2001, Jaffre et al., 2009). These are classical cardiac stimuli and these studies suggest that fibroblasts may be important in mediating the cardiac response to these stressors through paracrine communication. However, the effect on the function of myocytes was not examined in these studies. Furthermore, these studies provide an interesting insight in to the importance of fibroblasts in the initial response to overload and biochemical insult, but not into the effects of fibroblast paracrine factors in more established pathological settings.

5.1.1. **Chapter aims:**

The work reported in this chapter aims to investigate the effects of adult fibroblasts from pressure overloaded hearts on myocytes structure, Ca^{2+} transients and contractility. These effects are compared with control fibroblasts from sham operated hearts.

5.2. Methods

A schematic of the experimental protocol is shown in Figure 5.1.

5.2.1. **Animal model**

A 10 week model of TAC was used to produce pressure overload in rats. Adult male Lewis rats of 200-250g were used and the aorta was constricted to a diameter of 0.9mm for 10 weeks as described in the Section 2.2.1.

The effect of TAC was assessed using echocardiography and measurements of heart weight and LV weight to body weight ratios. The effect of TAC on the fibroblasts was examined by staining for α-SMA expression in fibroblasts 48 hours after isolation.

Sham operated animals were used as a control.

5.2.2. **Cell isolation and co-culture**

Fibroblasts were isolated from TAC and sham operated animals 10 weeks after the operations were performed and co-culture was set up as described in the Section 2.4.1.

Fibroblasts were isolated from 6 TAC hearts and 5 sham hearts. Myocytes were isolated from 12 normal 200-250g Lewis rat hearts.

5.2.3. **Assessment of myocyte phenotype**

The paracrine effects of the fibroblasts on the myocytes were assessed by measuring the following parameters after 24 hours of co-culture:

- Cell viability
- Cell volume
- T-tubule density
- Ca^{2+} transients
- Sarcomere shortening

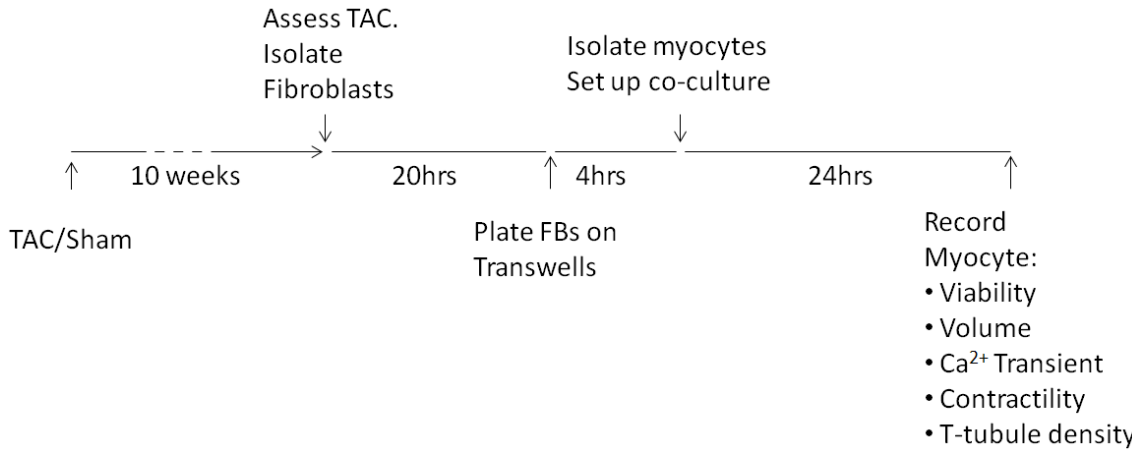


Figure 5.1: Schematic of the experimental protocol

5.3. Results

5.3.1. **10 week thoracic aortic constriction produced a compensated hypertrophic response of the heart**

The *in vivo* effects of TAC on heart function were examined using echocardiography (Figure 5.2). Left ventricular posterior wall thickness was increased by 12% indicating hypertrophy of the left ventricle. The ejection fraction was also increased, which is indicative of compensated hypertrophy. The hearts have not begun to fail as once the compensatory response is exhausted, and the heart begins to fail, ejection fraction drops (Wei et al., 2010).

The hypertrophic phenotype was also assessed using measurements of heart weight, LV weight and body weight (Figure 5.3). The heart weight to body weight ratio (HW:BW) and LV to body weight ratio (LV:BW) were increased confirming the hypertrophic response.

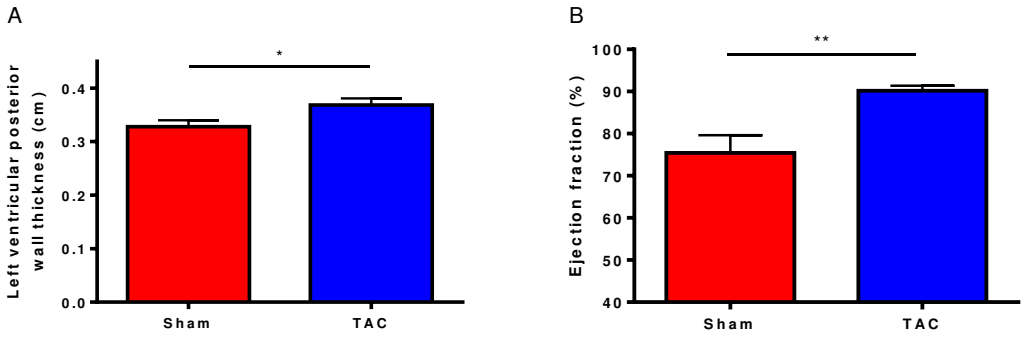


Figure 5.2: Echocardiography shows compensated hypertrophy in TAC animals
A) Left ventricular posterior wall thickness was increased with TAC. B) Ejection fraction was also increased with TAC. These results are indicative of a compensated hypertrophic response. Sham n=5 animals; TAC n=6 animals.

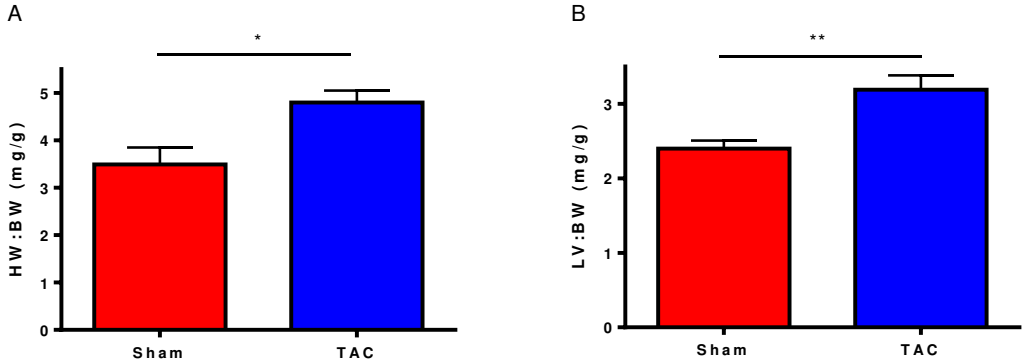


Figure 5.3: Heart and left ventricular to body weight ratios confirm a hypertrophic response to TAC
A) Heart weight to body weight and B) left ventricle weight to body weight ratios were increased after TAC compared to sham operated animals. Sham n=5 animals; TAC n=6 animals.

5.3.2. **TAC induces α-SMA expression in cardiac fibroblasts**

Pressure overload of the heart has previously been shown to increase fibroblast migratory and proliferative properties (Stewart et al., 2010). To assess whether fibroblasts were altered by the 10 week model of TAC used here, the cells were stained for the presence of α-SMA 48 hours after isolation. The majority of TAC fibroblasts stained positive for α-SMA, whereas at this time point fibroblasts from normal hearts were α-SMA negative (Figure 5.4). This is only a single marker of fibroblast activation,

and not enough cells were imaged to allow quantification of this parameter. However, this shows that TAC is activating fibroblasts to express α-SMA which is indicative of fibroblasts in pathology.

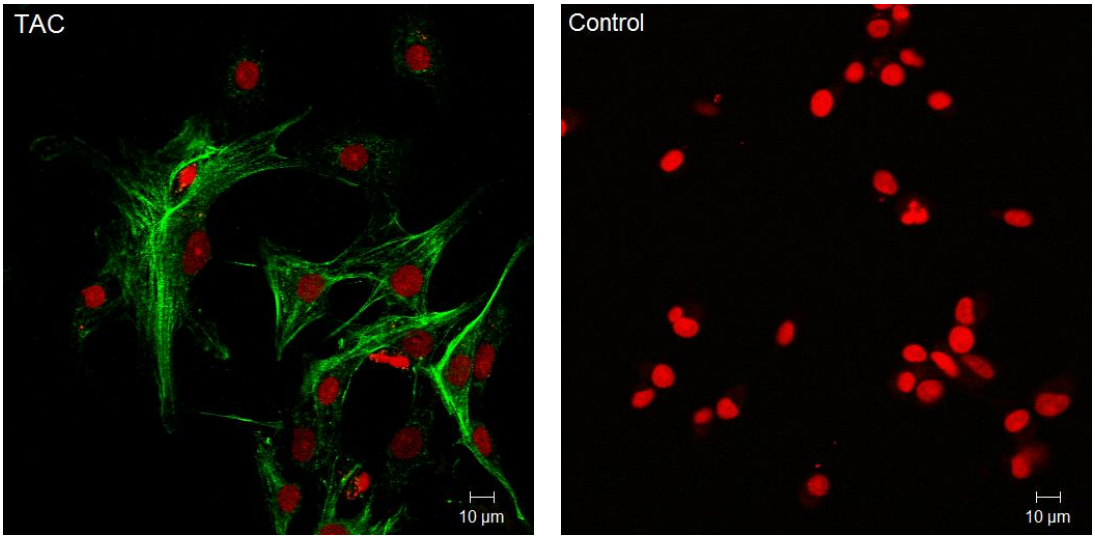


Figure 5.4: α-SMA staining in fibroblasts 48 hours after isolation from TAC and normal hearts
TAC fibroblasts largely stained positive for α-SMA, although not ubiquitously. Normal fibroblasts were negative for α-SMA. Green: α-α-SMA. Red: Nuclear stain.

5.3.3. **Fibroblasts from both TAC and sham operated rats reduce myocyte viability**

The % of rod shaped cells was reduced in myocytes cultured with either sham or TAC fibroblasts (Figure 5.5). Co-culture with either group of fibroblasts reduced viability to a similar extent; co-culture with sham fibroblasts caused a 23% reduction and co-culture with TAC produced a 20% decrease in the % of cells that were rod shaped compared to control.

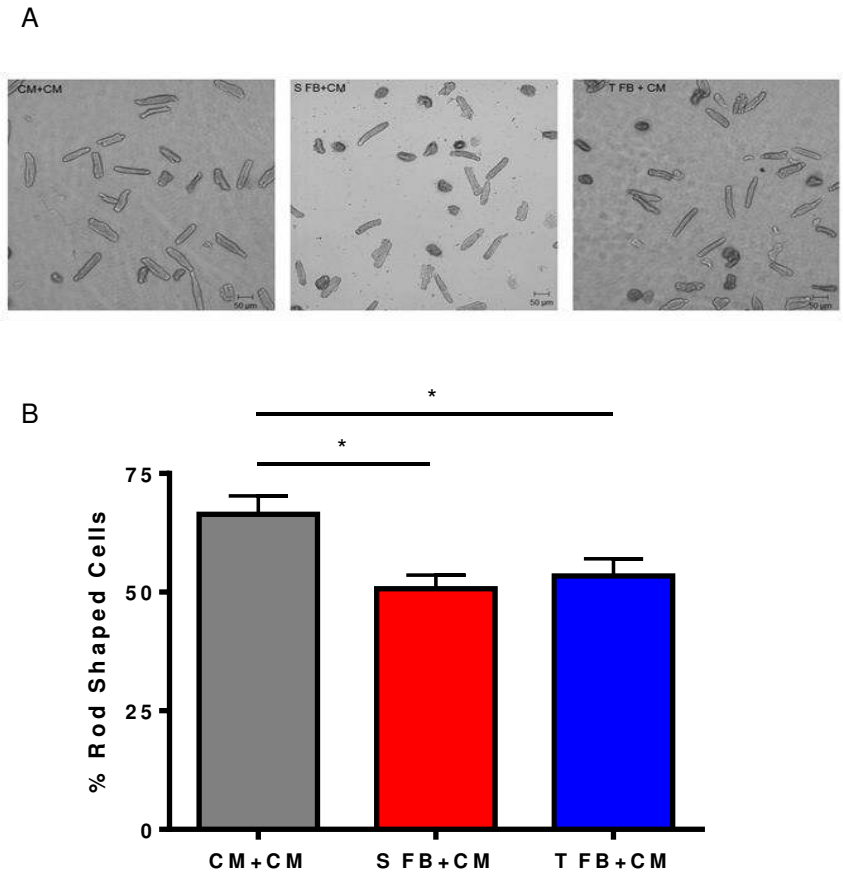


Figure 5.5: Co-culture with either TAC or sham fibroblasts reduced myocyte viability
A) Representative recording of myocyte viability. B) The percentage of myocytes retaining their rod shape was reduced by co-culture with TAC or sham fibroblasts compared to control. CM+CM n= 6 set ups; S FB+CM n=7 set ups; T FB+CM n=8 set ups.

5.3.4. **TAC and sham fibroblasts both induced myocyte hypertrophy**

The volume of myocytes was used as a marker of cellular hypertrophy, and assessed with 3-dimensional reconstruction of di-8-ANEPPS staining. Myocyte volume was increased when they were co-cultured with TAC or sham fibroblasts compared to control (Figure 5.6). The extent of the increase in myocyte volume was similar in both groups.

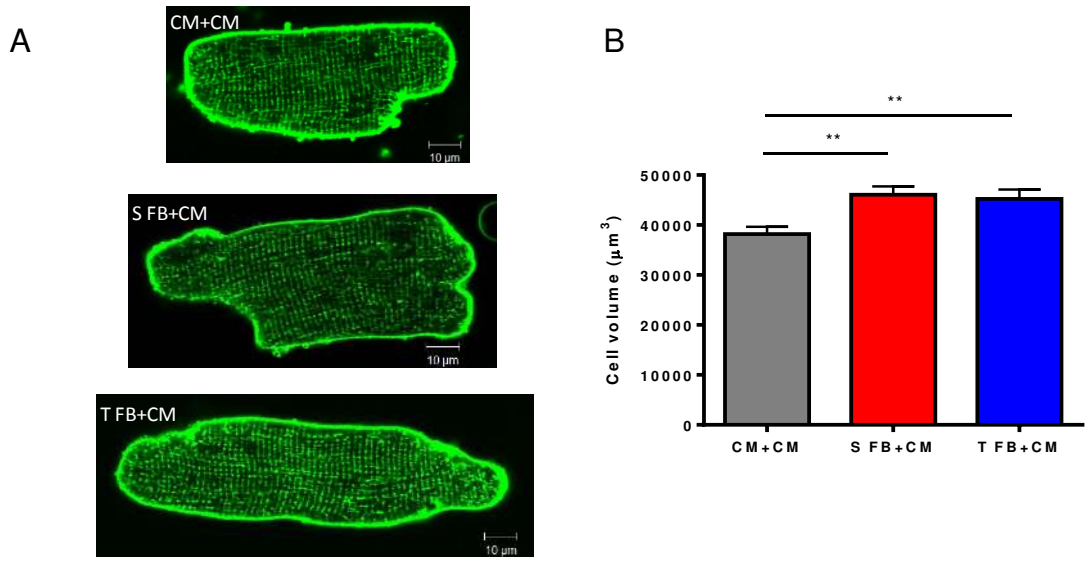


Figure 5.6: Co-culture with TAC or sham fibroblast led to myocyte hypertrophy
A) Representative recordings of di-8-ANEPPS staining. B) Myocyte volume was significantly increased after co-culture with either TAC or sham fibroblasts compared to control. CM+CM n=68; S FB+CM n=44; T FB+CM n=41.

5.3.5. **Sham and TAC fibroblasts have different effects on myocyte Ca^{2+} transients**

Stimulated Ca^{2+} transients were recorded using the Ca^{2+} sensitive dye Fluo-4 (Figure 5.7). The transient amplitude was greater after co-culture with sham fibroblasts and smaller after co-culture with TAC fibroblasts. Time to peak and time to 50% decay were unchanged in any set up. Time to 90% decay was reduced in both fibroblast groups.

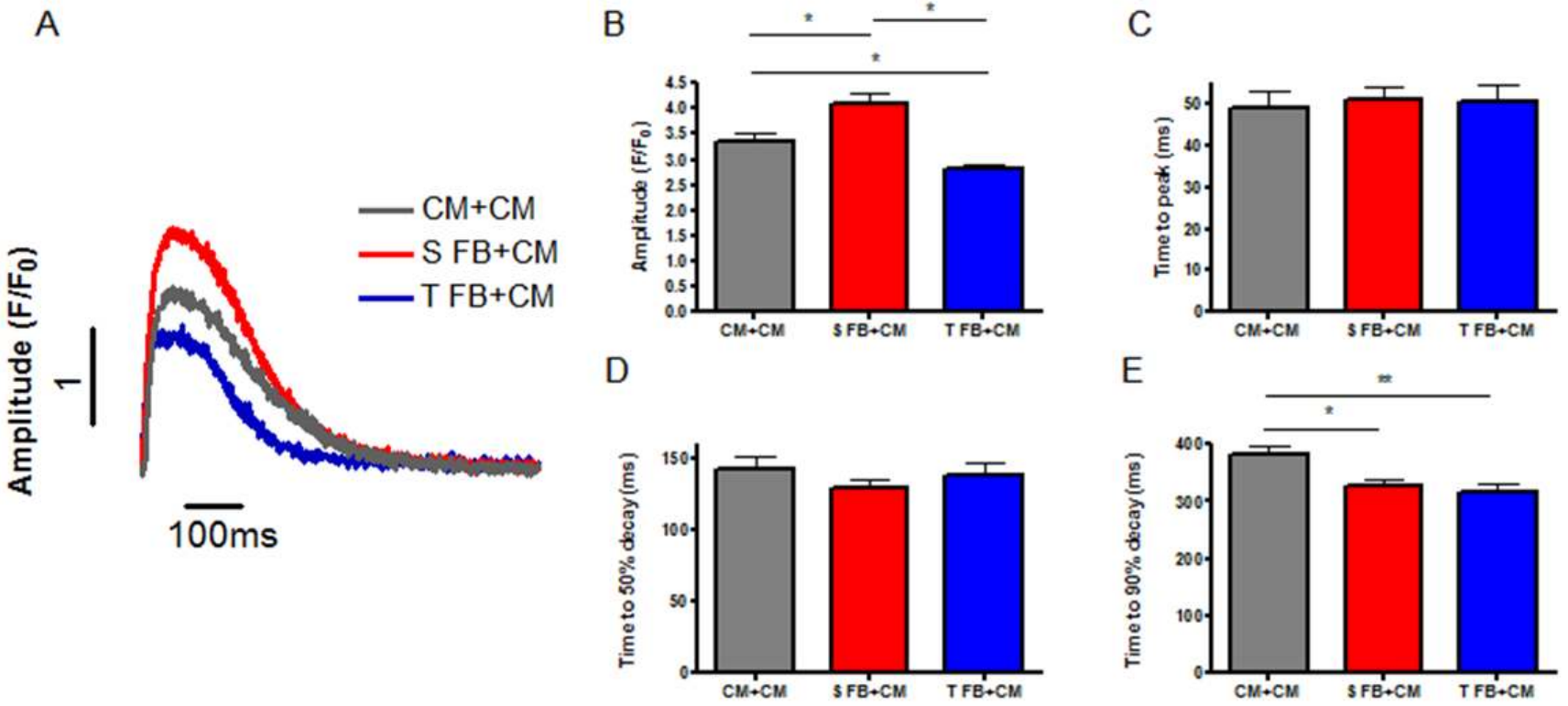


Figure 5.7: Sham and TAC fibroblasts have opposing effects on myocyte Ca^{2+} transient amplitude

A) Representative Ca^{2+} transients. B) Myocyte Ca^{2+} transient amplitude was larger in myocytes co-cultured with sham fibroblasts compared to those co-cultured with TAC fibroblasts. C) Time to peak and D) time to 50% decay were not affected by co-culture with fibroblasts. E) Time to 90% decay CM+CM n=48; S CF+CM n=56; T CF+CM n=40.

5.3.6. **Myocyte contractility was affected by both TAC and sham fibroblasts**

The Ca^{2+} transient drives the contraction of myocytes which is ultimately responsible for the function of the heart as a pump. To assess myocyte contractility stimulated sarcomere shortening was tracked (Figure 5.8). The amplitude of sarcomere shortening was not affected by either group of fibroblasts. Time to 90% peak was reduced in the sham fibroblast group but not altered in the TAC fibroblast group compared to control. The relaxation times of sarcomere contraction were altered by both fibroblast groups. Time to 50% and 90% decline to baseline were reduced, with TAC fibroblasts having a greater effect on time to 50% decline. The increased speed of relaxation can be partly explained by shortening of the Ca^{2+} transient, although the effect on relaxation of contractility appears to be greater than on the decline of the Ca^{2+} transient.

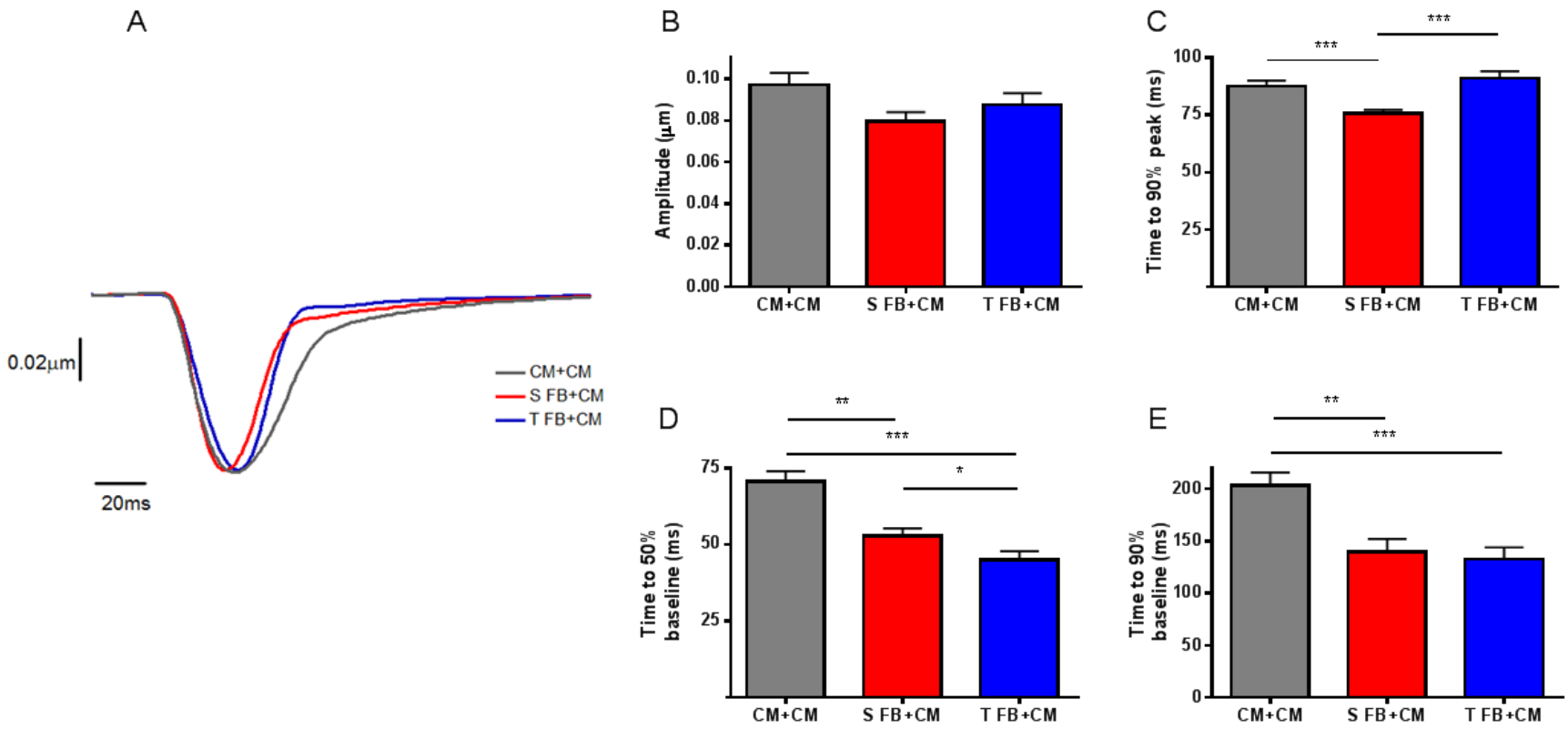


Figure 5.8: Myocyte contractility is altered by co-culture with sham or TAC fibroblast

A) Representative recordings of sarcomere shortening. B) Absolute amplitude of sarcomere shortening is not different between the groups. C) Time to 90% peak is reduced in myocytes co-cultured with sham fibroblasts compared to control myocytes and myocytes co-cultured with TAC fibroblasts. The time for sarcomere length to return D) 50% and E) 90% to baseline is reduced by both sham and TAC fibroblasts. The effect of TAC fibroblasts on time for 50% return to baseline is greater than that of sham. CM+CM n=65; S FB+CM n=51; T SB+CM n=65.

5.3.7. **Neither TAC or sham fibroblasts affect t-tubule density**

The t-tubule density of myocytes was measured as the percentage di-8-ANEPPS stained area. T-tubule density was not significantly different between any of the three groups (Figure 5.9).

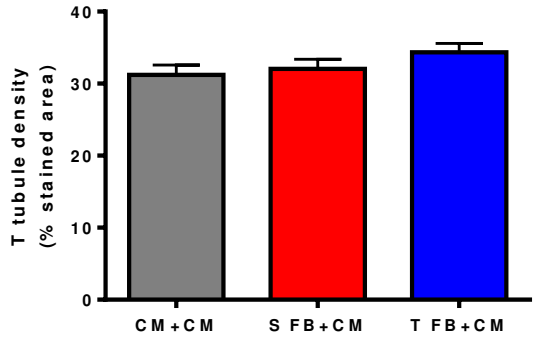


Figure 5.9: Fibroblasts did not affect the t-tubule density
T-tubule density was unaffected by co-culture with fibroblasts. CM+CM n=53; S FB+CM n=39; T FB+CM n=37

5.4. Discussion

The results presented in this chapter compare the paracrine effects of fibroblasts from pressure overloaded hearts with fibroblasts from sham operated hearts. Both sham and TAC fibroblasts reduced myocyte viability and induced myocyte hypertrophy in co-cultured myocytes. The response of the Ca^{2+} transient was different. Sham fibroblasts led to an increased transient amplitude whereas TAC fibroblasts caused a diminished transient amplitude. The decay times of the Ca^{2+} transient were reduced by co-culture with either group of fibroblasts. This effect was also seen in the sarcomere relaxation time.

This is the first comparison of the effects of adult fibroblasts from normal and pathological hearts on adult myocytes and is therefore important in our understanding of the paracrine effects of fibroblasts on myocyte function. Previously Vasquez e*t al* (2010) compared the paracrine effects of fibroblasts from myocardial infarction and control hearts on the electrophysiology of neonatal cells and also showed a difference

between normal and pathological fibroblasts. They found that only fibroblasts from a rat model of myocardial infarction induced changes in conduction velocity and action potential duration despite keeping the fibroblasts in culture for up to 1 month. This is slightly different to the observations here, where we see that both normal and TAC fibroblasts affect myocytes, but with differences between the two groups in the effects on Ca^{2+} transient amplitude. Despite this, our results and those of Vasquez et al (2010) show the importance of comparing pathological and control fibroblasts when trying to understand the role of fibroblasts in the heart, rather than just using a single source as has been done in other experiments.

5.4.1. **TAC was a suitable source of *in vivo* activated fibroblasts**

TAC and coronary artery ligation were considered as the source for pathological fibroblasts. Both have been shown to affect fibroblast phenotype (Squires et al., 2005, Stewart et al., 2010). However, coronary artery ligation produces different populations associated with the scar and remote regions (Squires et al., 2005) whereas TAC produces a homogeneous effect on the LV and was therefore used (this is discussed in more detail in Section 2.2.). Characterisation of the model here shows that TAC successfully induced pressure overload of the heart. It resulted in a compensated hypertrophy, as evident from the echocardiography and the size of the heart.

The effect of pressure overload on fibroblasts has been shown previously. *In vivo* there is an increase in the ECM which is associated with activation of fibroblasts (Doering et al., 1988, Weber et al., 1988) and the proliferation and migration of isolated fibroblasts are also altered (Stewart et al., 2010). Although we have not assessed these parameters, the model used here induced the expression of α-SMA, a marker of fibroblast alteration.

It is important to note that α-SMA expression does not define a single cell type. In culture, although two populations of fibroblasts may both be α-SMA positive other characteristics can be different. Jarvis et al (2006) found that although fibroblasts from control and infarcted hearts both express α-SMA in culture, their response to cytokines

was still different. This may also be apparent *in vivo* and therefore just because the cells used here are α-SMA positive does not mean the results would be necessarily the same using α-SMA positive cells from other settings such as myocardial infarction. The relevance of our results to these settings would itself need to be studied.

5.4.2. **Similar effects of TAC and sham fibroblasts on myocyte viability and volume**

The reduction in viability and the increase in cell volume were similar after co-culture with either sham or TAC fibroblasts. The mechanisms underlying these changes have been discussed in Section 4.4.

Although these measurements are comparable between the two groups, it is possible that they are produced by different pathways. For example, cardiac hypertrophy can be defined as either physiological or pathological (Bernardo et al., 2010) and a whole array of growth factors, signalling molecules and cytokines can produce myocyte hypertrophy (Suzuki et al., 1990, Palmer et al., 1995, McMullen et al., 2004). Therefore, whether these changes are the result of the same pathways with sham and TAC fibroblasts needs to be addressed, and is investigated in Chapter 6.

5.4.3. **Effects on Ca^{2+} cycling and contraction**

The major difference between the effects of TAC and sham fibroblasts was the change in Ca^{2+} transient amplitude. Myocytes co-cultured with sham fibroblasts had a greater Ca^{2+} transient amplitude whereas myocytes co-cultured with TAC fibroblasts had a reduced Ca^{2+} transient amplitude. Both groups also had an increased speed of decline of the Ca^{2+} transient.

The decline of the Ca^{2+} transient is predominantly controlled by NCX and SERCA. NCX removes Ca^{2+} from the cell and SERCA takes Ca^{2+} into the SR. Increased activity of SERCA raises the level of SR Ca^{2+}, which in turn means that more Ca^{2+} is available for the following transient (Periasamy and Huke, 2001). Therefore increased SERCA activity is associated with a larger Ca^{2+} transient and may explain the observations in

the sham fibroblast group. SERCA mRNA levels have previously been shown to be sensitive to cytokines such as Il-6 (Prabhu, 2004, Tanaka et al., 2004). SERCA activity is not only mediated by altering SERCA levels but also through post translational modification; for example, phosphorylation (Xu et al., 1993), modification by small ubiquitin-related modifier (Kho et al., 2011) and interaction with phospholamban (Rodriguez and Kranias, 2005) alter SERCA activity. These processes may also represent targets of paracrine mediators (McTiernan et al., 1997).

Despite the increased rate of Ca^{2+} transient decline in the TAC group, increased SERCA activity does not fit due to the reduction in Ca^{2+} transient amplitude without other changes in SR Ca^{2+} handling parameters. If the storage of Ca^{2+} within the SR was altered, such that there was increased leakage of SR Ca^{2+} this could explain the reduced Ca^{2+} transient along with increased SERCA activity. Ca^{2+} leakage through RyR has been linked to the reduced SR Ca^{2+} and reduced Ca^{2+} transient amplitude in heart failure. This reduction in the Ca^{2+} transient amplitude was seen despite the level of SERCA activity not falling (Ai et al., 2005, Belevych et al., 2007). The increased Ca^{2+} leak may be due to alterations of the RyR or through changes in the storage of Ca^{2+} within the SR. Ai et al (2005) showed that Ca^{2+} leak was increased due to phosphorylation of RyR channels and Chopra et al (2007) showed that small decreases in calsequestrin, the main Ca^{2+} binding protein in the SR, also increased Ca^{2+} leak.

An alternative explanation for the decreased transient amplitude is a reduction in ICa,L, due to a shorter action potential (Cannell et al., 1987). This could also explain the shortening of the Ca^{2+} transient as the stimulus for Ca^{2+} release would be removed sooner, although this may be expected to also reduce the time to peak. More rapid removal of Ca^{2+} through NCX could also explain the effect on transient decline. A combination of these changes is likely in the effects seen and further work is required to understand the mechanisms behind the observed changes.

The increased speed of myocyte relaxation observed may be due to the faster Ca^{2+} transient decay. However, the amplitude of contraction does not follow the changes that are seen in the Ca^{2+} transient amplitude. The amplitude of the sarcomere

shortening is affected by the Ca^{2+} transient amplitude but also other factors such as the sensitivity of the myofilaments to Ca^{2+} (Puceat et al., 1990). Therefore changes in Ca^{2+} transient amplitude do not necessarily result in similar changes in contractility (Bing et al., 1991).

5.4.4. **Comparison with previous studies**

Previous studies of the paracrine effects of fibroblasts on myocyte function are limited. Cultured fibroblast conditioned medium reduced the contractility of neonatal myocyte (LaFramboise et al., 2007). A reduced contractility was also induced in adult myocytes in co-culture with cultured fibroblasts (Cartledge et al., 2011). This was matched by a reduction in the Ca^{2+} transient. Although a decreased amplitude of contraction was not observed in these experiments, the reduction in Ca^{2+} transient is evident with TAC fibroblasts where as sham fibroblasts have a comparably larger transient. In this regard it would appear that cultured fibroblasts are more similar to the fibroblasts from TAC hearts rather than fibroblasts from the normal heart.

Other reports have looked at the effects of fibroblasts on action potential duration. This is the stimulus for the Ca^{2+} transient, as it produces Ca^{2+} influx through ICa,L. As the action potential lengthens, ICa,L increases and therefore the Ca^{2+} transient is longer. Fibroblasts have been shown to affect the action potential of neonatal myocytes. Guo *et al* (1998b) showed that the time-to-25% repolarisation of the action potential was prolonged (although time-to-80% was unchanged) and Pedrotty *et al* (2009) found that time-to-90% repolarisation was prolonged by cultured fibroblast conditioned medium. The prolongation of the action potential in adult myocytes by fibroblast conditioned medium has also been suggested by Kaur *et al* (2013). This would be expected to cause a prolongation of the Ca^{2+} transient, which is the opposite of the effect we observe. Interestingly, Vasquez *et al* (2010) found that the action potential of neonatal myocytes was shortened by fibroblast conditioned medium when using fibroblast from hearts after myocardial infarction. A shorter action potential would be expected to shorten the Ca^{2+} transient and therefore is more in keeping with our results. All of these studies have used fibroblast conditioned medium and whether

the effects are different in a co-culture system is unknown. It would therefore have been interesting to look at the action potential under our experimental set up.

5.4.5. **Investigating other aspects of excitation contraction coupling**

Although it would have been interesting, myocyte electrophysiology was not successfully investigated here. Single myocyte action potentials can be recorded to look at myocyte excitation using whole cell perforated patch clamping. This technique was established in the lab (Ibrahim et al., 2010). However, patch clamping of cultured myocytes was found to be difficult and, due to the limited number of co-culture set ups it was not possible to record an adequate number of action potentials to analyse this parameter. The paracrine effect of fibroblasts on the electrical properties of the heart was therefore studied using myocardial slices (see Chapter 7).

Ca^{2+} handling properties can also be studied in more detail by looking at Ca^{2+} sparks and SR Ca^{2+} content. Ca^{2+} sparks were not consistently detectable in culture (as demonstrated in Section 3.3.5.). SR Ca^{2+} content has previously been measured in cultured myocytes within the lab (Cartledge et al., 2011) but attempts to measure it here were unsuccessful. Some recordings were achieved but only 1 or 2 recordings are possible per co-culture set up and due to the limited number of set ups available and the inconsistent success it was not possible to produce enough data to provide meaningful results.

5.4.6. **A role of fibroblast paracrine mediators in disease progression**

Characterisation of the 10 week model of TAC used in these experiments show that the myocytes have a larger Ca^{2+} transient amplitude compared to control (Ibrahim et al., 2012). Other models of hypertrophy in rats have found differing effects, showing no changes in Ca^{2+} transient amplitude or reduction in the transient amplitude (Bing et al., 1991, Moore et al., 1991, Zhang et al., 1995). What is more consistently observed is the reduction in Ca^{2+} transient amplitude with the progression into heart failure but the cause of these changes is unclear (Balke and Shorofsky, 1998). Fibroblasts are

sensitive to mechanical strain and biological mediators that are increased in pressure overload and coupled with the results reported here it is therefore interesting to hypothesise that fibroblasts may drive the changes in Ca^{2+} handling associated with the progression to heart failure.

5.4.7. **Summary**

The results reported in this chapter show that fibroblasts activated *in vivo* by pressure overload of the heart have different effects on myocytes compared to fibroblasts from the normal heart, particularly in the regulation of cytoplasmic Ca^{2+}.

It is important to consider the implication of these results for fibroblast and myocyte interaction *in vivo*. These results suggest that fibroblasts have the capacity to modulate myocyte function, with an improved time to peak contraction and increased Ca^{2+} transient in myocytes co-cultured with sham fibroblasts. However, these effects are different with TAC fibroblasts, which resulted in a decreased Ca^{2+} transient amplitude. This raises the possibility that fibroblasts may switch in disease states to drive some of the negative effects in myocardial function.

The next phase of this investigation was to look at potential mechanisms involved in the effects observed. The role of TGF-β, a major factor in fibroblast and myocardial physiology was therefore investigated.

CHAPTER 6.

Investigating the role of TGF-β in the paracrine communication between fibroblasts and myocytes

6.1. Introduction

In Chapters 4 and 5 the paracrine effects of fibroblasts on myocyte properties have been examined. In this chapter we set out to investigate possible mediators involved in these effects. Among the possible mediators in the paracrine communication between fibroblasts and myocytes (explored in detail in Section 1.6.) I have focused on TGF-β for the following reasons:

Firstly, TGF-β is released by fibroblasts (Eghbali, 1989, Zhao and Eghbali-Webb, 2001). Secondly, myocytes express TGF-β receptors (Engelmann and Grutkoski, 1994) and TGF-β has effects on myocytes which include stimulating hypertrophy, driving apoptosis and altering Ca^{2+} handling and contractility (Roberts et al., 1992, Neylon et al., 1994, Schluter et al., 1995, Avila et al., 2007, Li et al., 2008, Heger et al., 2011). These are potential explanations for the observations reported in this Thesis and make TGF-β a good candidate to explore. Thirdly, TGF-β has been previously shown to be a possible paracrine mediator. *In vitro,* the myocyte hypertrophy induced by fibroblast conditioned medium has been blocked with TGF-β neutralising antibodies (Gray et al., 1998) and *in vivo* TGF-β knockout mice do not develop hypertrophy in response to Ang II infusion (Schultz Jel et al., 2002). Fibroblast-derived TGF-β has also been shown to affect myocyte electrophysiology, increasing the voltage gated Na^+ current and decreasing the transient outward K^+ current (Kaur et al., 2013).

SB 431542 is a potent and specific TGF-β type 1 receptor antagonist (Inman et al., 2002). It has been shown to block the effect of TGF-β on cardiomyocyte (Heger et al., 2011) and therefore is an ideal tool for investigation the role of TGF-β in the paracrine effects of fibroblasts on myocytes.

6.1.1. **Chapter aims**

The work described in this chapter set out to test the hypothesis that TGF-β is responsible for the paracrine effects of fibroblasts on myocyte viability, volume and Ca^{2+} transients. The role of TGF-β in the effect of TAC and age-matched control fibroblasts was examined.

6.2. Methods

6.2.1. **Measurement of potential paracrine mediators**

The level of TGF-β1 was measured in the co-culture supernatant using R&D systems TGF-β Quantikine® ELISA kit (R&D systems, UK; Cat. No. MB100B).

6.2.2. **Blocking TGF-β type 1 receptors in co-culture**

A schematic of the experimental protocol is shown in Figure 6.1A.

Fibroblasts were isolated from 10 week TAC animals and age matched controls (without sham operation) and set up in co-cultures with normal rat ventricular myocytes. 10μM SB 431542 (Sigma Aldrich, UK) in DMSO (final DMSO concentration 0.1%) was added to the co-culture to block the TGF-β type 1 receptors. 0.1% DMSO was added to control set-ups.

The final set ups were:

- Myocytes + Myocytes
- Myocytes + Myocytes + SB 431542
- Control fibroblasts + Myocytes
- Control fibroblasts + Myocytes + SB 431542
- TAC fibroblasts +Myocytes
- TAC fibroblasts +Myocytes + SB 431542

6.2.3. **Blocking TGF-β type 1 receptors in conditioned medium**

A schematic of the experimental protocol is shown in Figure 6.1B.

Fibroblasts were isolated from 10 week TAC and age matched control rats. They were cultured for 20 hours in FB medium, and then plated in Transwells at 10,000 cells in 200μl ITS medium per Transwell. After 4 hours the Transwells were suspended in 2ml

ITS medium in a glass bottom dish. This is the same set up as for the co-culture, except that myocytes were not added to the glass bottom dish. After 24 hours, the medium was collected and any cells were removed by centrifuging at 620*g* for 5 minutes. The medium was then frozen at -20°C ready for use.

To assess the effects of conditioned medium freshly isolated myocytes were plated on laminin coated glass bottom dishes at 5,000 cells per dish. The conditioned medium was thawed at 37°C and 2ml was added to the myocytes either in the presence of 10µM SB 431542 or 0.1% DMSO.

The final sets up were:

- Myocytes + myocyte conditioned medium
- Myocytes + control fibroblast conditioned medium
- Myocytes + control fibroblast conditioned medium + SB 431542
- Myocytes + TAC fibroblast conditioned medium
- Myocytes + TAC fibroblast conditioned medium + SB 431542

6.2.4. **Assessment of myocyte phenotype**

After 24 hours of co-culture or exposure to conditioned medium, the following properties of the myocytes were examined:

- Viability
- Volume
- Ca^{2+} transients
- T-tubule density (for co-cultures only)

The protocols are described in the Section 2.4.

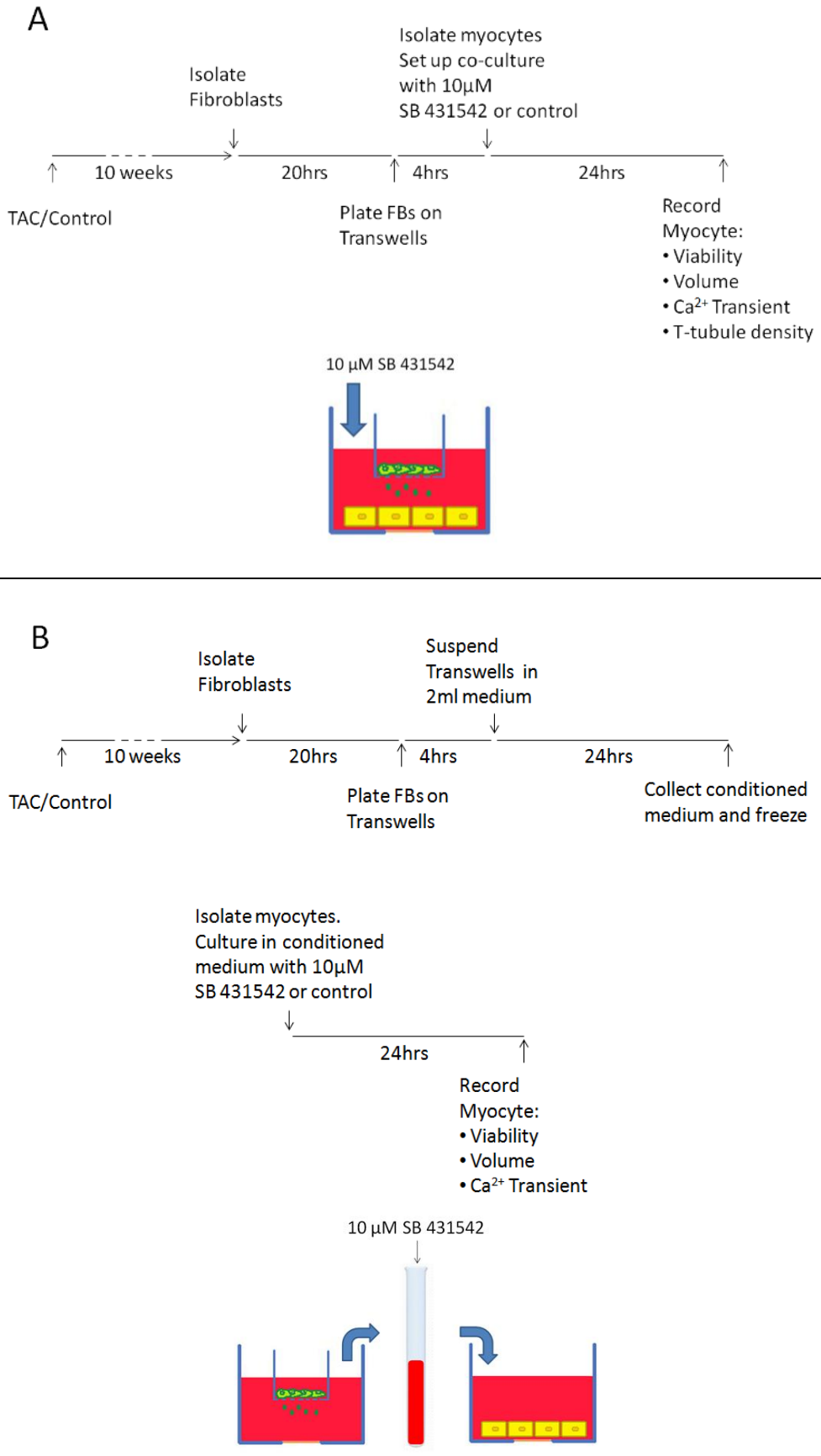


Figure 6.1: Schematic and illustration of the experimental protocol

A) Blocking TGF-β in co-culture. B) Blocking TGF-β in conditioned medium.

6.3. Results

6.3.1. **TGF-β1 was increased in co-culture medium from fibroblast co-cultures**

The level of TGF-β1 was measured in the co-culture medium using ELISAs (Figure 6.2). TGF-β1 was increased in both the sham and TAC fibroblast groups compared to control. The level in the sham fibroblast group was higher compared to the TAC fibroblast group.

Due to the raised levels of TGF-β1, we investigated its role in the changes in myocyte phenotype using the TGF-β type 1 receptor antagonist SB 431542.

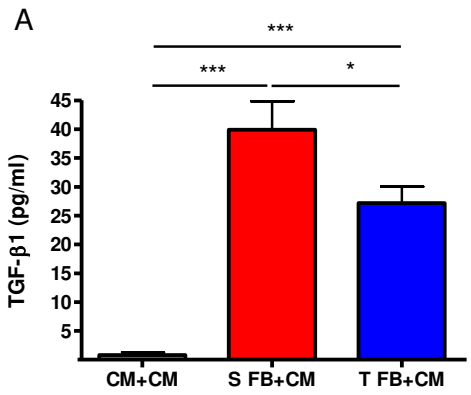


Figure 6.2: The levels of TGF-β1 in co-culture supernatant

A) TGF-β1 was increased in co-culture with either sham or TAC fibroblasts compared to the control. The level was higher in the sham fibroblast co-culture compared to the TAC fibroblast co-culture. CM+CM n=8; S FB+CM n=10; T SB+FM n=10 (number of ELISA measurements).

6.3.2. **Blocking TGF-β type 1 receptors prevented the loss in myocyte viability**

Both normal and TAC fibroblasts reduced the viability of co-cultured myocytes (Figure 6.3). This reproduced the results found in Chapters 4 and 5. SB 431542 blocked the reduction in myocyte viability in both fibroblast groups. In the presence of the

antagonist, the viability in both the fibroblast groups was not different from control levels.

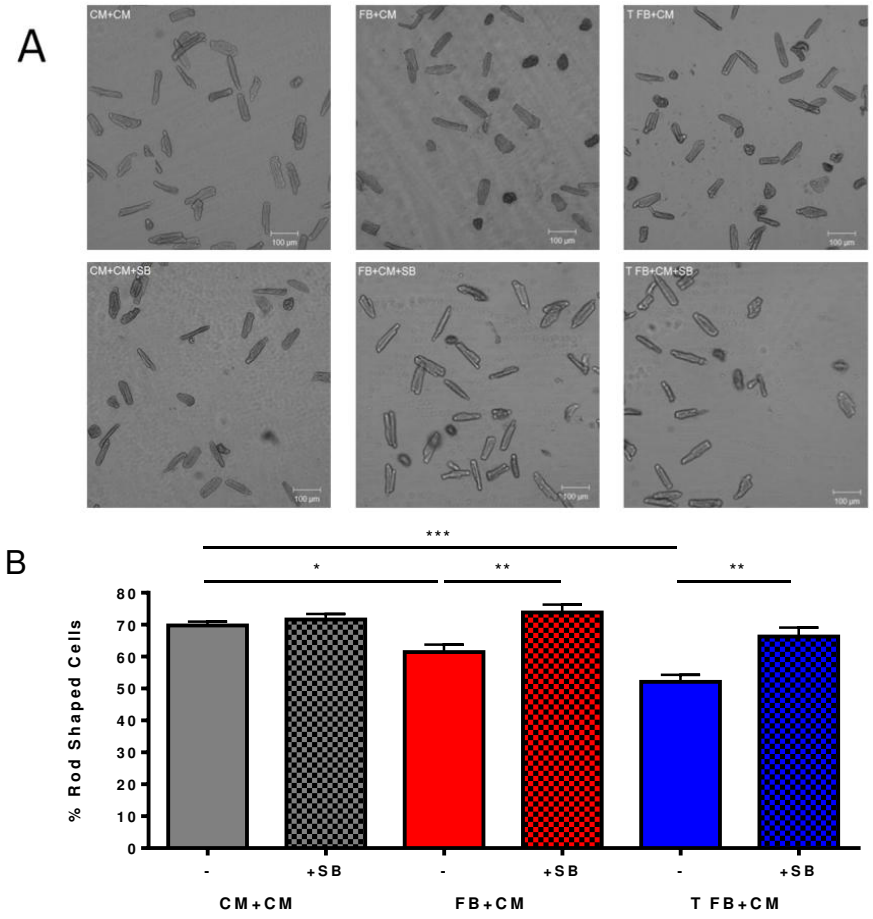


Figure 6.3: Antagonism of TGF-β type 1 receptors blocked the reduction in viability

A) Representative recordings of myocyte viability. B) Co-culture with normal or TAC fibroblasts reduced myocyte viability but this was blocked by SB-431542. CM+CM n=5; CM+CM+SB n=5; FB+CM n=6; FB+CM+SB n=6; T FB+CM n=6; T FB+CM+SB n=6 (number of co-culture set ups).

6.3.3. **SB-431542 blocked the hypertrophic response in myocytes**

Myocytes co-cultured with normal fibroblasts or TAC fibroblasts were larger than the controls (Figure 6.4), as was previously observed in Chapters 4 and 5. This hypertrophic response was blocked by the addition of the TGF-β type 1 receptor antagonist. In the presence of the antagonist cell volumes were not different from control.

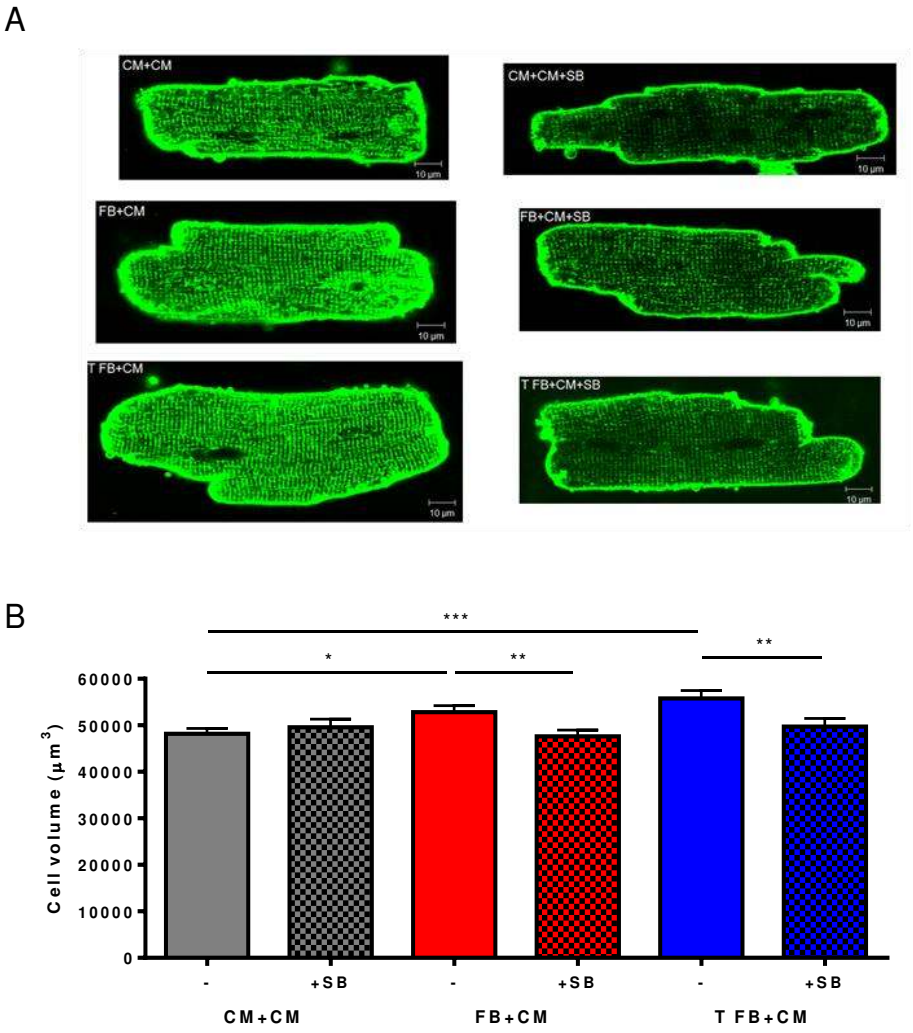


Figure 6.4: Antagonism of TGF-β type 1 receptor blocked the hypertrophic response

A) Representative images of di-8-ANEPPs stained myocytes, B) Both normal and TAC fibroblasts induce a hypertrophic response in myocytes. This is blocked by the presence of SB-431542 in the co-culture medium. CM+CM n=60; CM+CM+SB n=43; FB+CM n=48; FB+CM+SB n=47; T FB+CM n=44; T FB+CM+SB n=42.

6.3.4. **Blocking TGF-β type 1 receptors prevented the changes in myocyte Ca^{2+} transients**

The changes in the Ca^{2+} transient amplitude were prevented by SB 431542 (Figure 6.5). The transient amplitude was increased in the normal fibroblast group and decreased in

the TAC fibroblast group, as had previously been observed in Chapters 4 and 5. The amplitude was normalised to the control levels when the TGF-β type 1 receptor antagonist was added to the co-culture. The time to 90% decay of the Ca^{2+} transient was reduced in both fibroblast co-culture groups. This was not affected by blocking TGF-β type 1 receptors.

There appears to be some direct effects of the antagonist on the time to peak of the Ca^{2+} transient. Although the time to peak is unchanged by the fibroblasts, it is shortened in the presence of the TGF-β type 1 receptor antagonist. This causes a significant difference in the normal fibroblast group but has a similar, although non-significant effect in the TAC fibroblast and control groups.

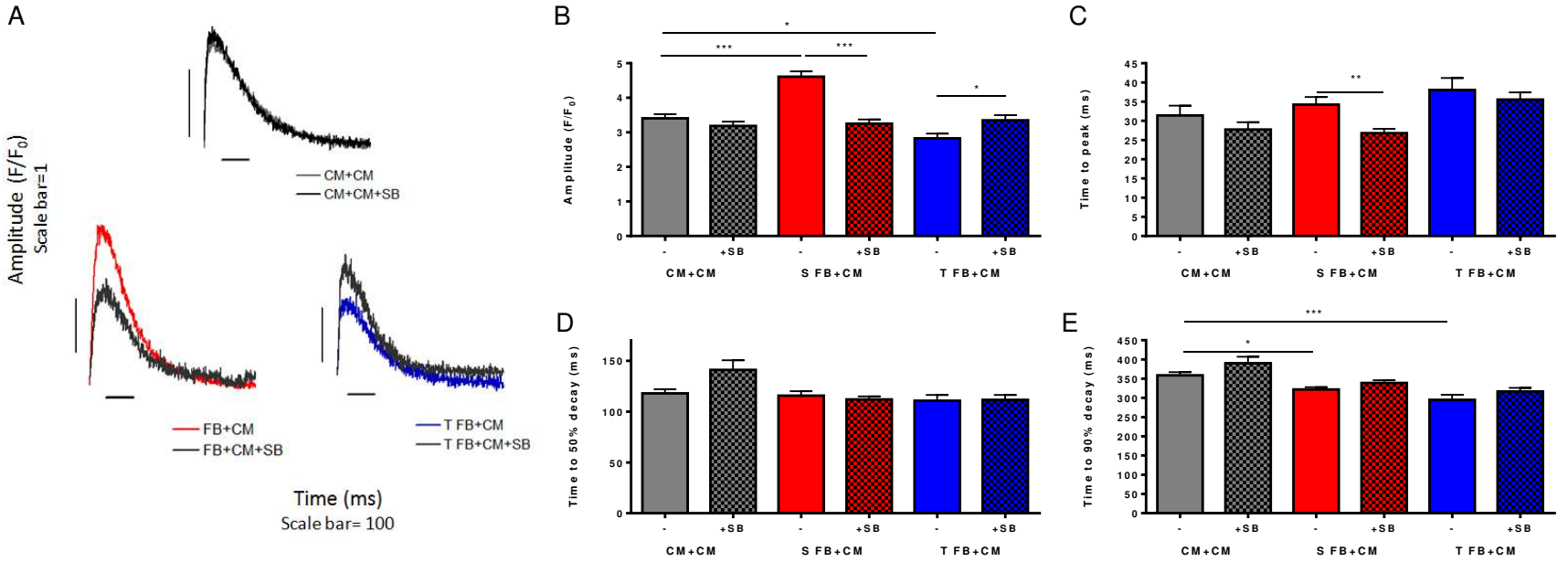


Figure 6.5: Changes in Ca^{2+} transient parameters were blocked by SB 431542

A) Representative Ca^{2+} transients recordings. B) The amplitude of the Ca^{2+} transient is increased by normal fibroblasts and reduces by TAC fibroblasts. SB 431542 blocks both of these changes. C) Time to peak is unaffected by co-culture with fibroblasts, although SB 431542 shortens the time to peak in normal fibroblast co-cultures. D) Time to 50% decay is unchanged in any experimental group. E) Time to 90% decay is reduced by co-culture with either normal or TAC fibroblasts and this effect is unchanged in the presence of SB 431542. CM+CM n=42; CM+CM+SB n=33; FB+CM n=50; FB+CM+SB n=60; T FB+CM n=36; T FB+CM+SB n=45.

6.3.5. **T-tubule density was unchanged in any group**

The t-tubule density was unaffected by either the fibroblasts or the addition of the TGF-β type 1 receptor antagonist (Figure 6.6). This is similar to the previous findings in Chapter 4 and 5.

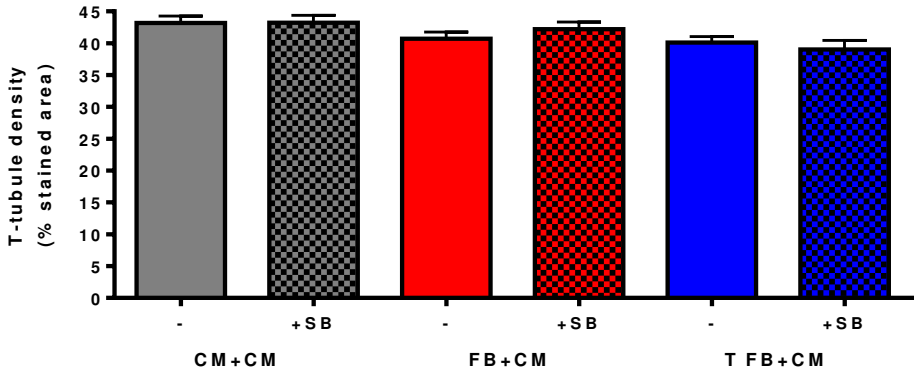


Figure 6.6: T-tubule density was unchanged by fibroblasts or TGF-β antagonism

T-tubule density is not altered in any experimental set up. CM+CM n=53; CM+CM+SB n=43; FB+CM n=48; FB+CM+SB n=47; T FB+CM n=42; T FB+CM+SB n=42.

When added to the co-culture set ups, SB 431542 blocks nearly all of the paracrine effects observed including the opposite effect of normal and TAC fibroblasts on the Ca^{2+} transient amplitude. This may be explained by the different levels of TGF-β. Alternatively, the TGF-β may be acting on the fibroblasts and inducing the release of different downstream paracrine mediators. To test this hypothesis we used conditioned medium to test the effects of blocking the TGF-β type 1 receptors on the myocytes only.

6.3.6. **SB 431542 does not block the reduction in myocyte viability with conditioned medium**

Normal or TAC fibroblast conditioned medium reduced the viability of myocytes (Figure 6.7). The addition of SB 431542 to the conditioned medium did not significantly block these effects as observed when it was added to the co-culture, although there is

a trend towards an increased viability in the presence of the antagonist (FB+CM vs. FB+CM+SB p=0.135; T FB+CM vs. T FB+CM+SB p=0.389).

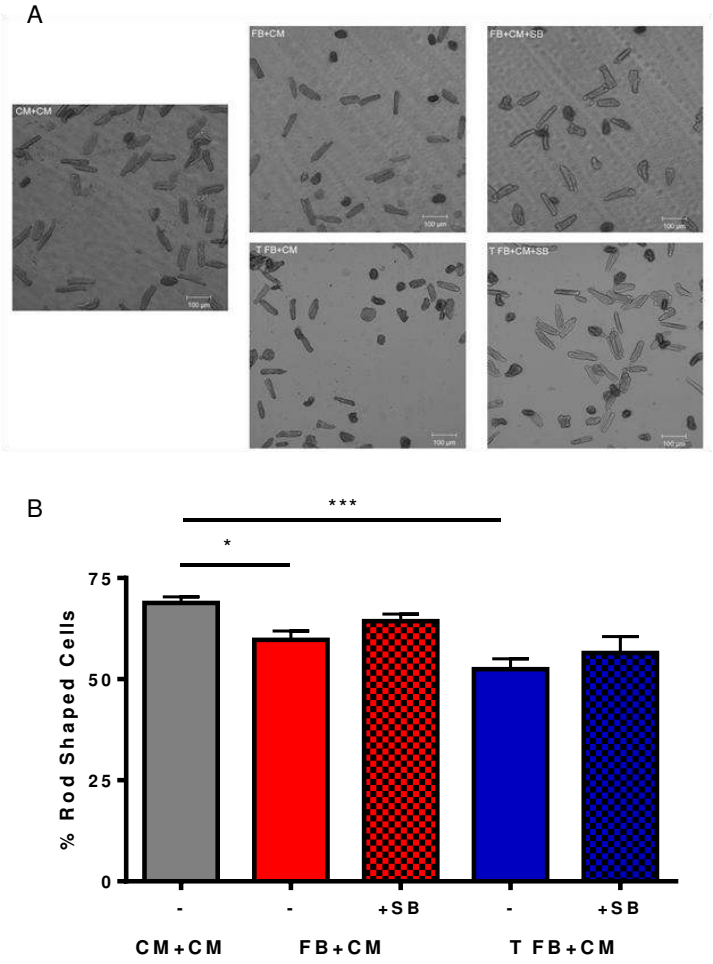


Figure 6.7: Myocyte viability was reduced by conditioned medium, but unaffected by SB 431542

A) Representative recordings of myocyte viability. B) Myocyte viability is reduced by either normal or TAC fibroblasts conditioned medium. The addition of SB 431542 to the conditioned medium does not have a significant effect on the viability of myocytes. CM+CM n=9; FB+CM n=12; FB+CM+SB n=9; T SB +CM n=10; T FB+CM+SB n=6.

6.3.7. **TAC fibroblast conditioned medium induced hypertrophy, and this was blocked by SB 431542**

TAC fibroblast conditioned medium induce myocyte hypertrophy, but normal fibroblasts conditioned medium did not (Figure 6.8). This is different to when co-culture was used where both normal and TAC fibroblasts induced hypertrophy (see Figure 6.4). Blocking the myocyte TGF-β type 1 receptors prevented the hypertrophic response, which suggests fibroblast-derived TGF-β is directly driving myocyte hypertrophy.

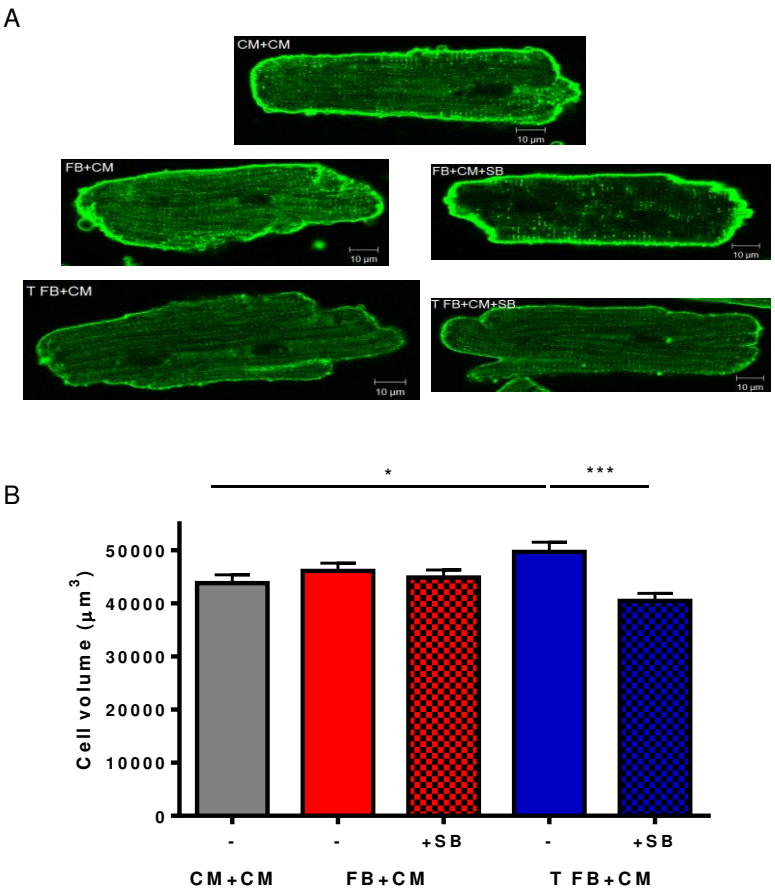


Figure 6.8: Myocyte volume was increased in TAC fibroblast conditioned medium, and this was blocked by SB 431542

A) Representative recording of di-8-ANEPPS staining. B) TAC, but not normal, fibroblast conditioned medium increased the size of the myocytes. This effect was blocked by SB 431542. CM+CM n=49; FB+CM n=48; FB+CM+SB n=49; T SB +CM n=50; T FB+CM+SB n=50.

6.3.8. **Myocyte Ca^{2+} transient were unaffected by conditioned medium**

The Ca^{2+} transients were unaffected by fibroblast conditioned medium (Figure 6.9). Unlike in co-culture, the Ca^{2+} transient amplitude and time to 90% decay were the same for control and myocytes exposed to normal or TAC fibroblast conditioned medium. These parameters are also not affected by the presence of the TGF-β type 1 receptor antagonist, although there is a direct effect of the drug on the time to peak which is increased in the presence of the antagonist.

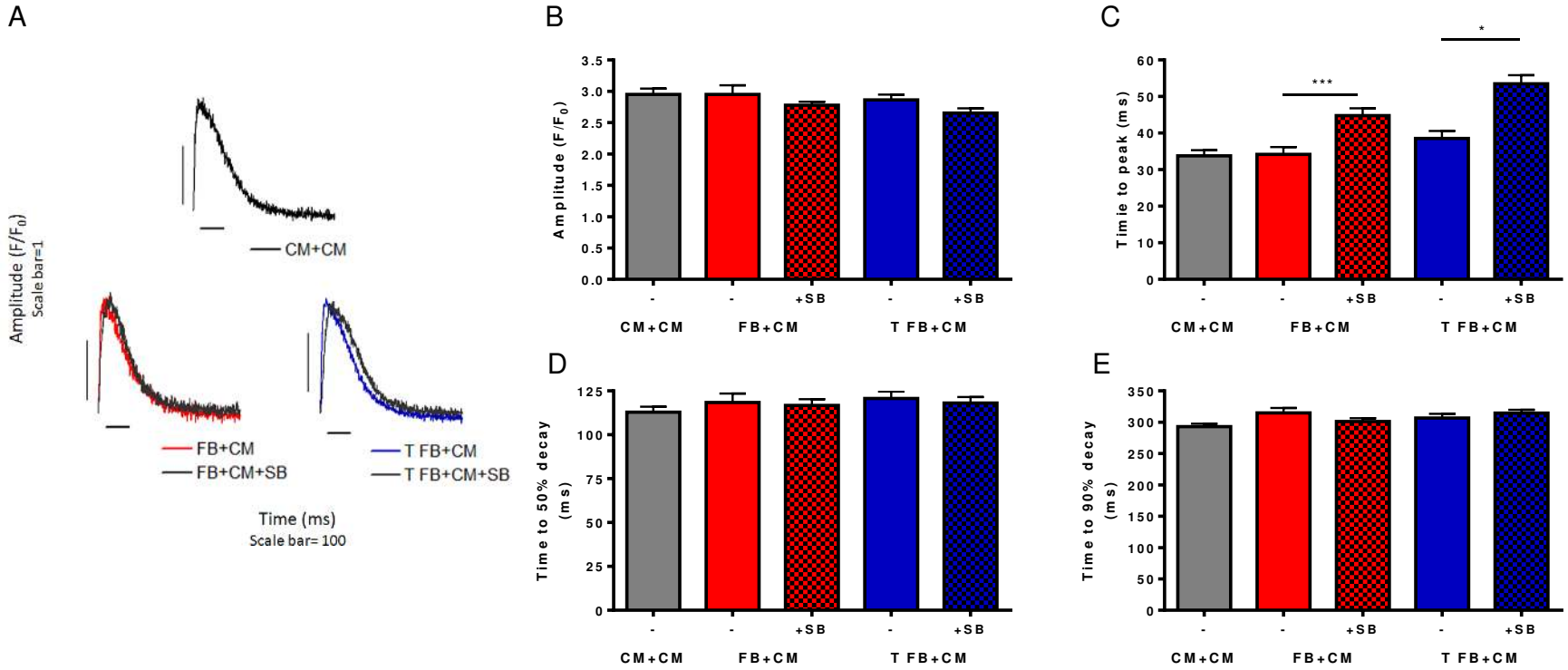


Figure 6.9: Myocyte Ca transients were unaffected by fibroblast conditioned medium

A) Representative recording of Ca^{2+} transients. Neither TAC nor normal fibroblasts conditioned medium affected the Ca^{2+} transient of myocytes normal myocytes over 24 hours. B) The amplitude, C) time to peak, D) time to 50% decay and E) time to 90% decay are all unaffected. SB 431542 caused a prolongation of time to peak. CM+CM n=49; FB+CM n=43; FB+CM+SB n=49; T SB +CM n=52; T FB+CM+SB n=47.

6.3.9. **TGF-β1 is present at different levels in fibroblast conditioned medium**

Due to the different effect of fibroblast conditioned medium to co-culture of fibroblasts and myocytes, the level of TGF-β1 in conditioned medium was measured. TGF-β1 was lower in shamfibroblast conditioned medium compared to the TAC fibroblast conditioned medium (Figure 6.10). These levels can be compared to the levels measured in co-culture (previously shown in Figure 6.2A). The level of TGF-β1 in sham fibroblast conditioned medium was lower than the level in sham fibroblast co-culture with myocytes ($p<0.01$) and also the TAC co-culture ($p<0.05$). The level of TGF-β1 was not different between TAC fibroblast conditioned medium and co-culture. The TGF-β1 expression in sham fibroblasts therefore appear to be modulated by the presence of the myocytes.

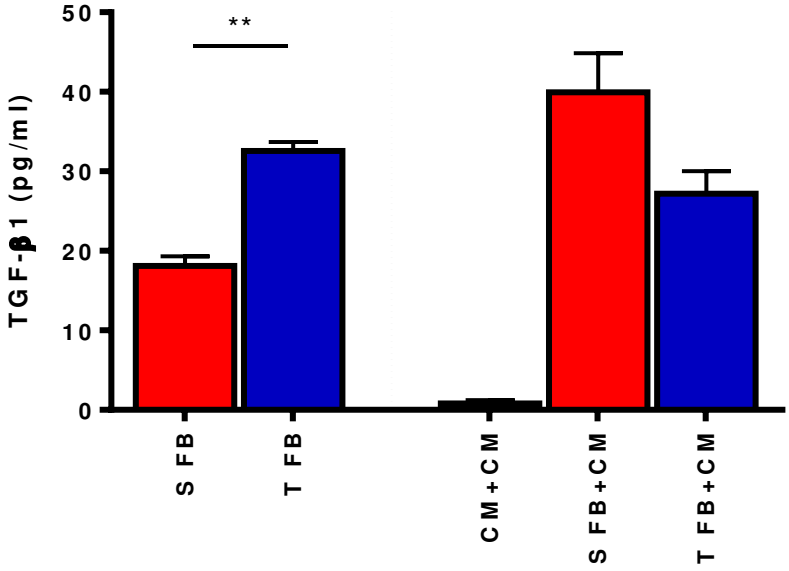


Figure 6.10: TGF-B1 is lower in sham fibroblast conditioned medium compared to TAC fibroblast conditioned medium

Sham fibroblast conditioned medium has a lower amount of TGF-β1 compared to TAC fibroblast conditioned medium. It is also lower than the level previously observed in the sham fibroblast co-culture and the TAC fibroblast co-culture. The level of TGF-β1 was not different between TAC fibroblast conditioned medium and TAC co-culture. S FB n=6; T FB n=6.

6.4. Discussion

This chapter has explored the role of TGF-β in the paracrine effects of fibroblasts on myocytes. When an antagonist to the TGF-β type 1 receptor was added to the co-cultures it blocked the reduction in viability, the increase in volume and the changes in the Ca^{2+} transient amplitude of the myocytes. Whether this was due to a direct effect of TGF-β on the myocytes or an indirect effect altering downstream mediators from the fibroblasts was investigated by adding the TGF-β type 1 receptor antagonist to fibroblast conditioned medium. In this experimental set up, only the myocyte TGF-β type 1 receptors are blocked. The results of these experiments show that TGF-β is directly responsible for some of the effects, for example the hypertrophic response, but is acting via the release of downstream mediators from the fibroblasts in other effects, for example the reduction in myocyte viability.

Firstly the level of TGF-β1 in the co-culture set ups was measured. It was raised in both the sham and TAC fibroblast co-cultures compared to control, with higher levels in the normal group. In a mouse model of TAC, TGF-β1 mRNA was initially raised although over time this returned to normal levels (Villarreal and Dillmann, 1992). It is possible that in established hypertrophy TGF-β1 protein levels drop below normal levels. It is also important to note that the ELISA used measures both latent and active forms of TGF-β1 and therefore the levels of active TGF-β1 in the co-culture may actually be different to those measured. MMPs can activate the latent form of TGF-β1 (Imai et al., 1997, Yu and Stamenkovic, 2000) and as the level of MMPs are altered following TAC (Nagatomo et al., 2000) the percentage TGF-β1 that is active may be higher in the TAC co-culture compared to the sham group. Also, the ELISA measured the level of TGF-β1, and not the level of TGF-β2 or 3. The three isoforms are translated from distinct genes and are regulated differently. For example, after myocardial infarction, elevated levels of TGF-β3 persist longer than TGF-β1 and 2, and TGF-β3 was localised to the scar region where as TGF-β1 and 2 were found throughout the LV (Deten et al., 2001). The levels of each isoform after TAC have not been characterised but may be different compared to control. Although only TGF-β1 levels have been measured, all three

isoforms can act via the TGF-β type 1 receptor and therefore are affected by the use of SB 431542.

6.4.1. **Viability**

The reduction in viability of myocytes in response to fibroblast paracrine mediators has previously been linked to apoptosis (Shivakumar et al., 2008), although necrosis and hyper-contraction may also explain these effects (discussed in Section 4.4.1). TGF-β is pro-apoptotic in various cells (Schuster and Krieglstein, 2002). The reports on the effect of TGF-β on myocyte apoptosis are conflicting. TGF-β has been shown to mediate myocyte apoptosis in response to Ang II (Schroder et al., 2006), but others studies have reported that TGF-β is protective against myocyte apoptosis in a model of ischemia–reperfusion injury (Yang et al., 1999). During ischemia-reperfusion in ferrets, TGF-β has been shown to be protective against necrosis by reducing neutrophil recruitment (Lefer et al., 1993). As there are no immune cells within the culture set up this is unlikely to contribute to the reduced viability. However, TGF-β can also increase SR Ca^{2+} release in neonatal myocytes (Neylon et al., 1994), which could result in excess Ca^{2+} release into the cytoplasm. This can trigger hyper-contraction of the myocytes which would also reduce their viability.

In the experiments reported here, the blockade of TGF-β type 1 receptors prevented the loss in myocyte viability in co-culture. However, when the antagonist was added to fibroblast conditioned medium the effect was diminished. There may be a smaller effect but it is not as pronounced and does not reach significance. TGF-β may therefore be having some direct effects, possibly by promoting apoptosis or hyper-contraction, but mainly appears to be having an effect by stimulating the release of secondary mediators from the fibroblasts. An example of a downstream mediator of TGF-β is ET-1 (Shi-wen et al., 2007). There are reports that ET-1 is pro- and anti-apoptotic in myocytes (Ogata et al., 2003, Mel'nikova et al., 2004, Ren et al., 2008a). ET-1 also raises the diastolic level of Ca^{2+} in myocytes and this may result in spontaneous hyper-contraction and reduced viability (Touyz et al., 1996). Therefore, the release of ET-1 from the fibroblasts in response to autocrine TGF-β signalling may

partially explain these results. There are many other mediators that can drive myocyte apoptosis that are released by fibroblasts, such as TNF-α (van Empel and De Windt, 2004).

6.4.2. **Hypertrophy**

In co-culture both normal and TAC fibroblasts induce myocyte hypertrophy and this is blocked by the TGF-β type 1 receptor antagonist. When using fibroblast conditioned medium, only the TAC fibroblasts induced significant hypertrophy, but this was also blocked by the TGF-β type 1 receptor antagonist.

TGF-β has been previously shown to induce myocyte hypertrophy (Gray et al., 1998, Rosenkranz et al., 2002). Our results are consistent with a direct hypertrophic effect of TGF-β, although it cannot be excluded that there is a further autocrine signalling pathway in the myocytes that is evoked by TGF-β and causing the hypertrophy, as described by Fredj *et al* (2006). The absence of a hypertrophic effect in the normal myocyte conditioned medium may be due to the lower levels of TGF-β in this medium compared to the other set ups used. This adds further support to the hypothesis that myocyte hypertrophy is a direct effect of TGF-β in these experiments.

The difference in the level of TGF-β1 in normal fibroblast conditioned medium and normal fibroblast co-culture suggests that fibroblasts are responding to a paracrine signal from the myocytes which induces a response from the fibroblasts altering their paracrine signalling, which in turn affects the myocytes (Figure 6.11). Ang II is the main mediator that has been investigated in the paracrine effects of myocytes on fibroblasts (Burstein et al., 2007, Tsai et al., 2011) and has been shown to induce TGF-β expression in fibroblasts (Campbell and Katwa, 1997, Tsai et al., 2011). It is possible that it is involved in the effects observed here. This paracrine signalling from the myocytes to fibroblasts is also evident in the effects on the Ca^{2+} transients.

6.4.3. **Ca^{2+} transients**

TGF-β can modulate the different elements that are involved in the Ca^{2+} transient including the ICa,L and properties of the SR. TGF-β reduces the level of ICa,L in neonatal myocytes (Neylon et al., 1994) but improves Ca^{2+} release from the SR (Avila et al., 2007). In adult cells TGF-β slows the time to peak and time to decay of the Ca^{2+} transient (Li et al., 2008).

In the co-culture experiments the effect of fibroblasts on Ca^{2+} transient amplitude were blocked by blocking TGF-β type 1 receptors although the reduced time to decay was not blocked. Li *et al* (2008) found that the amplitude of the Ca^{2+} transient was unchanged by TGF-β. Also, the fact that both the increase in the Ca^{2+} transient amplitude with normal fibroblasts and the decrease in Ca^{2+} transient amplitude with TAC fibroblasts were blocked challenges the hypothesis that this is a direct effect of TGF-β. These opposing effects could be a result of different levels of TGF-β, but may also be due to the release of other mediators from the fibroblasts in response to TGF-β.

When using conditioned medium no effect on the Ca^{2+} transient was seen with either normal or TAC fibroblasts. The lack of change in response to the conditioned medium despite the presence of TGF-β shows that this is not a direct effect of TGF-β, but rather through downstream mediators. This also suggests that a cross talk between the myocytes and fibroblasts is necessary (as also observed for the hypertrophic response in the normal fibroblasts group) and that the fibroblasts are responding to a signal from the myocytes.

Further work is required to gain insight into the other pathways involved. Ang II is a possible upstream signalling mediator from the myocytes to the fibroblasts. Due to the different effects of the normal and TAC fibroblasts, it appears that different mediators are released downstream of TGF-β. One possibility is ET-1 which is released downstream of TGF-β (Shi-wen et al., 2007). ET-1 increases the Ca^{2+} transient amplitude in healthy myocytes (Endoh et al., 1998) but reduces the Ca^{2+} transient

amplitude in myocytes from heart failure (Suzuki et al., 1998). The effects of ET-1 have been linked to changes in the ICa,L (Endoh et al., 1998) but ET-1 also affects SR Ca^{2+} handling, downregulating SERCA2a mRNA levels (Hilal-Dandan et al., 2009). ET-1 may therefore be responsible for some of the downstream effects of TGF-β. These putative pathways are highlighted in Figure 6.11.

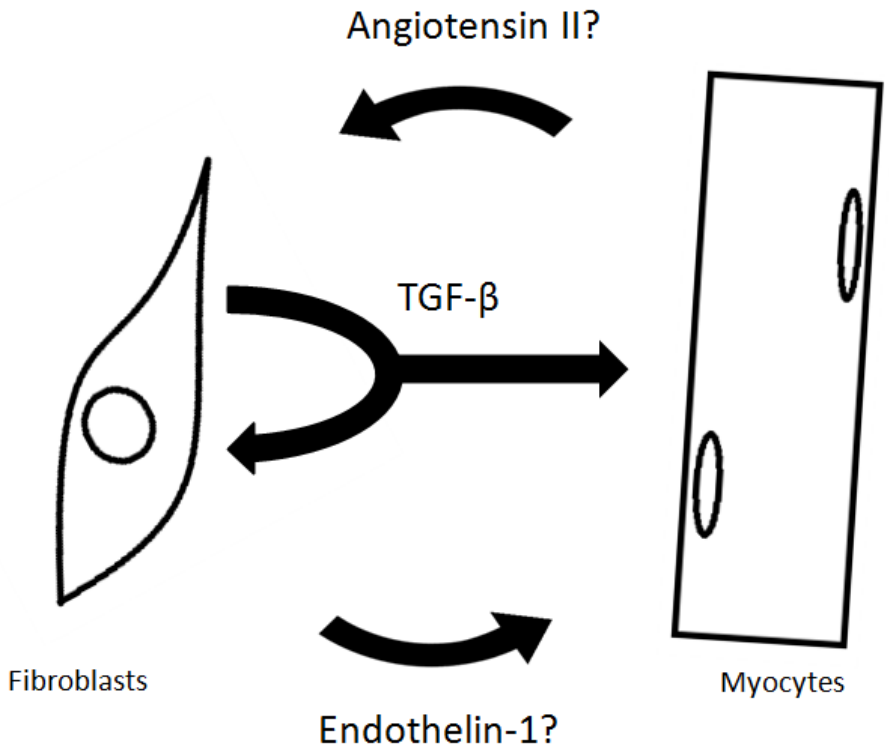


Figure 6.11: Possible pathways involved in the paracrine effects observed

A paracrine mediator released by myocytes upregulates TGF-β release in normal fibroblasts. This may be Ang II. Fibroblasts then release TGF-β which has direct effects on the myocytes but also stimulates the fibroblasts to release other paracrine mediators. Downstream of TGF-β, fibroblasts release other mediators which affect the myocytes. ET-1 is a candidate mediator that may be involved.

Alternatively, the absence of any effect in the conditioned medium experiments may be due to the degradation of a mediator within the conditioned medium. In co-culture there is a persistent production of mediators by fibroblasts throughout the myocyte

culture, whereas in the conditioned medium the mediators are produced by the fibroblasts which are then removed at the start of the myocyte culture. Therefore, the level of mediators throughout the myocyte culture may be lower due to degradation without continued production. It has been reported that over 24 hours in culture the levels of TGF-β and Ang II fall about 50% and the level of ET-1 falls about 10% (Squires et al., 2005). However, conditioned media have been used alongside co-culture previously and they have produced the same effects on myocyte hypertrophy and electrophysiology (Harada et al., 1997, Guo et al., 1999). Also in our experiments the effects of fibroblast derived mediators on myocyte viability and volume were still observed when using conditioned medium. Thus, the changes in the Ca^{2+} transient may be caused by a different mediator that is less stable and is degraded in the conditioned medium.

6.4.4. **Summary**

The experiments described in this chapter investigated the role of TGF-β in the paracrine effects of fibroblasts on myocytes using SB 431542, an antagonist of the TGF-β type 1 receptor. The results show that TGF-β directly mediates the hypertrophic response of the myocytes to fibroblasts. However, the reduction in viability appears to be at least in part due to a downstream mediator released from fibroblasts. The effects on the Ca^{2+} transient also appear to be mediated by a downstream mediator, although this is only released following a paracrine signal from the myocytes.

These results highlight the complexity of the paracrine interaction between fibroblasts and myocytes. TGF-β is an important mediator in these effects; however the effects are likely to be the result of a bi-directional dynamic interaction between the two cell types and involve a complex signalling network.

CHAPTER 7.

The paracrine effects of cultured fibroblasts on the electrical properties of myocardial slices

7.1. Introduction

Cardiac fibroblasts can affect the electrical properties of the myocardium through modulation of the ECM. Deposition of excess ECM produces regions of conduction slowing promoting conduction heterogeneity and therefore providing a substrate for unidirectional block and re-entry circuits leading to arrhythmias (Burstein and Nattel, 2008, Tan and Zimetbaum, 2011).

More recently interest has grown in the potential pro-arrhythmic effects of gap junctions between myocytes (Miragoli et al., 2006). The potential role of gap junctions between fibroblasts and myocytes has been discussed in detail in Section 1.5.1.1. Although fibroblasts are not excitable cells, they have a more positive resting membrane potential than myocytes (Jacquemet and Henriquez, 2007). Gap junctions between the two cells raise the resting membrane potential of myocytes. At low levels this brings the myocytes closer to the threshold of action potential initiation, increasing excitability and increasing conduction velocity. At higher levels, the depolarisation of the myocyte membrane potential reaches a level that leads to voltage inactivation and reduced availability of voltage gated Na^+ channels. This reduces action potential upstroke velocity and conduction velocity which promotes arrhythmias (Miragoli et al., 2006). Also, fibroblasts can couple myocytes over distances up to 300µm but this leads to delayed conduction and would further promote arrhythmias (Gaudesius et al., 2003). The potential effects of heterocellular gap junctions are large, leading to increased risk of arrhythmias (Nguyen et al., 2012), but conclusive evidence of their existence *in vivo* is still lacking.

Fibroblasts can also affect the electrophysiology of myocytes via paracrine mediators. *In vitro* fibroblast-derived paracrine mediators affect channel expression, currents and action potential morphology in neonatal and adult myocytes (Guo et al., 1998b, Guo et al., 1999, Kaur et al., 2013). However, the use of neonatal myocytes allows for the study of conduction velocity due to the production of a confluent monolayer (Pedrotty et al., 2009, Vasquez et al., 2010) whereas in isolated adult myocytes the study of conduction velocity is not possible. For this reason, whether fibroblast-derived

paracrine mediators affect the multicellular electrophysiological properties of adult myocardium has not been addressed. To examine the effects of fibroblasts on conduction velocity in the adult heart we utilised myocardial slices. These are thin viable sections of ventricular tissue (described in detail in Section 2.3.) that allow the field potential and conduction velocity of intact adult myocardium to be examined. Furthermore, previous studies have used rat myocytes, whereas the experiments in this chapter were conducted with dog tissue. The electrophysiology of the dog heart is more similar to human electrophysiology compared to other animals including the rat (Lompre et al., 1981, Su et al., 2003) making it a more relevant species to study.

7.1.1. **Chapter aims**

The experiments reported in this chapter aim to assess the paracrine effect of cardiac fibroblasts on adult dog myocardial tissue electrophysiology. Using myocardial slices, the effects on field potential duration and conduction velocity of adult cardiac tissue can be addressed. Possible mechanisms will be investigated using western blotting to assess protein expression.

7.2. Methods

A schematic of the experimental protocol is shown in Figure 7.1A.

7.2.1. **Cell, tissue and co-culture preparation**

Beagle dog hearts were provided by GlaxoSmithKline. Dogs were euthanised with pentobarbital and the heart was rapidly removed and couriered to the lab in ice cold cardioplegia.

Fibroblasts were prepared as described in Section 2.3.4. Due to the unpredictable supply of tissue it was not possible to use freshly isolated fibroblasts at an early time point of culture. Freezing the cells after 24 hours in culture was attempted but was unsuccessful. Therefore, the fibroblasts were kept in culture for 6-8 days in FB medium

and then frozen for long-term storage as follows. The cells were collected from culture using trypsin. They were centrifuged at 620*g* for 5 minutes and then resuspended in 3ml 90% foetal bovine serum, 10% DMSO at 4°C. This was frozen slowly in 1ml aliquots. They were kept on ice for 5 minutes, then at -20°C for 1 hour and then stored at -80°C overnight before being transferred to liquid nitrogen until they were needed. The fibroblasts were thawed two days before they were required. The frozen aliquot was rapidly warmed at 37°C and then added to 5ml pre-warmed FB medium. After 4 hours incubation at 37°C in 5% CO_2 the medium was replaced with fresh medium.

Myocardial slices were prepared as described and put into co-culture as described in Section 2.5.2. Slices were cultured on Transwells at the interface of the H.ITS medium and air, in co-culture with 40,000 fibroblasts (Figure 7.1B). Co-culture with 40,000 HEK cells or culture of the slice alone was used for the control. After 24 hours in culture, the MEA output of the slices was recorded again. The slice was then snap frozen for protein analysis by SDS PAGE and western blotting.

Fibroblasts were isolated from 3 dog hearts and slices were prepared from 4 dog hearts. Fibroblasts were used for co-culture between passages 2-4 and 9-12 days of culture.

7.2.2. **SDS PAGE and western blot analysis of connexin 43**

The level of connexin 43 protein in the slices after co-culture was analysed by SDS page and western blot. The SDS page was run as described in Section 2.8., using 0.125µg of protein for each sample. The primary antibody used for the detection of connexin 43 was rabbit anti connexin 43 antibody (Sigma, USA; Cat. No. C6219) at a dilution of 1:1500 in blocking solution, and the secondary antibody was donkey anti-rabbit alkaline phosphatase-conjugated (Pierce, USA; Cat. No. 31345) at a dilution of 1:2000.

The amount of connexin 43 was normalised to the level of actin to provide accurate comparison between each sample. Due to the similar molecular weight of actin and connexin 43 it was not possible to measure both on a single blot. Therefore actin was

measured on a separate gel. 0.25µg of protein was loaded per sample and the primary antibody used was goat anti-actin (Santa Cruz Biotechnology, USA; Cat. No. sc-1616) at a dilution of 1:1000 and the secondary antibody was an alkaline phosphatase-conjugated rabbit anti-goat antibody (Pierce, USA; Cat. No. 31300) at a dilution of 1:2000.

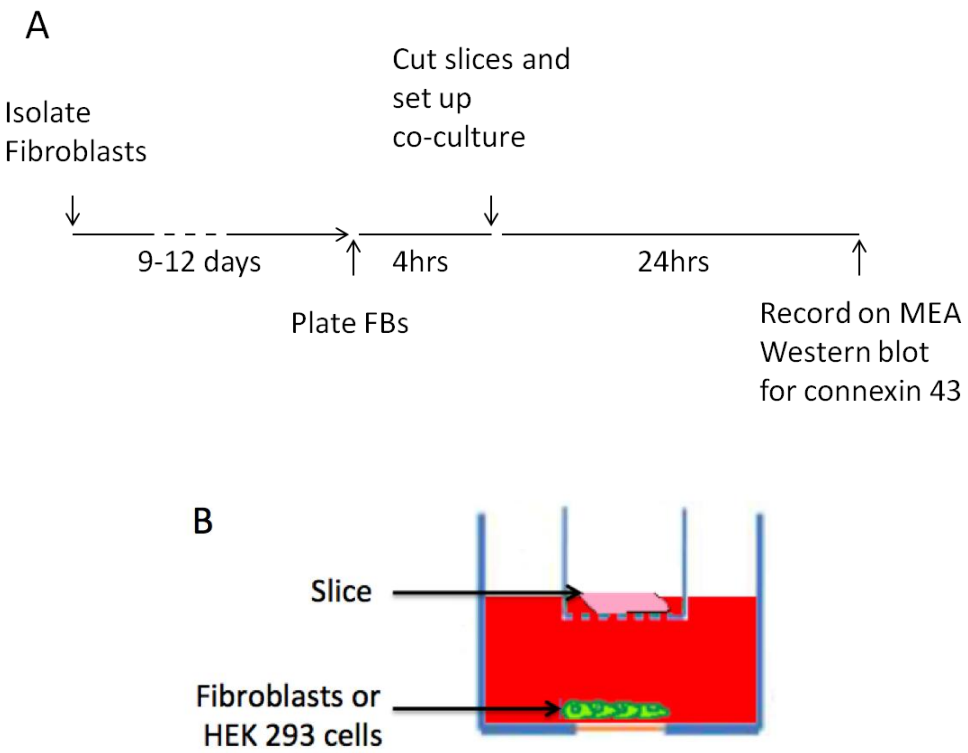


Figure 7.1: Schematic of the experimental protocol and co-culture set up

A) The experimental protocol. B) The co-culture set up.

7.3. Results

7.3.1. **Cultured fibroblasts express α-SMA**

Dog fibroblasts were used between 9 and 12 days after isolation, at passage 2 or 3. At this time point the cells were vimentin positive (184/184) and also DDR2 positive (140/140) and desmin negative (0/132) which showed that they were fibroblasts

without smooth muscle cell contamination. The cells were also stained for CD-31, a marker for endothelial cells, and were negative (0/88). The majority of the fibroblasts were also α-SMA positive (116/147) (Figure 7.2).

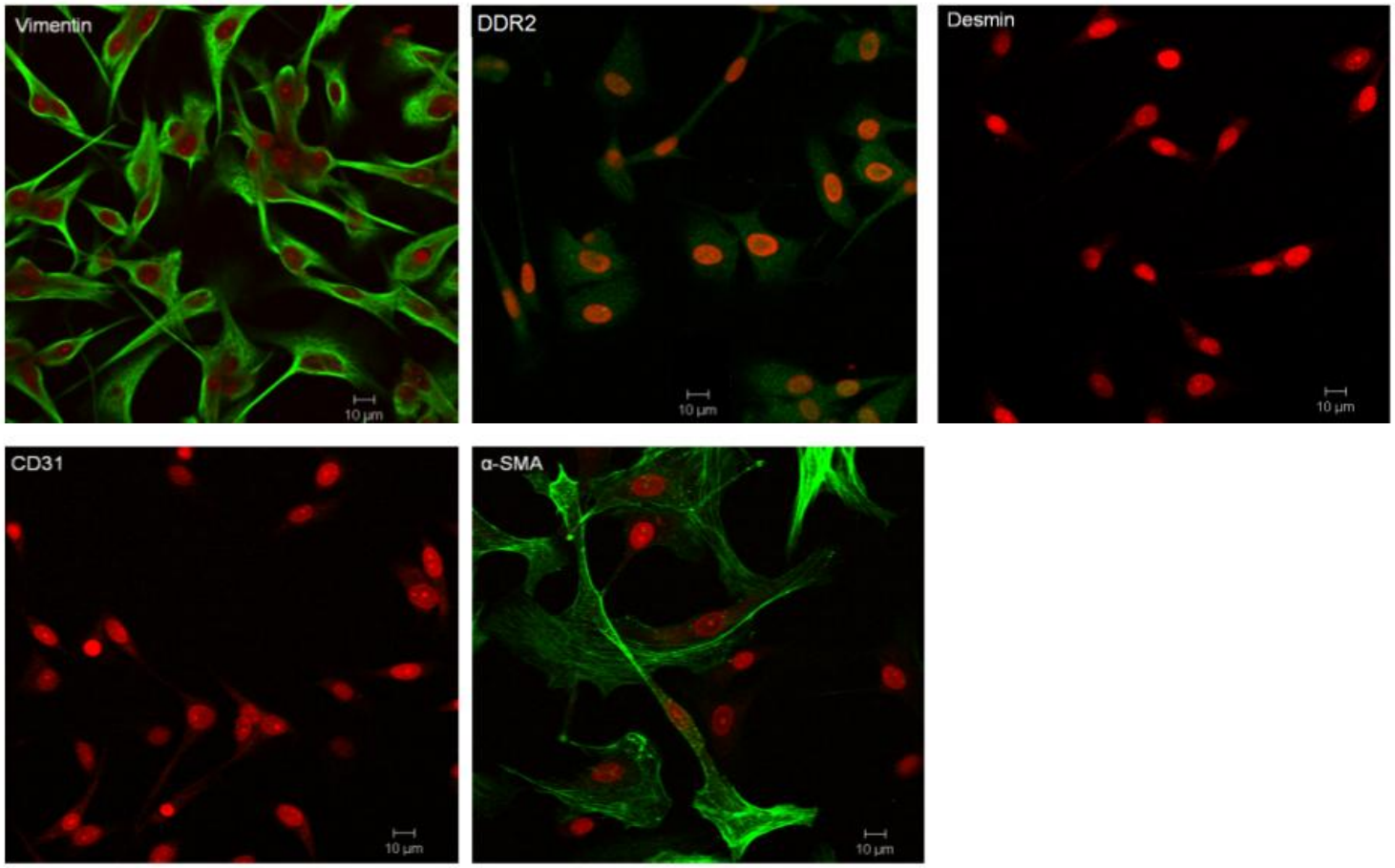


Figure 7.2: The dog fibroblasts used for co-culture express α-SMA

The isolated dog cells were characterised as fibroblasts by the expression of vimentin and DDR2 and the absence of desmin and CD31. The majority of the cells were also positive for α-SMA the time point of use (79%). Red: nuclear marker.

7.3.2. **A slice contains approximately 40,000 fibroblasts**

Slices were cut from a $1cm^3$ block of tissue with a thickness of 300µm, giving a final volume of 1cm by 1 cm by 300µm. There is no apparent quantification of the actual increase in the number of fibroblasts in cardiovascular disease in the literature, but it was decided the number of fibroblasts that was co-cultured with the slices was to match the number of resident fibroblasts within the slice. This would double the number of fibroblasts present within the healthy myocardium to represent the increase in fibroblasts in disease. It was therefore necessary to count the number of fibroblasts within the slice. To achieve this, the tissue was stained for vimentin and a nuclear marker. The number of fibroblasts (vimentin-positive cells) within a $1mm^2$, 15µm thick section was counted and used to estimate the number of fibroblasts in the whole slice. The number of cells within the slice was 41,571±2,303 (Figure 7.3). Therefore 40,000 fibroblasts would be co-cultured with each slice.

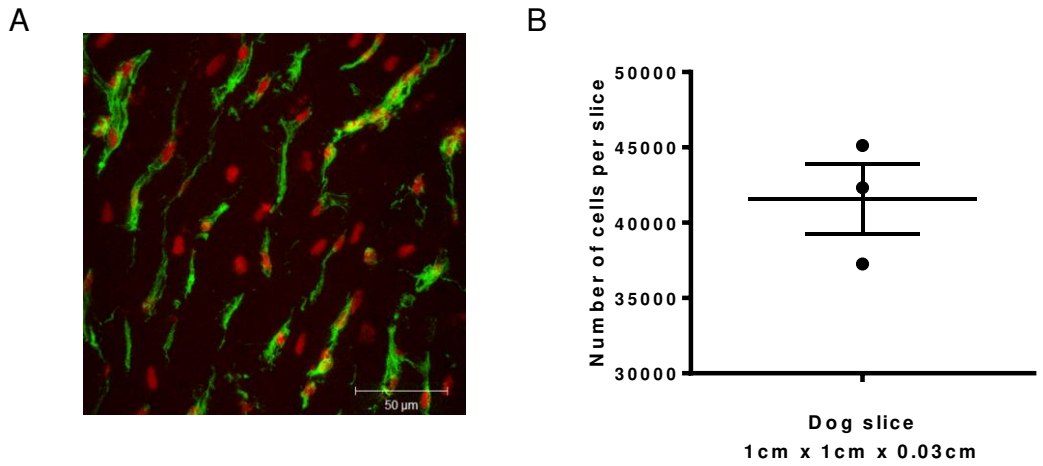


Figure 7.3: Each slice contained approximately 40,000 fibroblasts

A) Representative staining of a section of a slice. Green: Vimentin. Red: Nuclear marker. B) The number of fibroblasts within a slice, calculated by counting the number of cells in a $1mm^2$ section and multiplying up for the whole slice. n=3 sections from 1 slice.

7.3.3. **The field potential duration is not affected by co-culture with fibroblasts**

The field potential is the extracellular voltage change associated with the action potential. Field potential duration was not different between control slices and slices co-cultured with fibroblasts (Figure 7.4). Increased variability of action potential duration is pro-arrhythmic and as a measure of this the standard deviation of the field potential between 16 different points in a slice was calculated. The standard deviation of the field potential duration was not changed by fibroblasts.

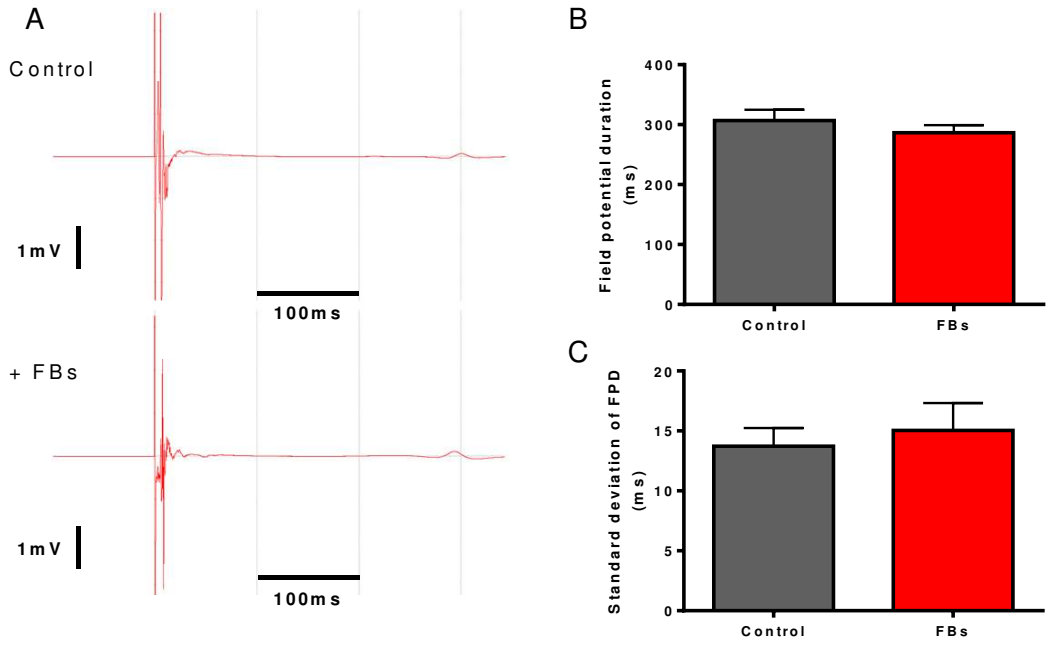


Figure 7.4: The field potential duration is unaffected by co-culture with fibroblasts

A) Representative recording of the field potential at a single electrode of the MEA system from a control slice and a slice co-cultured with fibroblasts. B) The field potential duration and C) the standard deviation of field potential duration within each slice were unchanged by co-culture with fibroblasts. Control n=7; FBs n=12.

7.3.4. **Longitudinal conduction velocity is lower after co-culture with fibroblasts**

The longitudinal conduction velocity (the conduction velocity in the direction of the muscle fibre orientation) was lower after co-culture with fibroblasts compared to

control slice culture (Figure 7.5). The transverse conduction velocity (the conduction velocity perpendicular to the direction of the muscle fibre orientation) was not significantly different between the two groups.

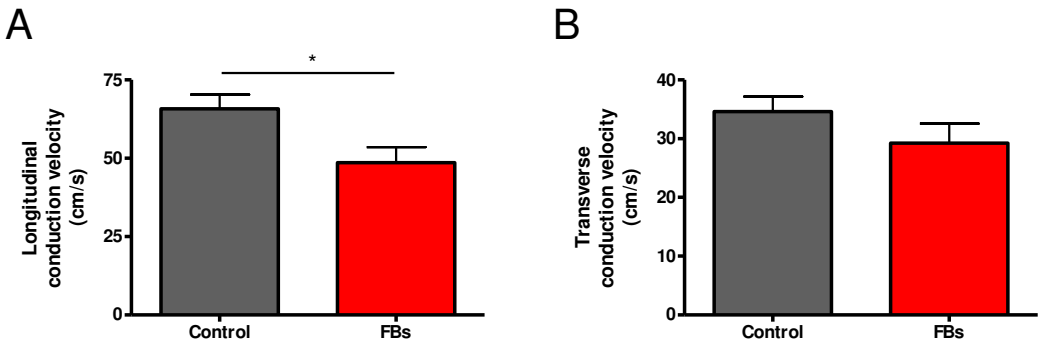


Figure 7.5: Longitudinal conduction velocity was lower after co-culture with fibroblasts

A) The longitudinal conduction velocity of slices was lower when co-cultured with fibroblasts compared to control cultures. B) The transverse conduction velocity was not significantly different between the two groups. Control n=8; FBs n=12.

Longitudinal conduction velocity is highly dependent on gap junctions at the intercalated disks allowing rapid electrical coupling between adjacent cells. In ventricular myocytes the predominant gap junction forming connexin is connexin 43 (Davis et al., 1995, Vozzi et al., 1999). A reduction in connexin 43 may explain the lower conduction velocity after co-culture with fibroblasts. Therefore, to investigate the potential mechanism underlying the change in conduction velocity, the level of connexin 43 was measured by western blotting.

7.3.5. **Connexin 43 is unchanged after co-culture with fibroblasts**

Connexin 43 was not significantly different in slices co-cultured with fibroblasts compared to control slice (Figure 7.6) although there was a non significant reduction in the mean connexin level.

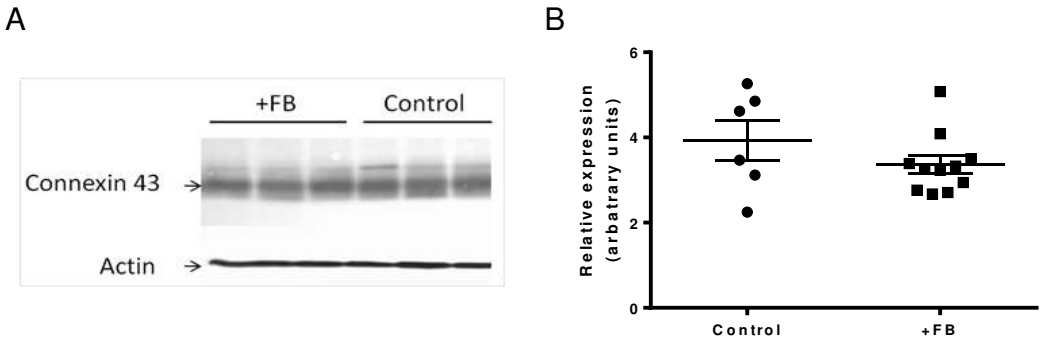


Figure 7.6: The level of connexin 43 protein is not significantly different after co-culture with fibroblasts

A) Representative western blot recording of connexin 43 and actin levels from 6 samples. B) Connexin 43 levels, normalised to actin, were not significantly different in myocardial slices after 24 hour co-culture with fibroblasts compared to control. Control n=6; FBs n=11.

7.4. Discussion

The results reported in this chapter show that cultured fibroblasts affect the electrophysiology of adult myocardial tissue through paracrine mediators. Co-culture of myocardial slices with fibroblasts slowed the longitudinal conduction velocity, although transverse conduction velocity and the field potential duration are unaffected. The level of connexin 43 protein was unchanged.

7.4.1. **Field potential duration**

The effects of fibroblasts on myocyte field potential/action potential have previously been investigated in neonatal myocytes and recently in isolated adult myocytes. Although we found that the field potential was not significantly different after co-culture with fibroblasts, previous reports have found changes in both the action potential duration and the underlying currents but the nature of these reported changes are conflicting. Guo *et al* (1999) found that the action potential of neonatal myocytes was prolonged at time-to-25% repolarisation, although not at time-to-80%

repolarisation, by fibroblasts conditioned medium. A decrease in the transient outward K^+ current was also observed that may explain this change. Pedrotty *et al* (2009) observed a prolongation of the action potential of neonatal myocytes at time-to-90% repolarisation with a reduction of the transient outward K^+ current and also the voltage gated Na^+ current and the inward rectifying K^+ current. In adult myocytes, fibroblast conditioned medium reduced the transient outward K^+ current, but increased the voltage gated Na^+ current. The overall effect on the action potential was not reported but these changes are consistent with a prolongation of the action potential (Kaur et al., 2013). However, Vasquez *et al* (2010) found that the action potential duration was reduced by medium conditioned with fibroblasts from a model of myocardial infarction, and found no effect with normal fibroblasts.

The lack of an effect observed with normal fibroblasts by Vasquez *et al* (2010) as well as the lack of change at time-to-80% repolarisation reported by Guo *et al* (1999) matches what was observed in our experiments. However this does not fit with the reports by Pedrotty *et al* (2009) or Kaur *et al* (2013). The underlying cause of the differences in these reports is not clear. All the previous studies used cells from normal rats kept in culture, although Guo *et al* (1999) and Pedrotty *et al* (2009) use neonatal cells, Vasquez *et al* (2010) uses adult fibroblasts and neonatal myocytes, and Kaur *et al* (2013) uses adult fibroblasts and myocytes. We have used adult dog cells and tissue. There are considerable differences in the electrophysiology and in fibroblasts between species and it is possible that the effects may be different (Lompre et al., 1981, Dawson et al., 2012). The previous reports have used conditioned medium whereas we have used co-culture. In the previous reports there are differences in the conditioning protocol used for the production of the conditioned medium and also in the medium used. Furthermore, the length of time the myocytes/myocardial slices were kept in co-culture or conditioned medium was different between the reports, and it has been previously shown that mediators can have transient effects on electrophysiology (Zhang et al., 2001). Whatever underlies the differences observed in these reports, they make it difficult to hypothesise about the effects of fibroblast in the intact heart. The use of the myocardial slices rather than isolated myocytes in our experiments is a better representation of the intact myocardium, as the myocytes are in an

environment closer to that of the intact heart, with the associated ECM, fibroblasts and other cell types. Therefore, this work examines the effect on whole myocardial tissue rather than just on myocytes including effects on non myocyte cells within the slice. Also, dog cardiovascular physiology is more similar to humans than rats are and therefore these results may be more applicable to the human heart.

A possible criticism is that field potentials are not accurate in representing action potential morphology. However, using the same set up as used here, pharmacological intervention to block K^+ currents, which are known to affect the action potential, prolonged the recorded field potential duration (Camelliti et al., 2011). Our work therefore supports some of the published data that reports no change in the field potential/action potential duration in response to fibroblast-derived mediators. However, this is an area of research that requires further investigation due to the conflicting nature of published data.

7.4.2. **Conduction velocity**

Neonatal myocytes have been essential for previous investigations of conduction velocity. When isolated and put into culture, neonatal myocytes form a confluent monolayer that is electrically continuous whereas adult myocytes remain as single cells. Therefore conduction velocity cannot be measured in isolated adult cells. However, myocardial slices keep myocardial tissue intact, and in particular maintain the electrical continuity between myocytes formed by gap junction. They therefore allow the measurement of conduction velocity with adult myocytes. The use of adult rather than neonatal myocytes had obvious advantages due to the maturity of the cells. Slices also have the advantage of maintaining anisotropy; that is the presence of the muscle fibres allows longitudinal and transverse conduction velocity measurements. In rat neonatal myocyte monolayers conduction is homogeneous and do not allow for longitudinal and transverse measurements. The conduction velocity of rat neonatal myocytes is approximately 18cm/s (Pedrotty et al., 2009, Vasquez et al., 2010). This is markedly different from the intact rat heart which has a longitudinal conduction velocity of 69cm/s and a transverse conduction velocity of 33 cm/s (Rossi

et al., 2008). The longitudinal conduction velocity of a dog heart is 58cm/s and the transverse conduction velocity is 25cm/s (Roberts et al., 1979). Our results show a longitudinal conduction velocity of 66cm/s and a transverse conduction velocity of 37cm/s. It is therefore clear that the results with slices are much more representative than neonatal myocyte monolayers. The presence of non myocytes and the intact ECM also makes myocardial slices a better model as these are important determinants of cardiac electrical activity and conduction velocity (Clayton et al., 2011).

Co-culture with cultured fibroblasts slowed the longitudinal conduction velocity, but did not affect the transverse conduction velocity. Both Pedrotty *et al* (2009) and Vasquez *et al* (2010) report that fibroblast conditioned medium slowed conduction velocity although Vasquez *et al* (2010) found that conduction was only significantly reduced with fibroblasts from a model of myocardial infarction. The paracrine slowing of conduction velocity may represent a pro-arrhythmic effect of fibroblasts. Heterogeneous increases in the number of fibroblasts in disease could slow the conduction velocity in areas of the myocardium and therefore produce conduction heterogeneity and a substrate for arrhythmia initiation (Baum et al., 2012).

The possible mechanisms of reduced conduction velocity include changes in the resting membrane potential of myocytes or alterations in gap junction coupling between cells, as well as changes in fibroblasts and ECM content interfering with conduction (Shaw and Rudy, 1997). Lowering the resting membrane potential of myocytes would reduce their excitability and therefore slow the conduction velocity. Alternatively, raising the resting membrane potential to the level that voltage-gated Na^+ channels were inactivated could also reduce conduction velocity (Buchanan et al., 1985). A reduction in gap junctions would decrease the rapid transmission of electrical signal between myocytes and would slow conduction velocity (Rohr, 2004). Gap junctions are predominantly located at the intercalated disk between myocytes within muscle fibres (Gourdie et al., 1991) and therefore are particularly important in the longitudinal conduction velocity. An increase in fibroblasts or ECM content would insulate between muscle fibres and be expected to affect transverse conduction. Therefore due to the specific reduction in longitudinal conduction velocity observed,

the level of connexin 43, the major connexin isoform in myocardial myocytes was measured.

7.4.3. **Connexin 43**

Previously, fibroblasts have been shown to reduce neonatal myocyte conduction velocity without a change in the level of connexin 43, but rather, the reduction in conduction velocity was linked to changes in the electrophysiology of these cells (Pedrotty et al., 2009). However, i*n vivo* it has been suggested that in the infarct border zone following myocardial infarction fibroblasts downregulate connexin 43 through paracrine release of interleukin 1β (Baum et al., 2012). Therefore, the effect of fibroblast-derived mediators on connexin 43 is still debated. We found that connexin 43 was not significantly reduced in slices after co-culture with fibroblasts. A post hoc power calculation was run[10] and the sample size required for an α error level of 5% and a β error level of 50% was found to be 16. It is therefore possible that this experiment is underpowered and producing a false negative result.

Despite the absence of a change in the level of connexin 43 protein, it is possible that a reduction in connexin 43 function was responsible for the reduction in longitudinal conduction velocity. In cardiovascular disease it has been shown that the localisation of connexin 43 to the intercalated disk is lost, and that the protein is found more ubiquitously around the cell membrane (Smith et al., 1991). This would reduce the transmission of the action potential across the intercalated disk and may explain the reduction in longitudinal conduction velocity observed. Also, connexin 43 function is altered by phosphorylation and this is another pathway by which connexin 43 function could be reduced despite the unchanged level of total protein (Lampe et al., 2000).

Canine ventricular myocytes also express connexin 40 and connexin 45 which are active in the formation of gap junctions (Kanter et al., 1992, Kanter et al., 1993). Down regulation of either connexin 40 or 45 is another alternative explanation for the reduced conduction velocity (Kirchhoff et al., 1998). Changes in the electrophysiology

[10] http://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/samplesizecalculators.aspx

of the individual myocytes within the slice, such as changes in resting membrane potential, could also underpin these observations and could not be excluded in this set of experiments.

7.4.4. **Summary**

In this chapter we have shown that co-culture with cultured fibroblasts reduces the longitudinal conduction velocity in adult myocardial tissue. This adds to data previously showing slowing of conduction in neonatal myocytes and represents a possible pro-arrhythmic effect of fibroblasts. The level of connexin 43 was measured to investigate a possible mechanism underlying these effects but was not significantly altered.

CHAPTER 8.

General Discussion

This project investigated the hypothesis that fibroblasts can modulate myocyte size and excitation contraction coupling via the release of paracrine mediators, and that these effects are altered in disease. We set out to investigate potential paracrine effects using adult rather than neonatal myocardium, to allow analysis in the setting of mature excitation contraction coupling, and adult fibroblasts used before they are activated to express α-SMA in culture, to improve the applicability of data to the *in vivo* environment (although this was not possible with the dog fibroblast work).

Initially the effects of normal rat fibroblasts were investigated, and these were found to reduce myocyte viability and increase the myocyte volume and Ca^{2+} transient amplitude. Next we showed that these effects are altered when using rat fibroblasts from pressure overloaded hearts. Although the loss of cell viability and increase in cell size was similar between pressure overload and control fibroblasts, the effect on the Ca^{2+} transient was different with fibroblasts from pressure overloaded hearts causing a reduction in the amplitude of the Ca^{2+} transient.

To examine the effects of fibroblasts on myocardial electrical activity in the multicellular setting, dog fibroblasts were co-cultured with myocardial slices. Co-culture with fibroblasts slowed the longitudinal conduction velocity without altering transverse conduction velocity or the action potential duration and variability.

The potential mechanisms of these observations were also investigated. In the cellular work, TGF-β type 1 receptors were blocked both in co-culture and in myocytes exposed to fibroblasts conditioned medium. TGF-β type 1 receptors appear to directly mediate the hypertrophic response of myocytes. They are also involved in the reduced viability and changes in the Ca^{2+} transient, however, these effects are mediated by downstream mediators released by fibroblasts in response to TGF-β. Signalling from the myocytes to the fibroblasts also modulate the effects of fibroblasts on the myocytes, as normal fibroblasts conditioned medium does not induce hypertrophy in normal myocytes and the effects on the Ca^{2+} transient observed in co-culture were absent when using conditioned medium.

The level of connexin 43 was measured in the myocardial slices as a reduction in connexin 43 could explain the changes in longitudinal conduction velocity. However, the level of connexin 43 was not significantly affected after co-culture with fibroblasts.

8.1. Strengths and limitations

One of the major aims at the outset of this thesis was to work with fibroblasts before their activation in culture. This was assessed by measuring α-SMA development and the fibroblasts were used before they had been activated to express α-SMA in culture. This is a strength of this work when compared to previous investigations into fibroblast paracrine activity that have used longer time periods and less tightly controlled fibroblast culture. Furthermore, adult myocytes and myocardial tissue was used throughout. Again, this is a major strength of this work as the majority of previous work has worked with neonatal cells. The use of adult myocytes and tissue allows a better insight into the effects on myocyte function due to the maturity of the systems involved in excitation contraction coupling. Finally, this thesis has directly compared fibroblasts from normal and pathological hearts, an essential element of understanding the potential roles of fibroblasts in cardiovascular health and disease. Vasquez et al (2010) is the only previous report to compare normal and diseased fibroblasts, and all other previous reports have only examined the effects of cultured fibroblasts from normal hearts.

It is important to note that these major attributes of this work come with inherent weaknesses. The use of fibroblasts within 48 hours of isolation also limits the depth at which it was possible to investigate the changes observed. By using the fibroblasts at this early time point the number of co-culture set ups was lower than if fibroblasts had been allowed to proliferate in culture. Therefore, the mechanisms behind the changes in Ca^{2+} handling such as SR Ca^{2+} content could not be fully investigated. Also, all co-culture set ups were used for functional experiments and therefore could not be used to investigate molecular changes using western blots, to look at protein changes, or polymerase chain reaction, to look at mRNA changes. This is a limitation that may be overcome by increasing the number of animals used, but was not feasible in this set of

experiments. Furthermore adult myocytes and myocardial slices are altered by culture, so although they are more mature than neonatal preparations they are limited by evident changes in the function of the myocytes and the slices over the 24 hours of culture used. It also cannot be excluded that there are changes in the fibroblasts despite the absence of α-SMA development.

Another limitation of this work was that only 1 ratio of fibroblasts to myocytes/myocardial slice was used. Furthermore, only 1 time point was measured after 24 hours. The effects with different ratios of fibroblasts to myocytes, which is something that changes in disease states (Zeisberg et al., 2007), requires further future studies. It would also have been interesting to look at a longer time course to see if the effects were different.

8.2. Implications and further work

This works add to the growing understanding of how fibroblasts can modulate myocyte structure and function. The exciting concept in this field of work is that fibroblasts may respond to pathological stimuli and then induce the myocardial response to these stimuli through paracrine communication. Fibroblasts are known to be sensitive to traditional pathological stimuli such as mechanical stress, biochemical mediators (such as Ang II) and hypoxia (Yokoyama et al., 1999, Roy et al., 2003, Porter and Turner, 2009). Increasing our understanding of what effects fibroblasts have on myocytes, and the potential pathways involved, may therefore eventually open up novel therapeutic targets to interfere with the development of cardiovascular disease.

We have shown that fibroblasts from pressure overloaded hearts induce hypertrophy in myocytes in co-culture or through conditioned medium, whereas fibroblasts from normal hearts only induce hypertrophy in response to signals from the myocytes. This backs up previous observations that fibroblasts can induce hypertrophy (e.g. (Harada et al., 1997)), and the *in vivo* models that show fibroblasts paracrine communication is important in myocyte hypertrophy in response to pressure overload or isoproterenol infusion (Jaffre et al., 2009, Takeda et al., 2010).

A novel finding of this thesis is the change in the paracrine effects of fibroblasts on myocyte Ca^{2+} transients after aortic constriction and pressure overload. Normal fibroblasts were found to increase the Ca^{2+} transient amplitude whereas fibroblast from TAC decreased the Ca^{2+} transient amplitude. Depression of Ca^{2+} transient amplitude is a characteristic of myocytes in failing hearts and it is therefore interesting to speculate that fibroblasts may be responding to pressure overload and contributing to myocyte dysfunction, which would have clear therapeutic implications.

Another relevant finding was the loss of myocyte viability in response to fibroblast-derived mediators. Myocyte loss is a characteristic of multiple cardiovascular diseases such as hypertrophic cardiomyopathy, atrial fibrillation and ischaemic heart disease (Olivetti et al., 1996, Aime-Sempe et al., 1999, Harvey and Leinwand, 2011). A possible role in ischaemic heart disease is particularly interesting when the observations of myocyte loss is coupled with the previous reports that fibroblasts are sensitive to changes in O_2 levels (Roy et al., 2003, Kang et al., 2004, Shivakumar et al., 2008). Again, it is interesting to hypothesise that fibroblasts may be responding to external stimuli and driving the changes in myocytes through paracrine communication.

We have also shown that fibroblasts can affect the electrophysiology of adult myocardial tissue. The reduction of longitudinal conduction velocity is potentially pro-arrhythmic, and it is important to ascertain whether these results are relevant to fibroblasts in normal and disease settings.

This work adds to the understanding of the communication between fibroblasts and myocytes, but raises many questions that will require further work to answer. Further work should be focused on two areas:

Firstly, future work should aim at providing a more detailed mechanistic insight into the effects that have been observed here. We have shown that there is a signal arising from the myocytes that affects the fibroblasts. Identification of what mediators are involved in this signal and the effects on the fibroblast phenotype is equally important as understanding the signalling from the fibroblasts to the myocytes. In this respect we

have shown that TGF-β is involved, but that there also appears to be downstream mediators released from the fibroblasts in response to TGF-β. Identification of these downstream mediators is essential in understanding this relationship. Finally, the mechanisms behind the changes observed require elucidating. Putative mechanisms have been discussed within each chapter but clarification of the mechanisms is important. Of particular interest are the level of SR Ca^{2+} and SERCA that may underlie the changes in Ca^{2+} transients. Finally, the effects of freshly isolated fibroblasts from normal and diseased hearts on myocardial slices would improve the applicability of the data.

Secondly, future work should look to translate these results into *in vivo* settings. The difficulties working with cultured fibroblasts, myocytes and myocardial slices have been discussed at length. This thesis has used fibroblasts at as early a time point of culture as possible when examining paracrine communication *in vitro*. This was important to provide the most applicable data to cardiac physiology possible. Therefore, to take this further *in vivo* work is required. The production of fibroblast-selective transgenic and knock out animals is a young field but has already shown that fibroblast-derived paracrine mediators are important in the hypertrophic response of the myocardium. It is through examination of myocyte excitation contraction coupling in these animals that the results here can be applied to *in vivo* physiology, ultimately improving our understanding of cardiovascular disease.

The use of transgenic animals will allow the investigation of the effects observed in this work to be investigated in an *in vivo* setting. Initially *in vivo* intervention could be used to alter fibroblast function before using the co-culture set up described in this thesis. Due to the time frame of the experiments involved *in vitro* modification of fibroblasts using shRNA is not feasible. However, through isolation of fibroblasts from genetically modified animals that either lack or overexpress TGF-β, its role in the relationship could be explored further by looking at the effect of these cells on isolated myocytes. This would still require an *in vitro* preparation but developing the use of myocardial slices to study fibroblasts may allow further transition towards *in vivo* understanding. As myocardial slices contain fibroblasts and myocytes they could be used as a model to

understand the paracrine interaction without the addition of external fibroblasts, and this would be a further step towards an understanding of *in vivo* paracrine interaction. Myocardial slices from genetically modified animals or the addition of pharmaceutical intervention would allow study of interaction between the *in situ* fibroblasts and myocytes. Again, genetic modification targeted specifically at fibroblasts, using a promoter such as the pro-2 α -collagen promoter would allow more targeted intervention at the level of the fibroblasts.

It is increasingly apparent that a greater understanding of the role of fibroblasts in terms of ECM regulation and inter cellular communication is important, and particularly with regards to the diversity of fibroblasts. Differences in fibroblasts from atria and ventricles have been found (Burstein et al., 2008), and therefore whether the paracrine effects of fibroblasts from different parts of the heart have different paracrine effects would be interesting to study. Fibroblasts from the atria appear more reactive, and therefore may have a role in the initiation of disease. Furthermore, it is possible that paracrine factors released from atrial fibroblasts in disease, such as atrial fibrillation, could signal to the rest of the heart and be involved in the progression of disease through the heart. Other differences may be present in fibroblasts from the left free wall, septum and right ventricles that may underlie some changes associated with these areas. For example, in hypertrophic obstructive cardiomyopathy, hypertrophy and fibrosis are both apparent in the left ventricle outflow tract and it is interesting to speculate that fibroblasts activation associated with the fibrosis may contribute to exacerbate the hypertrophy through soluble mediators (Noureldin et al., 2012).

Another areas where an understanding of fibroblasts is important is the development of direct reprogramming of fibroblasts to myocytes as a potential intervention to increase heart 'regeneration' after myocardial infarction (Ieda et al., 2010). The clear focus here is the increase in the number of myocytes to improve cardiac pumping function. However, the other side of the equation is the potential reduction of the number of fibroblasts. This could have implications on the function of fibroblasts, in terms of ECM modulation but also in terms of the interaction with myocytes. Although

our α-SMA positive fibroblasts are from a different setting to myocardial infarction they had a negative effect on myocyte function. Therefore this could possible present another beneficial effect of transformation of fibroblasts to myocytes, although this requires further investigation.

8.3. Concluding remark

We have shown that fibroblasts modulate myocyte size, viability and excitation contraction coupling through the release of paracrine mediators, and that TGF-β is important in driving these effects. Furthermore, these effects are altered in fibroblasts after pressure overload and this may represent a novel pathway in which fibroblasts drive the changes in myocardial function in cardiovascular disease. Further work is necessary to better identify the mechanism involved and the applicability of this data to cardiovascular physiology and disease.

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