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The role of cardiac perivascular cells in cardiac repair.

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Abstract

Ischaemic heart disease accounts for approximately 7 million deaths worldwide on a yearly basis and this figure is only set to rise as life expectancy in developing countries increases. Although no longer considered a post mitotic organ, the adult heart demonstrates only a very limited capacity for regeneration. Consequently ischaemic injury results in massive loss of contractile cardiomyocytes with damaged myocardium replaced by a non-contractile and poorly conductive collagen scar. This in turn often leads to the development of heart failure.

Enhancing or supplementing the myocardial regenerative capacity of the heart is thus a key goal in the development of effective therapies for the treatment of cardiac infarction. Several stem cell populations of non-cardiac origin have been investigated for their capacity to contribute to myocardial repair when therapeutically transplanted into injured hearts. Recent efforts have focused on the “next generation” of donor cells, endogenous cardiac progenitor cells, as these are thought to be better adapted to survival in the cardiac environment and to possess enhanced cardiomyocyte differentiation potential. Pericytes, proposed as the source of the elusive mesenchymal stem cells (MSC) within multiple tissues, are a potential new cell type for use in regenerative medicine. This study tests the hypothesis that pericytes and another perivascular progenitor population, the adventitial cell, from foetal cardiac tissue will positively contribute to the repair of the myocardium post injury.

Staining of human foetal ventricular myocardium confirmed the presence of large numbers of both cell types with pericytes tightly associated with capillaries and adventitial cells primarily located in the outer, adventitial layer of muscular arteries. CD146⁺ CD34⁻ pericytes and CD146⁻ CD34⁺ adventitial cells were isolated by FACS and expanded in culture. On examination of gene and protein expression both populations stably expressed a similar panel of pericyte markers, MSC markers and cardiac transcription factors as well as c-kit, a cardiac progenitor cell candidate marker. Co-culture with neo-natal rat cardiomyocytes induced expression of an additional cardiac progenitor marker Isl-1 and a mature cardiomyocyte marker ANP in adventitial cells but not pericytes. Labelled, co-cultured, perivascular progenitors

readily adhered to rat cells but did not appear to contract independently. De-methylation of perivascular progenitors prior to co-culture resulted in expression of sarcomeric proteins and spontaneous cytoplasmic calcium fluctuations in both populations but more commonly in pericytes. This suggests that cardiac perivascular cells contain a minor sub-population capable of cardiomyocyte differentiation.

When these populations were injected into the infarcted hearts of NOD/SCID mice, the animals treated with adventitial cells had significantly reduced cardiac function at 21 days post-surgery on ultrasound examination. An increased scar area and a non-significant trend towards increased scar length and a decreased wall thickness were also observed. Transplanted cells of both groups were detected in low numbers 21 days after injection. Adventitial cells were retained much more readily and in both populations retained cells exhibited three key morphologies: fibroblast type; macrophage type; and cardiomyocyte type. The majority of cells adopted a fibroblast type morphology, lesser numbers a macrophage like morphology and only rare cells a cardiomyocyte like morphology. Both fibroblast and cardiomyocyte type cells had single, human nuclear antigen positive nuclei suggesting true differentiation rather than cell fusion and pericytes exhibited an enhanced ability to differentiate into cardiomyocytes. This supports the *in-vitro* findings of a minor pro-cardiomyogenic subset within the perivascular cell population.

As a result of these findings the starting hypothesis was modified to propose that perivascular cells play a significant role in cardiac fibrosis, largely mediated through expression of surface integrin receptors. This was tested using mice expressing fluorescent proteins under the control of the PDGFR- β promoter and mice in which the α_v integrin subunit, common to 5 integrin receptors, had been deleted on the surface of PDGFR- β^+ cells. Immunostaining and flow cytometry revealed the PDGFR- β expression to be tightly restricted to perivascular cells and co-expressed with the fibroblast markers, vimentin, PDGFR- α , CD90.2 and CD34 in a subset of cells. Cardiac fibroblasts isolated from reporter mouse hearts revealed strong expression of PDGFR- α and CD34 but PDGFR- β expression in only approximately 20% of the population on flow cytometry. Following angiotensin II induced cardiac hypertrophy and fibrosis approximately 50% of fibroblasts expanding the interstitium

were PDGFR- β^+ . Genetic deletion of the α_v integrin subunit on PDGFR- β^+ cells resulted in a reduction in cardiac interstitial collagen content of about 50% compared to wild type controls. These findings suggest that the cardiac perivascular PDGFR- β^+ population is heterogeneous with a sub-population likely to be fibroblasts or fibroblast progenitors and that the development of cardiac interstitial fibrosis is in part modulated by integrin receptor expression on these cells.

In summary this study provides evidence of the existence of a pro-fibrotic progenitor population, which co-express pericyte and MSC markers, within the cardiac perivascular niche. These cells contribute to cardiac fibrosis both on transplantation and endogenously following cardiac injury with the latter mediated via α_v integrin expression. Within the perivascular progenitor population however there also appears to be a minor subset of pro-cardiomyogenic cells which are able to adopt a cardiomyocyte phenotype both *in-vitro* and *in-vivo*.

Declaration

I, James Edward Baily, declare that this thesis was composed by me and that all work described herein was performed by me except where otherwise acknowledged. This work has not been submitted for any other degree or professional qualification.

James E. Baily

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Finally I would also like to thank both my parents who from a child have provided me with all the support and encouragement I have needed to pursue my varied academic interests. My father gained his own PhD in 1973 and having been through the process myself now I admire his achievement even more.

Dedication

I dedicate this thesis to the very first Dr Baily,
my father, Ed.

I hope this makes you proud.

“After doing extensive research, I can definitely
tell you that single malt whiskies are good to drink.”

— Iain Banks, *Raw Spirit*

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Abbreviations

%	: percentage
°C	: degrees Celsius
α -SMA	: alpha smooth muscle actin
ACT-eGFP MHC-nLAC	: reporter targeting eGFP expression under the β actin promoter
ADAM12	: a disintegrin and metalloprotease 12
Adv	: adventitial cells
ALKP	: alkaline phosphatase
ANA	: anti-human nuclear antigen
AngII	: angiotensin II
ANOVA	: analysis of variance
ANP	: atrial natriuretic peptide
ATP	: adenosine triphosphate
BMP	: bone morphogenic protein
bp	: base pairs
BrdU	: bromodeoxyuridine
CCL4	: carbon tetrachloride
CD	: cluster of differentiation molecule
cDNA	: complementary DNA
c-kit	: CD117/stem cell factor receptor
CMLC2	: cardiac myosin light chain 2
CVD	: cardiovascular disease
CNS	: central nervous system
CO	: cardiac output
CO ₂	: carbon dioxide
Col1 α 1	: collagen 1 alpha 1
Col1 α 2	: collagen 1 alpha 2
Col3 α 1	: collagen 3 alpha 1
CT	: cycle threshold
CTGF	: connective tissue growth factor
DALYs	: disability-adjusted life years
DAB	: 3, 3'-diaminobenzidine

DAPI	: 4', 6-diamidino-2-phenylindole
DDR2	: discoid domain receptor 2
DMEM	: Dulbecco's modified eagle medium
DMSO	: demethyl sulphoxide
DNA	: deoxyribonucleic acid
DsRed	: red fluorescent protein from <i>Discosoma</i> sp.
EAC	: endocardial area change
EAd	: endocardial area diastolic
EAs	: endocardial area systolic
ECM	: extracellular matrix
EDTA	: ethylenediaminetetraacetic acid
EF	: ejection fraction
eGFP	: enhanced green fluorescent protein
EGM2	: endothelial cell growth media
ELISA	: enzyme-linked immunosorbent assay
Erk	: extracellular signal related kinases
ESC	: embryonic stem cell
FAC	: fractional area change
FACS	: fluorescence activated cell sorting
FAM	: 6-carboxyfluorescein
FAP α	: fibroblast activated protein alpha
FBS	: foetal bovine serum
Flk1	: foetal liver kinase 1
Flt 1 & 4	: fms-like tyrosine kinase 1 & 4 (vascular endothelial growth factor receptor)
FMO	: full minus one
FS	: fractional shortening
GAPDH	: glyceraldehyde 3-phosphate dehydrogenase
GFP	: green fluorescent protein
GTPase	: guanosine triphosphate hydrolase
H&E	: haematoxylin and eosin
HPRT1	: hypoxanthine phosphoribosyltransferase 1
IL-1	: interleukin 1

IL-6	: interleukin 6
iPS	: inducible pluripotent stem cells
Itgav	: alpha v integrin
Isl 1	: Islet 1/insulin gene enhancer
JNK	: c-Jun N-terminal kinase
LAD	: left anterior descending
LV	: left ventricle
MAPK	: mitogen activated protein kinase
Mef2c	: myocyte-specific enhancement factor 2c
MHC7	: myosin heavy chain 2
MLC2	: myosin light chain 2
mmHg	: millimetres of mercury
MMP	: matrix metalloproteinase
mTmG	: membrane-tomato membrane-green
mRNA	: messenger RNA
MSC	: mesenchymal stem cell
NBF	: neutral buffered formalin
NCD	: non communicable disease
ng	: nanograms
NG2	: neural/glial antigen 2/chondroitin sulphate proteoglycan
NOD/SCID	: non-obese diabetic/severe combined immune deficiency
NRCM	: neonatal rat cardiomyocytes
OCT	: optimal cutting compound
PAI-1	: plasminogen activator inhibitor 1
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
PDGF- β	: platelet derived growth factor beta
PDGFR- α	: platelet derived growth factor receptor alpha (CD140 α)
PDGFR- β	: platelet derived growth factor receptor beta (CD140 β)
Peri	: pericytes

PFA	: paraformaldehyde
P/S	: penicillin/streptomycin
qRT-PCR	: quantitative reverse transcriptase PCR
RGB	: red green blue
RGS5	: regulator of G-protein signalling 5
RNA	: ribonucleic acid
Rpl4	: ribosomal protein L4
RT-PCR	: reverse transcriptase PCR
SC	: subcutaneous
Sca-1	: stem cell antigen 1
SEM	: standard error of the mean
SIRPA	: signal regulator protein alpha
TBE	: tri/borate/EDTA
Tbp 1	: telomerase repeat binding protein 1
TGF- β	: transforming growth factor beta
Tie-2	: tyrosine kinase with immunoglobulin-like endothelial growth factor domains 1
TIMPS	: tissue inhibitor of metalloproteinase
TNF- α	: tumour necrosis factor alpha
Trop T	: troponin t
μ g	: micrograms
μ l	: microlitres
UPL	: universal probe library
UV	: ultraviolet
VE cadherin	: vascular endothelial cadherin
VEGF	: vascular endothelial growth factor
WT1	: Wilm's tumour 1

Chapter 1: Introduction

1.1 Ischaemic heart disease

1.1.1 Global incidence of ischaemic heart disease

Development and access to better health care combined with an aging population means that non communicable disease (NCD) now accounts for over half of the global annual human mortality. NCD is now the most frequent cause of death in most countries. Even in Africa where communicable and nutritional diseases have long dominated NCDs are rapidly increasing in incidence (WHO, 2013). Of the 57 million deaths worldwide in 2008 36 million (63%) were attributable to NCD and of these 17.3 million (30%) were due to cardiovascular disease (CVD). Within this category ischaemic heart disease is the main pathology accounting for 42% of cases (WHO, 2013). Importantly CVDs may not result in death but instead cause severe disability. Disability-adjusted life years (DALYs) represent years of healthy life lost and are a measure of the total burden of the disease. The number of DALYs resulting from CVDs is estimated at 151,377 million of which 62,587 million hours are attributed to ischaemic heart disease (WHO, 2013). It is clear therefore that ischaemic heart disease exerts a significant global cost both socially and financially.

1.1.2 Pathophysiology of ischaemic heart disease

In simple terms ischaemic heart disease results from the reduction in coronary blood supply to the myocardium with the development of ischaemia and irreversible myocardial damage (Cotran et al., 1993). This myocardial infarction results in reduced conductivity and contractility of the myocardium with the development of acute or chronic heart failure. By far the most common cause of impaired myocardial blood supply is severe atherosclerotic plaque development within the wall of the coronary artery with thrombus formation overlying the site of plaque rupture. Less frequent causes include emboli formation and vasospasm (Cotran et al., 1993). The consequences of hypoperfusion and thus the degree of myocardial damage are dependent on several factors including: the severity of the blood flow reduction; the length of the ischaemic period; the volume of cardiac muscle supplied by the affected vessel; and the presence of suitable collateral blood vessels in the infarcted region.

Possible sequelae of myocardial ischaemia are the development of reversible functional disturbances such as myocardial stunning, angina pectoris, myocardial hibernation, pre- or post-conditioning, or irreversible injury.

The normal myocardium has a high energy requirement and is extremely sensitive to ischaemia with the rapid cessation of aerobic glycolysis resulting in the inadequate production of high-energy phosphates (eg: adenosine triphosphate) and accumulation of potentially toxic metabolites (eg: lactic acid) (Opie, 1998). The shift to anaerobic metabolism is brisk and myocardial contractility is significantly reduced within 60 seconds of the onset of ischaemia (Tennant and Wiggers, 1935). The subsequent reduced ATP concentrations result in inhibition of the sodium-potassium pump leading to increased intracellular Na^+ and Cl^- and cell swelling. Increased cytosolic Ca^{2+} , activation of proteases and changes in contractile myofilaments result from altered ion transport in the sarcolemma and the sarcoplasmic reticulum whilst elevation of cytoplasmic lactate concentrations reduce contractile function (Burke and Virmani, 2008).

1.1.3 Microscopic appearance of ischaemic myocardial injury

The earliest microscopic lesions identifiable within myocardial infarcts are hyperacidophilia of the myocyte sarcoplasm seen between 12 and 24 hr post onset. Myocyte striations however appear normal at this stage. By 24 hr mild neutrophil infiltrates are present at the ischaemic border. Between 24 and 48 hr coagulative necrosis of myocytes develops with nuclear pyknosis, early karyorrhexis and karyolysis whilst striations are preserved. Border neutrophil infiltrates become more intense. By days 3 to 5 necrotic central infarct regions exhibit loss of both nuclei and striations. Neutrophil invasion of necrotic tissue is evident with degeneration and formation of abundant karyorrhectic debris. Macrophages and fibroblasts start to appear in the border region. Macrophages may contain nuclear debris and pigment as they phagocytose necrotic myocytes. By 7 days numbers of intact neutrophils decline and granulation tissue with neovascularisation develops at the margins. Lymphocytes and plasma cells infiltrate the infarct margins in low numbers. In the minority of cases eosinophils may be present. By 10 to 14 days fibroblasts are prominent and

deposition of collagen increases. Differentiation of fibroblasts to myofibroblasts allows contraction and remodelling of the scar over the following weeks (Cotran et al., 1993). Repair may take from 4 to 8 weeks to complete and the rate depends on multiple factors including systemic blood pressure, cardiac output and collateral blood flow.

Within the infarcted myocardium two zones of injury occur: a central zone in which there is no or only low blood flow and an outer border zone which is supplied by collateral blood vessels. Indeed the degree of coronary collateral flow is a key determinant of infarct size and individuals suffering from coronary occlusion who have well developed collateral vessels have a low risk of developing acute myocardial infarction following sudden thrombosis and coronary occlusion (Miwa et al., 1999).

1.2 Cardiac Regeneration

The heart of adult mammals has long been considered a postmitotic organ with injury resulting in the permanent loss of cardiomyocytes and replacement by fibroblasts and a collagen rich scar. This scar tissue has a markedly reduced ability to conduct electrical impulses and contract compared to normal myocardium. The self-renewal capacity of fish and amphibian cardiac tissue in contrast is well established with complete regeneration and return to normal function of the myocardium in adult zebrafish within 60 days of resection of 20% of the apex of the ventricle (Poss et al., 2002).

The terminally differentiated status of the cardiomyocyte was challenged recently by the discovery that carbon-14, resulting from atomic bomb tests carried out before the Limited Nuclear Test Ban Treaty in 1963, could be detected in the DNA of cells. By using carbon dating the investigators were able to determine the age of cardiomyocytes and calculate annual turnover using cardiac tissue from recently diseased humans. The results indicated that at 25 years of age the annual cardiomyocyte turnover is 1% and this decreases to 0.45% by the age of 70. Over an entire lifespan less than 50% of cardiomyocytes are replaced. (Bergmann et al., 2009). Although encouraging this degree of limited turnover in the adult human heart

is clearly unable to replace sufficient myocardium following ischaemic damage. Further hope however has been provided by the finding that following surgical resection of approximately 15% of the left ventricular apex neonatal mice were able to fully regenerate their myocardium (Porrello et al., 2011). In this study the investigators found that if the resection was carried out in mice of 1 day of age complete myocardial regeneration occurred within 21 days. Conversely if the ventricle of mice of 7 days of age was resected then a collagenous scar formed at the site of injury instead, resembling the repair process seen in adult humans. This work, for the first time, suggested that the potential for substantial myocardial regeneration also exists in the mammalian heart. One potential limitation of this initial work is that in both the zebrafish and mouse the injury was surgical resection rather than ischaemia, as seen following myocardial infarction. This was addressed recently with the publication of a study in which the left anterior descending coronary artery was ligated in neonatal mice (Haubner et al., 2012). Mice operated on at 1.5 days of age exhibited complete myocardial regeneration 28.5 days post-surgery whereas those subjected to coronary ligation at 7.5 days exhibited no regeneration by 28.5 days. This confirms that cardiac regeneration in mammals is dependent on age rather than the cause of the initial myocardial damage.

1.2.1 Cardiac progenitors

Opinion is split regards the source of new cardiomyocytes post injury. Some researchers favour de-differentiation and proliferation of existing cardiomyocytes whilst others consider proliferation and differentiation of an endogenous progenitor cell pool within the heart a more likely mechanism. Credible evidence exists for both. A transgenic zebrafish model, in which the *cmlc2* (cardiac myosin light chain 2) promoter drives the expression of a tamoxifen inducible Cre recombinase and a second constitutive β -actin promoter drives a floxed DsRed sequence and stop sequence followed by a GFP sequence, has been used to investigate cardiac regeneration. Prior to Cre induction the DsRed but not GFP sequence is transcribed in all cells and thus all cells fluoresce red. Pulsing the fish with tamoxifen results in induction of Cre recombinase expression, excision of the floxed DsRed and stop sequence in cardiomyocytes with the result that these cells fluoresce green.

Cardiomyocyte progenitors not expressing *cmlc2* however will remain red. Applying the apical amputation model of injury to this transgenic fish model resulted in the generation of new myocardium that fluoresced 100% green indicating that it originated from the pre-existing mature cardiomyocyte pool (Kikuchi et al., 2010). Work published around the same time using a similar technique agreed with these findings (Jopling et al., 2010).

The alternative cardiac regeneration hypothesis focuses on the endogenous cardiac progenitor cell population, adult stem cells that reside within post natal hearts that are purported to provide a source of regenerating cardiomyocytes. These cells can be identified by several means including expression of surface markers (eg: *Isl1*, *c-kit* and *Sca-1*), their ability to efflux dyes or form spheroids (Laflamme and Murry, 2011). It appears, as the work in this field matures, that there is substantial overlap between these populations. Of these progenitors, *c-kit*⁺ cells have been the most intensely studied. *c-kit* (CD117) expression is seen in a variety of cells including telocytes, mast cells, haematopoietic stem cells and endothelial cells, smooth muscle cells and cardiomyocytes following myocardial injury (Tallini et al., 2009). Transgenic mice studies in which eGFP is under the control of the *c-kit* locus demonstrated eGFP⁺ cells in developing hearts with numbers peaking 2 days postnatally and dropping to only rare eGFP⁺ cells in adult hearts. Isolated eGFP⁺ cells from neonatal mice were able to differentiate into endothelial cells, cardiomyocytes and smooth muscle cells. Cryo-ablation of the ventricle in adult mice resulted in *c-kit*-eGFP expression in endothelial cells, smooth muscle cells and mature cardiomyocytes at the margins of the infarct (Tallini et al., 2009). In further work *c-kit*⁺ cells were isolated from the hearts of neonatal and adult ACT-eGFP MHC-nLAC double transgenic mice in which eGFP expression is targeted to the *c-kit*⁺ cells whilst nuclear β galactosidase is restricted to cardiomyocytes. Culturing of these cells with neonatal cardiomyocytes and injecting them into normal or infarcted hearts revealed cardiac differentiation in a low percentage (2.4%) of co-cultured neonatal *c-kit*⁺ cells but no differentiation in adult *c-kit*⁺ cells (Zaruba et al., 2010). Both studies suggest that *c-kit*⁺ cells from neonatal mammalian hearts have cardiomyocyte differentiation potential but that potential is lost in the adult heart.

1.3 Stem cell transplantation as a method of enhancing cardiac regeneration

Because intrinsic cardiac repair mechanisms alone are unable to regenerate sufficient amounts of myocardium intensive efforts are underway to enhance the cardiac regeneration process. Several potential therapies have been investigated including cell therapy/transplantation, cellular reprogramming and tissue engineering (Laflamme and Murry, 2011). Of these, cell therapy has progressed furthest in terms of translational development and several donor cells types have been studied. The first attempts at cell based repair of damaged myocardium however began in the early 1990s with the use of autologous myoblasts/skeletal muscle satellite cells (Marelli et al., 1992, Taylor et al., 1998, Menasche et al., 2001). These cells are considered to be skeletal muscle progenitors and as such are responsible for regeneration of damaged skeletal muscle. Direct injection of these cells into infarcted myocardium in several species resulted in cell survival but unsurprisingly led to the development of skeletal muscle rather than cardiac muscle phenotype. In addition intercalated disk proteins N-cadherin or connexin 43 were not detected and electromagnetic coupling or synchronous beating did not occur (Reinecke et al., 2002, Leobon et al., 2003). The next big group of cells to be studied were bone marrow cells. Initially investigators focused on the ability of these cells to contribute to repair in skeletal muscle and genetically labelled bone-marrow cells transplanted into immuno-deficient mice migrated to areas of muscle degeneration and underwent myogenic differentiation (Ferrari et al., 1998). This stimulated interest in these cells as potential donor population for myocardial repair. At first it appeared that bone marrow cells transplanted into injured myocardium differentiate into mature cardiomyocytes (Toma et al., 2002, Orlic et al., 2003). However this was shortly contradicted by work which suggested transplanted bone marrow derived cells fuse with cardiomyocytes rather than differentiate into new cells (Alvarez-Dolado et al., 2003). Two years later an in-depth study repeated this work using GFP expressing c-kit⁺ bone marrow cells from transgenic mice and found that transplanted cells differentiated into cardiomyocytes with no evidence of cell fusion (Kajstura et al., 2005).

Latterly pluripotent stem cells and endogenous cardiac progenitors have been investigated as potential candidates for cell therapy. Pluripotent stem cells, both embryonic stem cells (ES cells) and inducible pluripotent stem cells (iPS cells), unlike adult stem cells can propagate indefinitely whilst retaining their ability to differentiate into all cell types (Puri and Nagy, 2012). These cells can be readily transformed in culture into immature cardiomyocytes and when transplanted into the hearts of mice and rats human ESC derived cardiomyocytes form islands of myocardium within the scar zone (Caspi et al., 2007). Significant hurdles however need to be overcome before they can be used in human studies. ES cells are derived from pre-implantation blastocysts and thus there are ethical concerns regarding their use (Daley et al., 2007). They are also allogeneic and thus will require concurrent immune-suppressive therapy. iPS cells in contrast can be derived from re-programmed autologous fibroblasts through treatment with four factors (Oct3/4, Sox2, c-Myc and Klf4) and hold great promise as transplantable population (Takahashi and Yamanaka, 2006). Standard re-programming however involves the use of retroviral transduction and raises the risk of neoplastic transformation. Another issue concerning both cell types is that their pluripotency which, though of benefit regards cardiomyocyte differentiation, also risks transformation into teratomas once transplanted (Nussbaum et al., 2007).

Endogenous cardiac progenitor cells would not, in theory, suffer from these issues and thus efforts have been focused on identifying populations of dormant resident progenitors that might be isolated and expanded or triggered to differentiate *in-situ*. Various studies have described isolation of c-kit⁺ cells from mammalian heart and a positive response to their transplantation post ischaemic injury (Tallini et al., 2009, Bearzi et al., 2007, Tang et al., 2010) with cells differentiating into cardiomyocytes and endothelial cells. These findings are not without controversy however and several published studies have suggested that c-kit⁺ cells fail to differentiate into significant numbers of cardiomyocytes *in vitro* or after transplantation (Zaruba et al., 2010, Tallini et al., 2009). Another cardiac progenitor cell type under investigation is the cardiosphere forming cells. These migrate out of cardiac tissue explants grown in culture and form spheroids. The resultant cell population is mixed but have been

shown to positively contribute to repair of the myocardium upon transplantation and differentiate into cardiomyocytes *in vitro* (Messina et al., 2004, Chimenti et al., 2010). As with c-kit⁺ progenitors these cells have been adopted for use in clinical trials however recent work has thrown doubt on their purity and stemness suggesting that they are predominantly cardiac fibroblasts contaminated with cardiomyocytes from the initial sample (Andersen et al., 2009).

1.3.1 Paracrine effects versus differentiation

Regardless of the origin or type of injected donor cell the numbers retained post injection in all studies is small compared to the numbers of myocytes lost. This raises questions as to whether sufficient cells survive to account for the functional benefits reported. Many investigators now consider that their predominant mode of action may be through a paracrine effect due to the release of inflammatory cytokines or microvesicles containing microRNAs (Ratajczak et al., 2012, Laflamme and Murry, 2011). It is proposed that soluble factors released from the stem cell populations act on the resident cells of heart including cardiomyocytes, endothelial cells, smooth muscle cells, fibroblasts and cardiac progenitors to enhance cardiac function through neovascularisation, cardiac remodelling, cardiac regeneration, increased contractility, myocardial protection and altered cardiac metabolism (Gnecchi et al., 2008). In particular it is thought that MSCs secrete antagonists of Wnt ligands and that this pathway may play a significant role in their mechanism of action (Mirotso et al., 2007). Recently the paracrine potential of c-kit⁺ and Sca-1⁺ cortical bone derived stem cells and cardiac derived stem cells from eGFP⁺ mice has been compared. These cells produced 8 paracrine factors associated with cardio protection, angiogenesis and stem cell function *in vitro*. After injection only 2 of these paracrine factors (basic fibroblast growth factor and vascular endothelial growth factor) were detectable in bone marrow derived cells and associated with neovascularisation of the border zone. Bone marrow cell transplantation improved survival, cardiac function and reduced infarct size compared to cardiac derived stem cell treated animals and controls and after 6 weeks eGFP⁺ cardiomyocytes were seen in the bone marrow treated group but not the cardiac cell treated group suggesting an enhanced therapeutic potential of the former (Duran et al., 2013).

1.4 Perivascular cells: a source of progenitor cells for regenerative medicine?

1.4.1 The perivascular cell niche

The perivascular cell population as distinct from the endothelium was first identified by Rouget and Eberth in the late nineteenth century. This population is highly heterogeneous and several types of adult stem and progenitor cells have been identified within the walls of the arteries, veins and capillaries in a variety of organs (Tilki et al., 2009). Several names have been used to describe cells from this perivascular location including Rouget cells, mural cells, adventitial cells and pericytes. Pericytes are associated with capillaries, post capillary venules and terminal arterioles. These cells have both primary and secondary cytoplasmic processes. Whilst the primary processes extend along the length of the vascular endothelium the secondary processes project perpendicularly to wrap around the vessel (Armulik et al., 2011). The body of the cell is closely opposed to the underlying endothelium and is surrounded by a basement membrane (Figure 1-1). *In vitro* studies have demonstrated that synthesis of the basement membrane is carried out by both endothelial cells and pericytes (Mandarino et al., 1993).

Another subset of less well characterised perivascular cells are located predominantly within the adventitia of larger vessels (arterioles, arteries and veins) (Crisan et al., 2009). These adventitial cells reside within the loose collagenous stroma surrounding the vessel potentially in close proximity to capillaries of the *vasa vasorum*, fibroblasts, leukocytes and nerve cells (Zengin et al., 2006) (Figure 1-2). Unlike pericytes the relationship of these cells with the endothelium of vessels is poorly defined, however these cells have been demonstrated to possess multilineage differentiation potential (Campagnolo et al., 2010, Corselli et al., 2012).

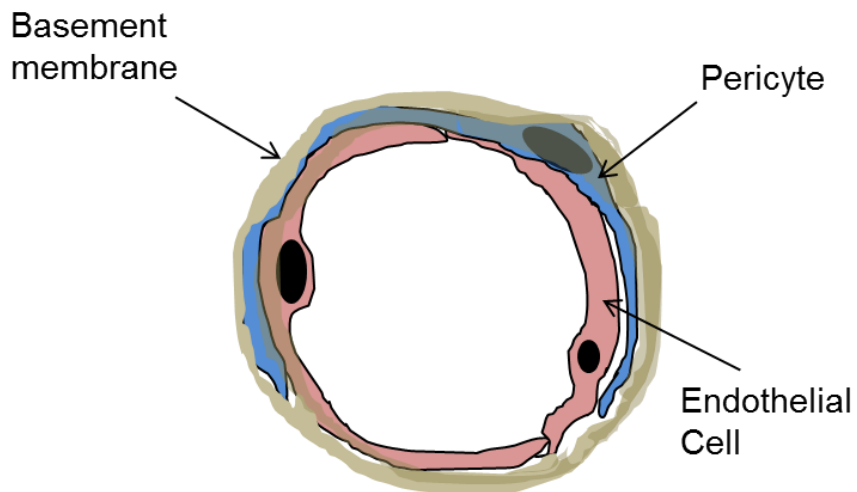


Figure 1-1 Schematic illustrating the close association of pericytes and endothelial cells relative to the basal lamina (basement membrane) within the wall of capillaries.

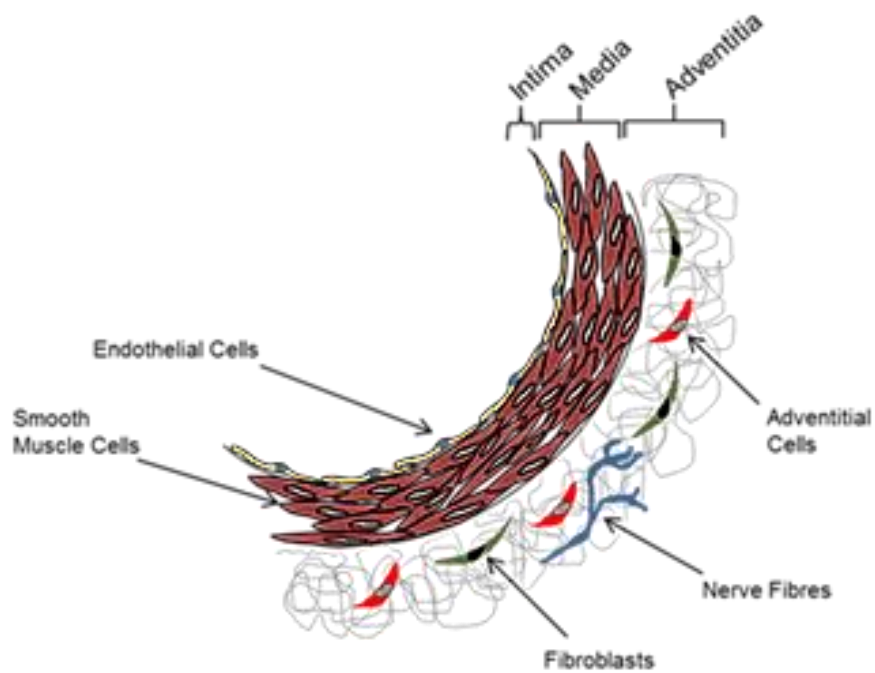


Figure 1-2 Schematic illustrating the location of adventitial cells within the wall of muscular arteries.

A third perivascular population are the mesoangioblasts. These progenitor cells are associated with the perivascular niche of small vessels and derive from the mesoderm. They were first identified in explants of the embryonic mouse aorta from where they were isolated and shown to co-express endothelial and myogenic markers whilst demonstrating an ability to differentiate into skeletal muscle *in-vitro* (De Angelis et al., 1999). The name mesoangioblast was used to denote a common progenitor capable of contributing to both the vascular system and the extravascular mesoderm. Freshly isolated mesoangioblast clones express early endothelial markers such as CD34, foetal liver kinase 1 (Flk1), stem cell antigen 1 and vascular-endothelial (VE) cadherin with a variable proportion expressing α -SMA (Minasi et al., 2002) and in culture they can also be induced to osteogenic and adipogenic lineages (Cossu and Bianco, 2003).

1.4.2 The origins of perivascular cells

Within coronary vessels perivascular cells are thought to arise from epicardial mesothelium (Mikawa and Gourdie, 1996). A mesothelial origin is also suspected in other organs such as the gut (Wilm et al., 2005), lung (Que et al., 2008), 2008) and liver (Asahina et al., 2011). It is proposed that mesothelial cells undergo epithelial-to-mesenchymal transition then separate before migrating into organs to develop into fibroblasts, vascular smooth muscle cells and perivascular pericytes. Several key signaling pathways are involved in the recruitment and stabilization of pericytes during organ development. PDGF- β is produced by endothelial cells undergoing angiogenesis and binds the extracellular matrix from where it mediates recruitment of pericytes through binding of the PDGF- β receptor (Armulik et al., 2011). TGF- β signaling also plays a role in vasculogenesis and has been implicated in both perivascular cell and endothelial cell proliferation and maturation (Gaengel et al., 2009). Angiopoietin 1 is produced by pericytes amongst other mesenchymal cells and interacts with the Tie-2 receptor on endothelial cells to promote vascular stability and reduced permeability (Armulik et al., 2011).

1.4.3 Perivascular cell markers

The study of perivascular cell populations is significantly complicated by the lack of a single specific marker identifying these cells in tissue or on fluorescence activated cell sorting. The current markers not only label subsets of the perivascular population but also a minority of non-perivascular cells. Established pericyte and perivascular cell markers include CD146 (cluster of differentiation 146 or melanoma cell adhesion molecule), NG2 (chondroitin sulphate), PDGFR- β (platelet derived growth factor receptor beta), desmin, ALKP (alkaline phosphatase), α -SMA (alpha smooth muscle actin), RGS5 (regulator of G-protein signalling 5) and CD34 (cluster of differentiation 34). None of these markers however is 100% specific for pericytes and depending on species and tissue will also label cells of other lineages. CD146 labels, in addition to pericytes, a subset (proportion is species dependent) of endothelial cells (Bardin et al., 2001) and also mesenchymal stem cells in which it has been linked to vascular smooth muscle differentiation potential (Espagnolle et al., 2014). It is a transmembrane glycoprotein and part of the immunoglobulin superfamily involved in calcium independent cell adhesion (Shih, 1999). NG2 is a membrane spanning protein which interacts with the cytoskeleton and plays a role in cell migration and proliferation. As well as labelling pericytes it also identifies neural progenitors, glial cells and chondroblasts as well as neoplastic cells in melanomas, glioblastomas and chondrosarcomas (Stallcup and Dahlin-Huppe, 2001). PDGFR- β labels pericytes and is important in the development of mesenchymal tissue and plays a role in the function of fibroblasts in tissue homeostasis and regeneration (Hewitt et al., 2012). Desmin is expressed in smooth and striated muscle cells in addition to the perivascular cell population (Chan-Ling et al., 2004). α -SMA is reported in a subset of pericytes (Crisan et al., 2009) but also labels smooth muscle cells of arteries, the wall of tubular organs such as the gut and uterus and in pathological situations some myofibroblasts. Ganglioside 3G5 was initially considered a specific pericyte marker (Nayak et al., 1988) however it has also been found to react with T lymphocytes and neurons as well as endothelial and epithelial cells in the kidney (Nayak et al., 1992). CD34, as well as identifying adventitial cells also labels haematopoietic stem and progenitor cells, endothelial progenitor cells and

mature endothelial cells where it facilitates trans-endothelial cell migration (Nielsen and McNagny, 2008).

Because of the lack of a single specific marker for perivascular progenitor cells a panel of markers used in combination is required when identifying these cells in by flow cytometry or immunohistochemistry.

1.4.4 Perivascular cell physiology

Pericytes are the most studied and the best described of the cells within the perivascular niche and will be the focus of this review of cell physiology.

Ultrastructurally pericytes have a heterochromatic nucleus, large numbers of plasmalemmal vesicles, abundant rough endoplasmic reticulum and cytoplasmic contractile microfilaments (Diaz-Flores et al., 1991). Although the majority of the pericyte and endothelial cell surfaces are separated from one another by basement membrane matrix, direct contact between cells occurs at discrete points (Armulik et al., 2011). Several types of contact are present. Holes in the basement membrane allow pericytes and endothelial cells to form peg-socket type contacts in which cytoplasmic projections from pericytes interdigitate with endothelial invaginations (Rucker et al., 2000). Elsewhere close apposition of cell membranes at the edge of pericyte processes form occluding contacts. Adhesion plaques containing fibronectin and N-cadherin (Gerhardt et al., 2000) provide anchorage and finally gap junction-like structures, likely to be involved in ion transfer, have been reported (Diaz-Flores et al., 2009, Bergers and Song, 2005).

Pericyte density is not fixed and varies significantly between organs and vascular beds with the area of endothelial pericyte coverage also exhibiting marked variability. Of the organs studied the CNS is considered to exhibit the highest pericyte density with an endothelial to pericyte ratio of 1:1 to 3:1 and approximately 30% abluminal endothelial coverage present (Mathiisen et al., 2010). Pericytes on venous capillaries and post capillary venules are generally the most numerous with the most extensive cytoplasmic processes whilst those on larger venules are less frequent, larger and have greater endothelial contact area (Hirschi and D'Amore, 1996). In general however conservative estimates suggest an endothelial-to-pericyte

ratio of 1:1 to 10:1 with endothelial abluminal surface coverage of 70% to 10% in most normal tissues (Sims, 1986). It has been postulated that there is a positive correlation between pericyte density and coverage and endothelial barrier properties, reduced endothelial turnover and orthostatic blood pressures (Sims et al., 1994).

1.4.5 Perivascular cell function

Again the bulk of the knowledge of perivascular cell function relates to pericytes and it is these that will be discussed here. Initially it was thought that mature pericytes served simply as a scaffold to support endothelial cells and maintain vessel patency. However accumulated data in the last 30 years or so have confirmed that these cells have multiple complex functions.

Vasculogenesis is considered a key pericyte function. During embryonic development endothelial cells secrete proteases to degrade the basement membrane and invade the surrounding extracellular matrix. A migrating column of endothelial cells forms which is directed by a VEGF (vascular endothelial growth factor) gradient and it is thought that pericytes may also contribute this process by migrating in front of the endothelial column and expressing VEGF (Ozerdem and Stallcup, 2003). Behind the growth zone endothelial cells secrete growth factors that attract pericytes and promote vessel maturation, the key molecule being PDGF- β (Betsholtz, 2004). In turn pericytes induce endothelial differentiation and growth arrest (Gerhardt and Betsholtz, 2003).

Pericytes are also involved in the modulation of inflammation via remodelling of the capillary basement membrane and formation of pericyte to pericyte spaces that enable diapedesis of leukocytes (Proebstl et al., 2012, Wang et al., 2012). Furthermore brain pericytes have been shown to produce pro-inflammatory cytokines such as IL-1 and IL-6 (Fabry et al., 1993).

The regulation of capillary blood flow is perhaps the most universally promoted function of pericytes regardless of tissue of origin. This is achieved through vasoconstriction and vasodilation mediated via contractile filaments such as α -SMA, tropomyosin and myosin which are also present within smooth muscle cells. Work

has suggested that capillary vascular tone is regulated through the action of neurologic and small molecule pathways on pericyte receptors (Rucker et al., 2000) and that oxygen tension also affects pericyte contractility with relaxation in hyperoxic and dilation in hypoxic environments. However the challenges of reliable identification of pericytes *in vivo* and differences between *in vivo* and *ex vivo* findings have led other authors to question these conclusions (Armulik et al., 2011).

Within certain organs pericytes adopt specialist functions. For example in the brain they form the blood-brain barrier which regulates access of potentially toxic blood borne molecules to the CNS. A key part of this function is mediated by macrophage-like phagocytic and pinocytic activities (Thomas, 1999). In the liver hepatic stellate cells are considered the pericyte equivalent. These are located in the space of Disse between the sinusoid endothelium and the hepatocytes cords where they regulate extracellular matrix remodelling by producing matrix compounds and metalloproteinases. As such they are thought to play a key role in hepatic fibrosis and recruitment of inflammatory cells (Knittel et al., 1999).

1.4.6 Progenitor cell potential

Much of the current interest in the perivascular cell population and pericytes in particular is focused on their regenerative ability (Psaltis et al., 2011) and them being the origin of the mesenchymal stem cell in tissue (Crisan et al., 2008b).

Mesenchymal stem cells (MSCs) were first identified in bone marrow cultures as colony forming fibroblast like cells which, when transplanted, developed into cartilage, bone, adipose tissue and fibrous connective tissue (Friedenstein et al., 1974). Since their discovery MSCs or “MSC-like” cells have been demonstrated in multiple different tissue types including fat (Zuk et al., 2002, Campagnoli et al., 2001). Isolation of MSCs is primarily based on their rapid adherence to plastic in culture allowing removal of non adherent contaminating cells, such as haematopoietic cells in bone marrow samples. Cultures are then purified through expansion and passaging in deprivational media (Soleimani and Nadri, 2009). Phenotypic markers expressed by MSCs in culture include CD105, CD44, CD73 and CD90 (Thy-1) whilst markers not expressed include CD45, CD34, CD56 or CD31

(Chamberlain et al., 2007). As expected adhesion derived MSC populations are heterogeneous and although no single marker can be used to identify MSCs several studies have looked at isolating purified populations of these cells based on both positive and negative marker selection (Baddoo et al., 2003, Jones et al., 2002). One of the key features of MSCs is their ability to differentiate into adipogenic, osteogenic and chondrogenic phenotypes in culture (Chamberlain et al., 2007). MSCs are also thought to differentiate into different cell type's *in-vivo* with xenograft transplantation studies of human MSCs into foetal sheep demonstrating site specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes and bone marrow stromal cells (Liechty et al., 2000).

A role for perivascular cells as progenitor cells was first proposed in 1982 when it was suggested that pericytes could contribute to formation of adipocytes following thermal injury ((Richardson et al., 1982)). This work however was light and electron microscope based and involved no immunolabelling or *in vitro* culture. Subsequent studies, primarily using bovine retinal tissue, suggested that pericytes in culture could differentiate into adipocytes and chondrocytes (Farrington-Rock et al., 2004), osteoblasts (Doherty et al., 1998) or skeletal muscle (Dellavalle et al., 2007). The isolation of pericytes in these investigations however was carried out via digestion of tissue and adhesion of cells to plastic culture-ware. More recently isolation and purification of perivascular cell using antibodies against surface markers has been optimised by the Peault group. Immunostaining identified the emergence of haematopoietic stem cells in developing embryos from the vascular wall as well as a subset of perivascular cells in skeletal muscle that are able to contribute to skeletal myogenesis (Tavian et al., 2005, Dellavalle et al., 2007). This skeletal muscle perivascular pericyte population was further characterised, isolated and purified on immunohistochemistry and fluorescence activated cell sorting (FACS) using a panel of antibodies (CD146⁺, CD34⁻, CD56⁻ and CD45⁻) (Crisan et al., 2008a). Pericytes isolated in this manner were shown to be capable of long term culture, stable marker expression and osteogenic, adipogenic and chondrogenic differentiation potential. Shortly after this it was reported that the perivascular cell populations of multiple and varied tissues, including skeletal muscle, pancreas, placenta and adipose tissue,

express both the pericyte markers CD146, NG2 and PDGFR- β and MSC markers such as CD44, CD73, CD90 and CD105, *in situ* and following isolation and expansion in culture (Crisan et al., 2008b). This led to the now widely accepted proposal that perivascular cells are in fact the origin of MSCs in multiple human organs. Work since has confirmed the presence of cells with MSC phenotype and differentiation potentials within the perivascular niche of additional tissues such as the brain (Kang et al., 2010) and bone marrow (Mendez-Ferrer et al., 2010). Furthermore genetically modified mouse models have been used to identify perivascular cells as a source of white fat progenitor cells (Tang et al., 2008) and follicular dendritic cells (Krautler et al., 2012).

1.5 Perivascular cells and cardiac fibrosis

1.5.1 The cardiac fibroblast

Greater than 90% of the mammalian heart, by volume, is composed of specialised muscle cells or cardiomyocytes. By cell number however cardiomyocytes account for only approximately 25% of total cells in the human heart (Cotran et al., 1993). The remainder are endothelial cells, perivascular cells, fibroblasts, specialised nerve fibres (Purkinje fibres) and low numbers of leukocytes. The proportion of cardiomyocytes to supporting cells varies with species. Non cardiomyocytes account for approximately 45% of the population in mouse hearts and 70% in rat hearts. Of these, fibroblasts constitute approximately 27% in mice and 67% in rats (Banerjee et al., 2007). During embryonic development fibroblasts are thought to originate from mesenchymal cells in the pro-epicardium (Norris et al., 2008). In the foetal and neonatal heart fibroblasts are considered to develop from expansion of endogenous populations, epithelial to mesenchymal transition of the epicardium (Zhou et al., 2010) and fibroblastic differentiation of bone marrow derived cells (Visconti et al., 2006). However rather than being an uniform population, fibroblasts are markedly heterogeneous both between tissues and within individual organs (Fries et al., 1994) and given that no marker identifies all cardiac fibroblasts their full origin remains to be determined.

Within the heart fibroblasts form a complex three dimensional network surrounding individual and groups of myocytes. The interstitial connective tissue density and fibroblast content varies with region within the heart, with the sino-atrial node having a substantially higher component (Shiraishi et al., 1992). The role of cardiac fibroblasts in the healthy heart is thought to be in the provision of structural integrity (in particular collagen I, III and VI) and transmission of mechanical forces through the production of extracellular matrix, regulation of cell signalling and conduction of electro-mechanical impulses (Camelliti et al., 2005). Fibroblasts are also considered to play role in angiogenesis and will support formation of vascular structures when co-cultured with endothelial cells in serum free medium . This is likely in part to be a result of their secretion of angiogenic factors such as fibroblast growth factor and vascular endothelial growth factor (Powell et al., 1999). In cardiac pathology fibroblasts are important in both synthesis and degradation of extracellular matrix resulting in myocardial remodelling. Differentiation of fibroblasts to contractile and highly secretory myofibroblasts results in increased production of matrix metalloproteinases (MMPs), transforming growth factor beta (TGF- β), tumour necrosis factor (TNF- α), endothelin-1 and angiotensin II (Souders et al., 2009, Powell et al., 1999). These factors alongside increased extracellular matrix production promote remodelling of the myocardium.

Remodelling may be both beneficial and detrimental to cardiac function. Ischaemic heart disease for instance results in replacement of necrotic myocardium by fibrous tissue that prevents cardiac rupture but is also non-conductive and non-contractile. In hypertensive cardiac disease stretch of the myocardium results in interstitial fibrosis which combined with individual cardiomyocyte hypertrophy acts to increase ventricular stiffness and reduce contractility and ultimately cardiac function (Weber et al., 1995)

1.5.2 Perivascular cells: fibroblasts/fibroblast progenitors?

In addition to the established sources of pro-fibrotic cells mentioned above perivascular stem cells are now considered likely sources of fibroblasts following injury. The first study to suggest this investigated co-expression of pericyte markers

such as PDGFR- β and α -SMA with prolyl-4-hydroxylase beta-subunit, marker of pro-alpha I collagen synthesis, in dermal scars to describe several pro-fibrotic subsets of pericytes based on their anatomical location (Sundberg et al., 1996). More recently sophisticated genetically engineered mouse models have been used to further investigate this link between pericytes and fibrosis. Work in the kidney employed a collagen type 1 alpha 1 reporter mouse to show that pericytes were the major source of collagen expressing myofibroblasts in renal ischaemic injury (Lin et al., 2008). Investigators working on spinal cord repair developed a reporter mouse model in which a subgroup of pericytes expressing the Glast protein and termed type A pericytes were heritably identified by expression of a fluorescent protein. These cells were shown to contribute significantly to scar formation following injury to the cord and this failed to form when their ability to proliferate was inhibited (Goritz et al., 2011). More recently genetic fate mapping mouse models were used to identify a perivascular subset of PDGFR- α cells transiently expressing ADAM12 (a disintegrin and metalloprotease 12) that act as progenitors of a major fraction of collagen producing cells in the dermis and skeletal muscle. Knockdown or genetic ablation of ADAM12⁺ cells led to a reduction in interstitial collagen deposition (Dulauroy et al., 2012). Work in the liver using col-GFP mice has also demonstrated that hepatic stellate cells, considered to be a specialist subpopulation of pericytes, are the principle contributors to the myofibroblast population after hepatic injury with carbon tetrachloride (Kisseleva et al., 2012). Given these findings in disparate tissues it would not be surprising if the cardiac perivascular cell population was also a source of collagen producing cells.

1.5.3 The role of TGF- β in fibrosis

Transforming growth factor β (TGF- β) is thought to play an integral role in tissue fibrosis through its stimulation of fibroblast to myofibroblast transition (Hinz et al., 2007). TGF- β is part of a superfamily of growth factors which contains approximately 30 members. There are three isoforms of TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3) as well as inhibins, activins and BMPs. Of the three isoforms TGF- β 1 is both most widely distributed and conserved in mammals (Cotran et al., 1993). It is a homodimer synthesised and secreted into the extracellular matrix as a precursor

protein which is then cleaved into active growth factor and latent compound. Several molecules have been identified as TGF- β 1 activators and these include plasmin (Lyons et al., 1988), and matrix metalloproteases (MMP) 2 and 9 (Koli et al., 2001). In addition reactive oxygen species and integrin mediated interactions are capable of activating TGF- β 1 (Munger et al., 1999). It is produced by a wide variety of cells including platelets, endothelial cells, lymphocytes and macrophages and binds type II cell surface receptors which then recruit type I receptors with serine/threonine kinase activity triggering phosphorylation of Smad cytoplasmic transcription factors. As a result phosphorylated Smads form heterodimers with Smad 4 and enter the nucleus to inhibit or activate gene transcription. Given its ability to both promote and depress gene expression the dominant effect of TGF- β 1 depends on the tissue and the type of injury (Cotran et al., 1993). TGF- β 1 also activates non-canonical signalling pathways such as Erk, JNK, p38 MAPK and small GTPase pathways (Derynck and Zhang, 2003). In epithelia TGF- β 1 inhibits growth by blocking the cell cycle whilst in the mesenchyme of tumours it can promote invasion and metastasis. In immune cells TGF- β 1 may suppress inflammation whilst enhancing autoimmune responses in some circumstances. Perhaps most importantly TGF- β 1 is strongly pro-fibrotic stimulating fibroblast chemotaxis and increased production of collagen (Desmouliere et al., 1993), fibronectin and proteoglycans. By increasing the activity of protease inhibitors such as PAI-1 and TIMPS and decreasing the activity of matrix proteases it acts to prevent remodelling of deposited collagen (Mauviel, 2005). The synthesis of TGF- β 1 is markedly upregulated in animal models of cardiac hypertrophy and interstitial fibrosis and in models of myocardial infarct upregulation of TGF- β 1 and β 2 occur early whilst TGF- β 3 induction is delayed (Deten et al., 2001). The association between TGF- β 1 overexpression and cardiac hypertrophy is supported by findings in TGF- β 1 overexpressing transgenic mice which exhibit significant cardiac hypertrophy and interstitial fibrosis (Rosenkranz et al., 2002). Similarly heterozygous TGF- β 1 mice develop less age associated cardiac fibrosis and diastolic dysfunction (Brooks and Conrad, 2000) and in pressure overloaded rat hearts administration of anti-TGF- β 1 antibody results in reduced collagen accumulation and diastolic dysfunction without affecting cardiomyocyte hypertrophy (Kuwahara et al., 2002). Interestingly results of other studies using mutations of the TGF- β 1 type II

receptor to inhibit TGF- β 1 signaling have suggested that reduction in collagen deposition is also associated with ventricular dilation and systolic dysfunction (Lucas et al., 2010). Taken together these findings suggest excess TGF- β 1 results in excess myocardial fibrosis and diastolic dysfunction whilst a baseline level of TGF- β 1 activity is required to prevent excessive cardiac remodelling and dilation in cases of pressure overload.

1.5.4 The role of integrins in fibrosis

Integrins are transmembrane receptors that play a major role in both cell adhesion and the bi-directional transmission of signals between the extracellular matrix and the cytoskeleton. In addition they are involved in development, fibrosis, haemostasis, leukocyte trafficking, immune responses and cancer (Hynes, 2002). Integrins are heterodimeric receptors containing an alpha and beta subunits (Figure 1-3) and the various combinations of subunits results in a family of 24 receptors each of which is thought to have a specific function (Figure 1-4). Integrins are also expressed in both inactive and active states with the switch between the two a result of intracellular or extracellular signals, the latter often from integrins on other cells. (Hynes, 2002). Binding of the receptors leads to signal transduction which modulates a variety of cell functions including polarity, migration, proliferation, gene expression and differentiation. The role integrins play in fibrosis is by activating transforming growth factor- β (TGF- β) in the ECM (Cotran et al., 1993). The transforming growth factors 1, 2 and 3 are secreted in a complex into the extracellular matrix with their pro-peptides, latency associated peptides 1, 2 and 3. Here they are bound by specific integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$) and cleaved to release active TGF- β which binds cell surface receptors triggering phosphorylation of Smad cytoplasmic transcription factors and inhibition or activation of fibrosis associated gene transcription. (Annes et al., 2002, Aluwihare et al., 2009). In the mouse heart β_3 integrin plays a key role in extracellular matrix deposition in pressure overload hypertrophy (Balasubramanian et al., 2012). By increasing the activity of protease inhibitors such as PAI-1 and TIMPS and decreasing the activity of matrix proteases it also acts to prevent remodelling of deposited collagen. Myofibroblasts contain

several α_v subunit containing integrins and these play an important part in the development of fibrosis.

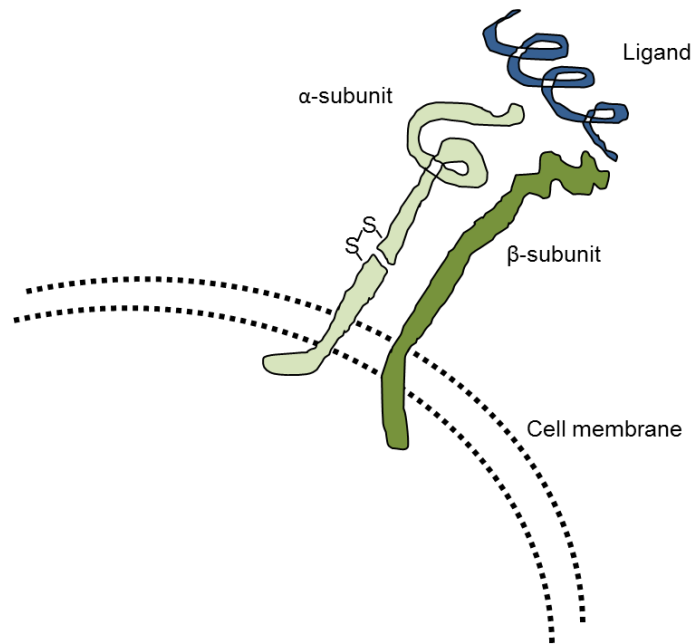


Figure 1-3 Structure of the integrin membrane receptor illustrating the alpha and beta subunits.

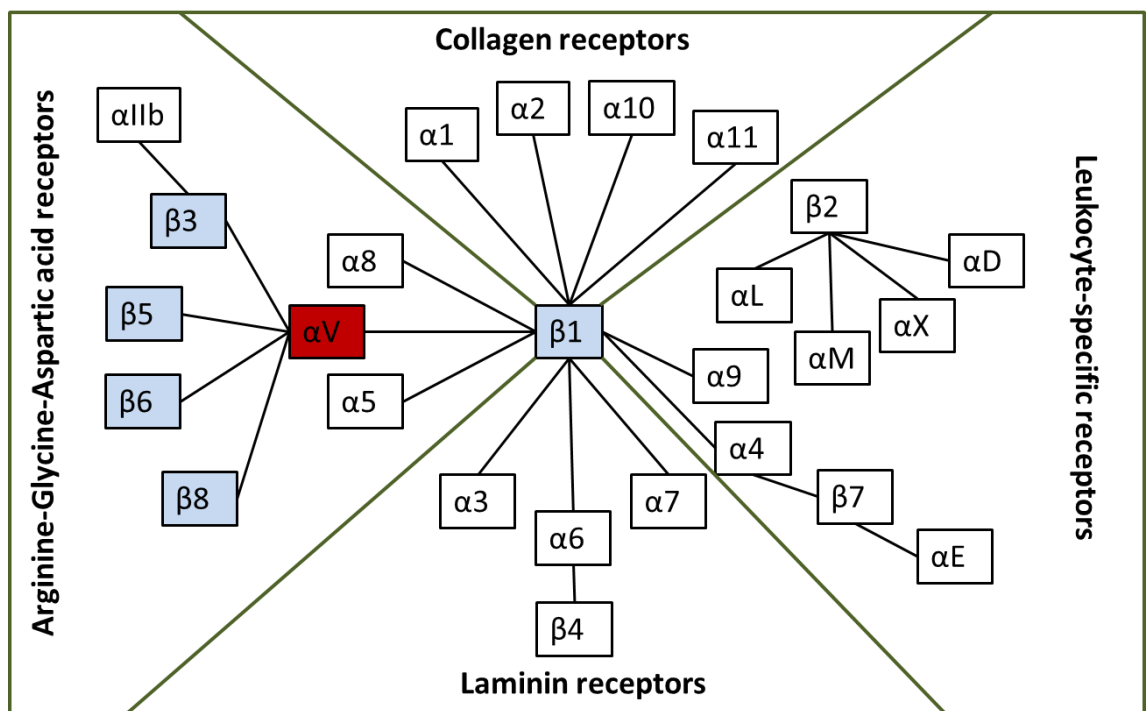


Figure 1-4 Schematic of the integrin receptor family. The α V subunit combines with the β 1, β 3, β 5, β 6 and β 8 subunits.

1.6 General hypothesis

The work in this thesis examines two populations of foetal cardiac perivascular cells as potential candidates for use in cell therapy in the injured murine heart post myocardial infarction. Pericytes isolated and expanded from human skeletal muscle and transplanted into infarcted mouse hearts have been demonstrated to improve cardiac function and reduce scar size (Chen et al., 2013). Stem cell niches are local microenvironments that play important roles in cell maintenance and regulation and are comprised of several components including stromal cells, extracellular matrix, blood vessels and neural inputs (Morrison and Spradling, 2008). It is thought that stem cells may be adapted to the specific niche microenvironments of their tissue of origin giving rise preferentially to cell types present within that tissue (Jones and Wagers, 2008). Supporting this suggestion is the finding that unlike pericytes from skeletal muscle cardiac pericytes are unable to differentiate into skeletal myofibres in culture (personal communication, W. Chen). It was therefore hoped that perivascular cells (pericytes and adventitial cells) from foetal cardiac tissue would be particularly suited to cardiac cell therapy given that they originate from the perivascular niche in the heart itself. Furthermore, foetal and neonatal mammalian tissue exhibits greater plasticity with regards repair processes than adult tissue, with reduced scar formation and enhanced regeneration (Porrello et al., 2011), and it was hoped that cells obtained from plastic foetal heart would readily differentiate into cardiomyocytes.

The working hypothesis for this study therefore was: “Human cardiac perivascular progenitor cells contribute positively to cardiac repair following ischaemic injury.”

1.7 General aims

The aims of the work were as follows:

- To identify and characterise two populations of human perivascular cells with regards their location in the heart, their expression of known pericyte, MSC and cardiac markers.
- To investigate the ability of these cells to differentiate into other cells types including osteogenic cells, adipogenic cells and cardiomyocytes *in-vitro*.

- To determine whether transplantation of these cells into ischaemically injured hearts results in improved function and repair.
- To determine the fate of these cells post injection into injured myocardium.

To investigate the mechanisms that may lay behind their differentiation into other cells types, if seen.

Chapter 2: General methods

2.1 Collection and preservation of human specimens for histology

Human foetal hearts were obtained from elective abortions under ethical consent approved by the University of Edinburgh ethics committee. Foetuses ranged from 9 to 20 weeks of age and both supply and tissue quality was highly variable. The hearts were dissected and the pericardium and great vessels removed then placed in chilled sterile tissue collection media (20% foetal bovine serum (FBS) in DMEM+ GlutaMax and 1% penicillin-streptomycin (P/S) - all Life Technologies). To remove excess blood the chambers were flushed with media under gentle pressure via a needle inserted into atria. Hearts for staining were blotted dry and bisected transversely through the mid-level of the ventricles before being immersed in optimal cutting temperature (OCT) compound (VWR) for 20 minutes then snap frozen in isopentane super cooled in liquid nitrogen. Freezing was carried out for less than 60 seconds to reduce the risk of the sample cracking. Samples were then wrapped in aluminium foil and stored at -80°C. Some heart samples were bisected and fixed in 4% paraformaldehyde (PFA) or 10% neutral buffered formalin (NBF).

2.2 Mouse necropsy

For mice in the cell transplantation study perfusion fixation was employed. Animals were anaesthetised via intra-peritoneal injection of medetomidine (0.5mg/kg) and ketamine (50mg/kg). The abdominal cavity was surgically opened, the abdominal aorta identified and catheterised with a 24 gauge catheter. Approximately 500µl of blood was withdrawn into a citrate buffer coated syringe and placed in a sterile 0.5ml Eppendorf tubes then immediately frozen on dry ice. The carotid vessels were severed and approximately 3ml of sterile ice cold saline was slowly infused via the catheter to flush out erythrocytes from the circulation. This was followed by approximately 3ml of ice cold 10% NBF. For animals in the fibrosis study euthanasia was carried out via rising levels of CO₂. The hearts were removed by severing the great vessels at the apex and careful dissection of any adhesions that had formed between the left ventricle pericardium and the parietal pleura lining the thoracic wall.

They were then blotted on tissue paper to remove excess fluid or blood and weighed. The lungs, spleen, kidneys, brain, skeletal muscle and femur were also collected in animals from the cell transplantation study.

2.3 Preservation and preparation of murine samples for histology

Perfusion fixed tissues were immersion fixed for a further 24 hrs in 10% NFB before being transferred to 70% ethanol for long term storage at 4°C. The hearts of fibrosis study mTmG or Ai14 reporter mice were transversely sectioned and either immediately frozen in OCT in liquid nitrogen or fixed in 4% paraformaldehyde (PFA) overnight before being passed through 30%, 20% and 10% sucrose solutions for 1hr, 1hr and 6 hours and then frozen in OCT on dry ice. The hearts of mice in the PDGFR- β α_v integrin angiotensin II fibrosis study were transversely sectioned into three equal thickness slices between the apex and base. The basal slice was fixed in 4% PFA overnight before being dehydrated in sucrose solutions and frozen. The middle slice was fixed for 24 hours in 10% NBF before being processed to wax sections. The apical slice was flash frozen on dry ice and stored at -80°C.

2.4 Cryosectioning of frozen tissue

Frozen OCT embedded tissue was allowed to come to temperature in the cryostat chamber for 20 minutes before being sectioned at 7 μ m thickness. Sections were adhered to a charged glass slide (Thermo Scientific), allowed to come to room temperature and then stained with haematoxylin and eosin (H&E) using the following protocol: immersion in haematoxylin solution for 5 minutes; washing under running tap water for 2 minutes; immersion in acid alcohol for 10 seconds; washing in Scott's tap water for 30 seconds; immersion in eosin for 30 seconds; washing under running tap water for 2 minutes; application of coverslips using water soluble mounting media (Cell Path). These slides were screened for evidence of autolysis or freeze crystal artefact. Sections from good quality tissue were stored at -80 °C for up to 4 weeks.

2.5 Preparation of paraffin sections from 4% PFA and 10% NBF fixed tissue

Paraformaldehyde and formalin fixed tissue was placed in biopsy cassettes (Leica Biosystems) before being dehydrated through sequential immersion in increasing concentrations of ethanol, cleared in xylene and infiltrated with paraffin wax using an automated tissue processor (Leica Biosystems). 6µm thick sections were cut from the upper surface of each slice by the histology department of the Shared university Research Facilities.

2.6 Immunohistochemical staining of tissue sections

2.6.1 Immunofluorescence

Frozen sections were post-fixed in chilled methanol:acetone (1:1) for 8 minutes before being dried at room temperature for 20 minutes. The tissue was encircled with a hydrophobic marker (PAP pen – Sigma Aldrich) and the slides were washed twice for 5 minutes each time with phosphate buffered saline (PBS) Tween 20 (0.2%, pH 7.2 – Sigma Aldrich) and placed in a humidified light tight tray. The secondary antibodies were raised in goat and non-specific antibody binding sites were blocked by incubating with 10% goat serum/PBS (Sigma Aldrich) for 1 hour. Excess blocking solution was tapped off the slide and the primary antibody diluted in 5% goat serum/PBS was applied. Slides were incubated overnight (16 hours) at 4°C and the following morning washed three times for 5 minutes each with PBS Tween 20 before being incubated for 1 hour at room temperature in the dark with the secondary antibody diluted in PBS. All secondary antibodies were Alexa Fluor dyes (Life Technologies) and were applied at a 1:600 concentration in PBS for 1 hour at room temperature. Slides were washed in PBS Tween 20 three times for 5 minutes each before being coverslipped using DAPI containing mounting media (Fluoromount-G DAPI – eBioscience). Autofluorescence in DAPI only stained controls was minimal in all channels. Isotype controls stained at the same concentration as the primary antibody, were used to assess the degree of non-specific primary antibody binding. Further controls in which the primary antibody was omitted were used to assess the degree of nonspecific secondary antibody binding if necessary. Stained sections were examined using a wide-field fluorescence microscope (Olympus BX-61) and images

were obtained using a Hamamatsu camera and Velocity imaging software (Velocity Inc.).

2.6.2 Chromagen immunohistochemistry

Unstained paraffin sections were de-paraffinised and rehydrated via the following protocol: xylene for 5 minutes; xylene for a further 5 minutes; 100% ethanol for 20 seconds; 100% ethanol for 20 seconds; 95% ethanol for 20 seconds; 80% ethanol for 20 seconds; 70% ethanol for 20 seconds then washing in tap water. Antigen was retrieved by immersing the slides in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH6.0) and heating in a microwave for 15 minutes. The slides were run under cold tap water for 10 minutes then washed twice for 5 minutes each time in washing buffer (PBS plus 0.025% Triton X-100) before being incubated for 20 minutes in 3% hydrogen peroxide (Sigma Aldrich) in methanol to quench endogenous peroxidases. Slides were then washed twice more for 5 minutes before being incubated for 30 minutes with 2.5% horse serum (Vector labs). Following incubation they were briefly rinsed with washing buffer before being incubated with the primary antibody diluted in 5% goat serum and PBS for between 1 hour and 16 hours.

Following primary antibody incubation the sections were washed three times for 5 minutes before being incubated for 30 minutes with ImmPRESS horse radish peroxidase conjugated anti-rabbit or anti-mouse antibody (Vector Labs). They were then rinsed once with washing buffer and incubated with 3, 3'-diaminobenzidine (DAB) horse radish peroxidase substrate solution for between 30 and 45 seconds before being rinsed under running tap water for 5 minutes. A counterstain was applied by incubating in haematoxylin solution (Sigma Aldrich) diluted 1:20 in H₂O for 1 minute. Excess stain was washed off under running tap water for 5 minutes and the sections were dehydrated via immersion in the following: 20 seconds in 70% ethanol; 20 seconds in 80% ethanol; 20 seconds in 95% ethanol; 20 seconds in absolute ethanol; 20 seconds in absolute ethanol; 5 minutes in xylene; 5 minutes in xylene. Finally coverslips were applied using pertex mounting medium (Cell Path) and slides were examined using an Olympus BX41 upright brightfield microscope.

2.7 Fluorescence Activated Cell Sorting/Flow cytometry of human foetal heart

Samples collected for fluorescence activated cell sorting (FACS) or flow cytometry analysis were stored in collection media overnight at 4°C. Atria were excised and the ventricles were placed in a sterile plastic petri dish and finely chopped with scissors and a single side razor blade. 1.5ml of collagenase I, 1.5ml of collagenase II and 1.5ml of collagenase IV (0.5mg/ml) were added to the dish and the contents were then aspirated with a Pasteur pipette and placed in a sterile small Nalgene pot. This was incubated in a shaking (120rpm) water bath (Grant Instruments) at 37°C for 30 minutes. Following digestion 5ml of 20% FBS/DMEM was added to quench further enzymatic activity and the digest was passed through a series of 100µm, 70µm and 40µm mesh filters (Fisher Scientific) to remove non digested tissue. The single cell suspension was then centrifuged at 1000rpm for 5 minutes, the supernatant aspirated and replaced with 1ml of red cell lysing buffer hybri-max (Sigma Aldrich). The pellet was re-suspended through gentle pipetting and incubated for 2 minutes to lyse erythrocytes before being diluted with 5ml of 2% FBS/PBS. The sample was again centrifuged at 1000rpm for 5 minutes and the pellet was re-suspended in 950µl of 2% FBS/PBS and incubated with 50ul of mouse serum (Sigma Aldrich) for 20 minutes to block non-specific binding sites. To count cells 10µl was removed and mixed with 10µl of Trypan Blue (Sigma Aldrich). 10µl of this suspension was placed under the coverslip of a haemocytometer and in each of the four quadrants the number of bright cells not taking up the stain was counted. This number was divided by 4 to give the mean per quadrant then doubled to account for the 2:1 dilution with the dye and finally multiplied by 10^4 to give the total number of cells in the sample.

Following blocking the suspension was divided between four tubes. Two thirds of the suspension was reserved for the stained sample and the remaining 1/3 divided equally between unstained, isotype and full minus one controls (FMO). These were centrifuged at 1000 rpm for 5 minutes, the supernatant removed and the pellets were re-suspended in 2% FBS/PBS at 100µl per 1,000,000 cells. All conjugated primary antibodies were added to the stained sample. All isotype antibodies were added to the isotype sample. All primary antibodies except for one were added to the FMO sample. Antibodies were incubated on ice in the dark for 20minutes then 5ml of 2%

FBS/PBS was added to each tube and the suspension centrifuged at 1000rpm for 5 minutes. Following aspiration a further wash of 5ml of 2% FBS/PBS was applied to ensure the removal of unbound antibody and the sample was centrifuged and re-suspended in 500µl in the case of the controls and 1000µl for the stained sample. 0.25µl of DAPI (Sigma Aldrich) diluted 1:100 in sterile distilled water was added to each tube to label nuclei immediately prior to sorting. Compensation beads (BD Pharmingen) for the species in which the primary antibodies were raised were prepared in parallel with the cells. One drop of positive beads was added to 100µl of 2% FBS/PBS in 5 separate tubes and 1µl of each antibody added to a tube. These were incubated on ice in the dark for 20minutes before being washed with 2% FBS/PBS, re-suspended in 100µl of 2% FBS/PBS. One drop of negative beads was added just prior to the sort.

Compensation was set using the stained and unstained beads for each antibody and the position of negative/positive gates were determined using the isotype controls. Cells were first identified from amongst debris via forward and side scatter parameters before the live (DAPI negative) singlet population was taken forward for analysis of antibody labelling. At the end of each FACS session a purity check was carried out in which approximately 600 cells from each population collected were re-run through the sorter and examined for antibody labelling. Fluorescence activated cell sorting was carried out by Shonna Johnston and Fiona Rossi of the Centre for Inflammation Research cytometry facility and samples were run on a FACS Aria II sorter (BD Pharmingen). Data was analysed using FlowJo software (FlowJo LLC).

2.8 Fluorescence Activated Cell Sorting/Flow cytometry of mouse heart

Hearts were dissected out and the atria excised. The ventricles were then finely chopped with a sterile scalpel blade before being suspended in 1.5ml per heart of 200 U/ml collagenase II (Gibco) in empty DMEM. Several digestions using 200, 300, 450 and 600 U/ml of Collagenase II and digestion periods of 20, 30, 45 minutes were trialled before the final protocol of was decided upon. Digesting tissue was continuously agitated in a rotatory agitator in a 37°C oven. After 10 minutes (15

minutes for 1st digestion and 10 minutes for all subsequent digestions) incubation the digest was gently triturated using a Pasteur pipette and the tissue was allowed to settle. The supernatant was then removed and placed in a new tube where the enzyme activity was quenched with 3mls of 10% FBS/DMEM before being placed on ice. Fresh enzyme solution was added and the process repeated 5 to 8 times until no obvious tissue remained. After the final digestion step all digest solutions were passed through a 40µl cell strainer before being centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the pellet re-suspended in 1ml of red blood cell lysing buffer hybrid-max (Sigma Aldrich) for 2 minutes before being diluted with 10% FBS/DMEM. Suspended cells were then stained as described in 2.7.

2.9 Culture expansion of human perivascular cells

Following FACS cells were maintained in EGM2 until they settled and adhered to the plastic well bottom (approximately 7 to 10 days). The old media was then removed and replaced with fresh pericyte growth media comprising DMEM + GlutaMAX 20% foetal bovine serum and 1% P/S and this was exchanged every 72 hours. Cells were passaged upon reaching approximately 90% confluence. Wells or flasks were washed twice with sterile PBS then 0.05% Trypsin EDTA (Life Technologies) was added and the cells returned to the incubator for 2 to 3 minutes. Dissociation was confirmed under the microscope with gentle percussion used to free any stubbornly adherent cells. 5ml of 20% DMEM was added to quench the trypsin and the cell suspension was centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and the pellet re-suspended in pericyte growth media before being re-seeded at a 3 to 1 new to old culture vessel area ratio.

2.10 Cryopreservation of cultured cells

After expansion in culture cells between passages 4 to 6, were cryopreserved for later use. Cultures were washed, detached with trypsin and pelleted as described. The pellet was re-suspended in 1ml of freezing media, comprising 90% foetal bovine serum and 10% EDTA (Sigma Aldrich), per million cells before being frozen at -80°C in cryovials (Fischer Scientific) in a Mr Frosty freezing container (Thermo Scientific). Frozen cells were moved to liquid nitrogen for longer term storage.

2.11 Thawing of cryopreserved cells

Cryovials were thawed in a 37°C water bath until only a small amount of ice remained. The outside of the vial was then cleaned with an ethanol soaked wipe. The content was pipetted into warmed thawing media comprising 50:50 FBS/DMEM and 1% penicillin/streptomycin and centrifuged at 1000rpm for 5 minutes. The supernatant was disposed of and the cell pellet re-suspended in 5ml of warmed 20% FBS/DMEM growth media before being transferred to culture flasks.

2.12 Preservation of RNA from cultured cells

At time of cryopreservation approximately 300,000 cells were preserved for RNA extraction by lysing with 0.5ml of TRIzol (Life Technologies). TRIzol preserved samples were stored at minus 80°C until required.

2.13 RNA extraction

Initial RNA extraction from human samples was carried out using a chloroform phase separation technique however poor 260:280 ratios and abnormal absorbance curves were frequently seen on sample assessment using a Nanodrop spectrophotometer (Thermo Scientific). To remove contaminating proteins and improve RNA quality a hybrid protocol was adopted combining TRIzol lysed samples with column extraction (Qiagen RNeasy Minikit). This resulted in significant improvement in product purity.

Frozen TRIzol preserved samples were thawed on wet ice and vortexed for 1 minute before being passed through a sterile 31 gauge needle and syringe five times to disrupt cell membranes. 0.2ml of chloroform (VWR) per ml of TRIzol was added to the homogenate and the sample shaken for 20 seconds before being centrifuged at 12,000 rpm for 18 minutes at 4°C. The uppermost aqueous phase was aspirated using a sterile 200µl pipette and transferred to a sterile 1.5ml Eppendorf tube. An equal volume of 100% ethanol (VWR) was slowly added and gently mixed by pipetting up and down. 700µl of sample was then transferred to an RNeasy mini column (Qiagen) seated within a 2ml collection tube. This was centrifuged for 60 seconds at 10,000 rpm. The flow-through was discarded and the spin column transferred to a new collection tube. 500µl of RPE buffer (Qiagen) was added and the column spun at 10,000 rpm for 60 seconds before discarding the flow-through. A further 500µl of RPE buffer was added again and the column centrifuged for 2 minutes at 10,000 rpm. The flow through was discarded and the column spun empty for 1 minute to remove any remaining buffer. Finally the column was transferred to a new 1.5ml collecting tube and 30µl of nuclease free water (Qiagen) was added directly to the membrane before being left to stand for 2 minutes at room temperature then spun at 12,000 rpm for 1 minute to elute the RNA. Eluted RNA was immediately transferred

to wet ice before being analysed on the Nanodrop. The absorbance curve was checked and the concentration of RNA, the 260:280 ratio and the 260:230 ratio were recorded. If the above values indicated good quality RNA (260:280 >1.85) then an RNA integrity gel was run. For this a 1.5% agarose gel was cast from 1.5g of agarose (Lonza) dissolved in 100ml of TBE buffer (89mM Tris base, 89mM boric acid, 2.5mM EDTA – all Sigma Aldrich) and containing 5µl of ethidium bromide (Life Technologies) or Gel Red (Cambridge Bioscience). 4µl of the RNA sample was mixed with 2µl of nuclease free water and 2µl of loading dye and a total of 6 µl was loaded into each well. 1µl of 100 base pair DNA ladder (New England Biolabs) was mixed with 1µl of loading dye and 4µl of nuclease free water and from this mixture 5µl was loaded into the gel. Gels were run in TBE buffer at 100V for 60 minutes and read on a UV transilluminator (Ultra Violet Products Ltd). RNA was considered to be good quality if both 28S and 18S bands were clearly seen with the former of approximately twice the density as the latter (Figure 2-1).

For the extraction of RNA from mouse tissue an RNeasy Micro Kit (Qiagen) was used. The RLT cell lysate buffer (Qiagen) was homogenised by repeated passage through a sterile, 23 gauge, RNase free needle. 350µl of 70% ethanol was added to the lysate and mixed by pipetting. The sample was then transferred to an RNeasy MinElute spin column in a 2ml collection tube and centrifuged for 45 seconds at 12,000rpm. The flow through was discarded, 350µl of RW1 buffer was added and the column centrifuged at 12,000rpm for 45 seconds. The flow through was discarded and 10µl of DNase I stock solution diluted in 70µl of RDD buffer was added directly to the membrane. This was incubated for 15 minutes at room temperature before a further 350µl of RW1 buffer was added and the column centrifuged for 45 seconds at 12,000rpm. The collection tube was discarded and the column placed in a new 2ml collection tube. 500µl of RPE buffer was added and the column was inverted and rotated several times to wash the walls before being centrifuged at 12,000rpm for 45 seconds. 500µl of 80% ethanol was added to the column and centrifuged for 2 minutes at 12,000rpm. The collection tube was discarded and the column placed in a new 2ml collection tube. The lid was removed and the open column spun at 13,000rpm for 5 minutes to dry the membrane. The

flow through and tubes were discarded and the column placed in a 1.5ml collection tube. 14µl of nuclease free water was added directly to the membrane and left to absorb for 2 minutes with the column lid closed. The column was then centrifuged for 1minute at 13,000rpm. The RNA sample was transferred to immediately ice and the RNA concentration was assessed using the Nanodrop and where sufficient RNA was available integrity gels were run as described above.

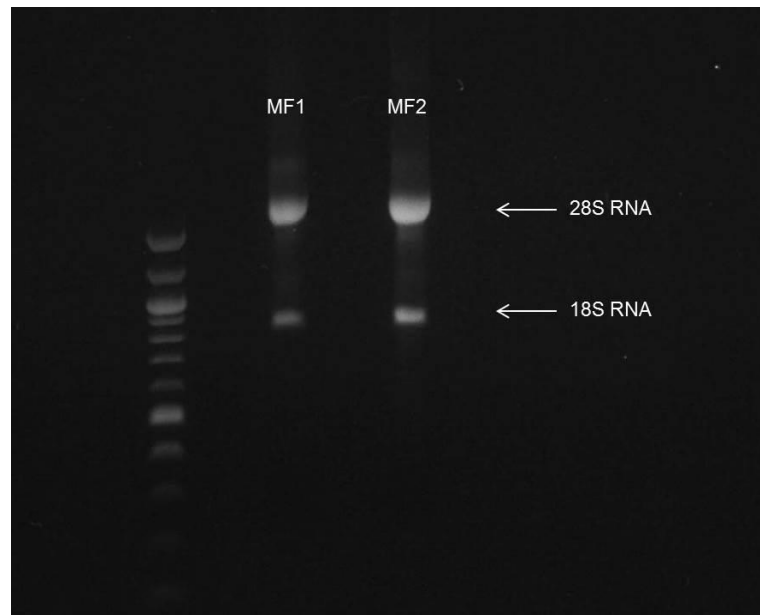


Figure 2-1 RNA integrity gel demonstrating 28S and 18S RNA bands in two populations of mouse fibroblasts, MF1 and MF2. DNA ladder bands represent 100bp.

2.14 Reverse-transcription of RNA and generation of cDNA

Reverse-transcription was performed immediately following extraction. In human samples the volume of RNA required to make 500ng was calculated based on the Nanodrop concentration measurement (mean of three 1µl samples) and sufficient nuclease free water added to reach a volume of 11µl. For mouse samples in the PDGFR-β fibrosis study the amount of RNA transcribed was limited to the maximum available in the most dilute sample. 1µl of random primers and 1µl of dNTP (Life Technologies) were added to the RNA, mixed by gentle pipetting and then heated to 65°C for 5 minutes in a thermocycler (Applied Biosystems) before being chilled on ice for 2 minutes. 4µl of 5x First strand buffer (Life Technologies) and 2µl of 0.1M DTT (Life Technologies) were added and mixed by pipetting. The sample was allowed to incubate for 2 minutes at room temperature before 1µl of Superscript enzyme (Life Technologies) was added and mixed by pipetting. A 10 minute incubation at room temperature followed and then the sample was heated to 42°C for 50 minutes and the enzyme inactivated by heating to 70°C for 1 minute. The resulting cDNA was stored at -20°C until required. Where RNA yields were sufficient a reverse transcription control, in which the Superscript enzyme (Invitrogen LifeTechnologies) was omitted, was included to assess for genomic DNA contamination.

2.15 Conventional Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Conventional reverse transcription PCR was performed using cDNA samples diluted 1:10 with nuclease free water. For each gene investigated a mastermix containing the following components was made:

Taq Buffer 5x (Life Technologies)	4µl
dNTP	0.2µl
Taq Polymerase (Life Technologies)	0.2µl
Primer Forward (10mM) (Eurofins)	0.5µl

Primer Reverse (10mM) (Eurofins)	0.5µl

For each gene strip tubes were prepared containing the following volume of reagents:

Nuclease free water	13.6µl

Mastermix	5.4µl

cDNA	1µl

Negative control sample contained 1µl of nuclease free water rather than template. The samples were mixed by gentle pipetting and then run in the thermocycler under the following PCR protocol: 94°C for 10 minutes; thirty cycles of 94°C for 10 seconds, 58°C for 30 seconds, 72°C for 50 seconds; 72°C for 7 minutes; 4°C for infinity.

PCR product was run immediately on a 1.5% agarose gel or stored at -20°C until required. 1.5% agarose gels were made as previously described and run in TBE buffer at 100V for 80 minutes. Gels were imaged as previously described.

2.16 Quantitative RT-PCR (qRT-PCR)

Forward and reverse primer sequences were designed using the online Universal Probe Library (UPL) database (Roche) which also specified the corresponding FAM hydrolysis probe. Initial assessment of primer efficiency was carried out by running a standard curve on serially diluted cDNA from positive control cells. The following dilutions in nuclease free water (Qiagen) were assessed: 1/10; 1/100; 1/1000; 1/10,000; 1/100,000. Efficiencies of between 1.85 and 2 were considered adequate and primers with efficiencies out-with these values were re-designed. The cDNA template was diluted 1:10 with nuclease free water. Each sample was run in duplicate or triplicate and for each gene a mastermix was made comprising, per well, of 4µl of LightCycler 480 probes master (Roche), 1.92µl of 2µM of forward and reverse primers (Eurofins) and 0.08µl of probe. For each gene 6µl of this mastermix was added per well with 2µl of cDNA template. The plate was then sealed with a clear adhesive film cover and centrifuged at 1000rpm for 30 seconds. Plates were

frequently stored overnight at 4°C in the dark before being run the following morning on a LightCycler 480 instrument (Roche). Data was analysed on the LightCycler 480 analysis software (Roche). CT values were calculated for each replicate and replicates with a deviation of greater than 0.5 of a cycle discarded or repeated. The mean of the remaining replicates was calculated for each gene. The relative quantification method was used to compare gene expression. The delta CT values were calculated for each sample by subtracting the mean CT value of the five housekeeper genes from the CT of the gene of interest. The mean delta CT value was calculated for the group selected to be the calibrator. The delta delta CT was then calculated by subtracting the mean delta CT of the calibrator group from the individual delta CT values in both groups. These values (X) were normalised by expression as 2^{-X} . The mean of the normalised values for each group was calculated as was the standard deviation and standard error of the mean (SEM = standard deviation/ $\sqrt{\text{sample number}}$).

2.17 Immunocytochemistry of cultured cells

Immunocytochemistry was carried out on cultured cells grown on sterile glass coverslips in 12 well plates. Growth media was aspirated and the cells were washed twice with sterile PBS before being fixed for 5 minutes with a 50:50 solution of methanol:acetone (VWR) or 4% PFA (Sigma Aldrich). Following fixation the fixative was aspirated and the cells again twice washed with PBS before being stored in PBS. Cells were then permeabilised with 0.025% Triton X-100 (Sigma Aldrich) for 8 minutes before being washed again with PBS. Non-specific antibody binding sites were blocked by incubation with 10% goat serum in PBS for 1 hour followed by primary antibody incubation. Primary antibodies were diluted in 5% goat serum. Coverslips were then washed three times with PBS for 5 minutes before the secondary antibody was applied. Three more 5 minute PBS washes were then carried out and the coverslips were carefully removed from the wells and placed cell side down onto a drop of Fluorochrome G DAPI mounting media on a glass slide and allowed to dry.

2.18 Labelling of cells for co-culture and injection studies

Two methods of cell labelling were used: CM-Dil (Life Technologies) cell tracker; and enhanced green fluorescent protein (eGFP) viral transduction.

CM-Dil dye was reconstituted in DMSO at 1µg/µl and diluted 1:500 in serum free DMEM. Media was aspirated from flasks and the cells washed three times with PBS before 5ml of dye solution was added. The cells and dye were incubated for 5 minutes at 37°C and then for 15 minutes at 4°C before being washed three times with PBS and the growth media replaced.

eGFP viral transduction used an eGFP expressing lentivirus under the control of a cytomegalovirus promoter which drives high constitutive levels of gene expression in mammalian cells and is particularly suited to stem cell transfection (Ward and Stern, 2002). Generation of genetically modified lentivirus particles was undertaken in Professor Andrew Baker's lab with the assistance of Nicola Britton at the Institute of Cardiovascular and medical sciences at the University of Glasgow. T150 flasks were seeded with 12×10^6 293T cells and allowed to settle over a 24 hour period in 10%FBS DMEM 1% P/S (complete DMEM) before being transfected with lentivirus. The following was added per 5ml of Opti-MEM media: 31.6µl of vector construct (PHRSIN); 50.9µl of envelope plasmid (PMDG); 99.1µl of packaging plasmid (INT). The media was then sterilised with a 0.22µm syringe filter. A separate quantity of Opti-MEM (Life Technologies) media containing 1µl of 10mM polyethylenimine (Sigma Aldrich) per 5ml was also filter sterilised. These two solutions combined at a 1:1 ratio to form the transfection media and incubated for 20 minutes at room temperature. Media was aspirated from the flasks and the cells were washed with Opti-MEM and 10ml of the transfection media mixture was added to each flask. Cells were incubated at 37°C, 5% CO₂ for 4 hours and the media was then removed and replaced with 20ml of fresh complete DMEM and the cells incubated for a further 3 days. Viral laden supernatant was removed at 48 and 72 hours. To concentrate the virus this media was filtered through a 0.22 µm filter and 33ml added to ultracentrifuge tubes that had been washed in ethanol then sterile PBS. Tubes were

centrifuged at 23,000rpm for 67 minutes at 4°C following which the supernatant was poured off and the tubes placed upside down for 5 minutes to drain. 100µl of Opti-MEM was then added to each tube and incubated on ice for 20 minutes. Viral particles were re-suspended by pipetting up and down and all aliquots were added to single tubes and the volume measured. Half the volume of Lenti-X Concentrator was added, the solution thoroughly mixed and then incubated on ice for 30 minutes. Following incubation the mixture was centrifuged at 1500g for 45 minutes at 4°C. The supernatant was removed and the pellet re-suspended in 50µl of Opti-MEM. The final viral solution was then aliquoted into 2µl batches in sterile 500ul Eppendorf tubes and frozen immediately at -80°C.

Viral titre was established via 72 hour transfection of 293T cells with serial dilutions of the virus followed by qRT-PCR analysis using a serially diluted expression plasmid as a standard. This step was carried out by Nicola Britton.

The final viral titre was calculated to be 4.35×10^6 infectious units/µl. To transfect perivascular progenitor media was aspirated from flasks and the cells washed with PBS twice. Half the volume of serum free media was replaced and 1µl of virus was added per 800,000 to 1×10^6 cells giving a multiplicity of infection of approximately 5. The cells were then incubated at 37°C for 5 hours before the remaining half volume of growth media was added and the cells incubated again overnight. The following morning the virus containing media was aspirated and replaced with growth media. The efficiency of transduction was assessed by immunofluorescence microscopy of several flasks of cells 24hrs post induction and was estimated at >95% (Figure 2-2). Brightness of the GFP signal varied between cells presumably as a result of number of copies of the GFP sequence integrated into the host cell genome but in all cases could be detected easily.

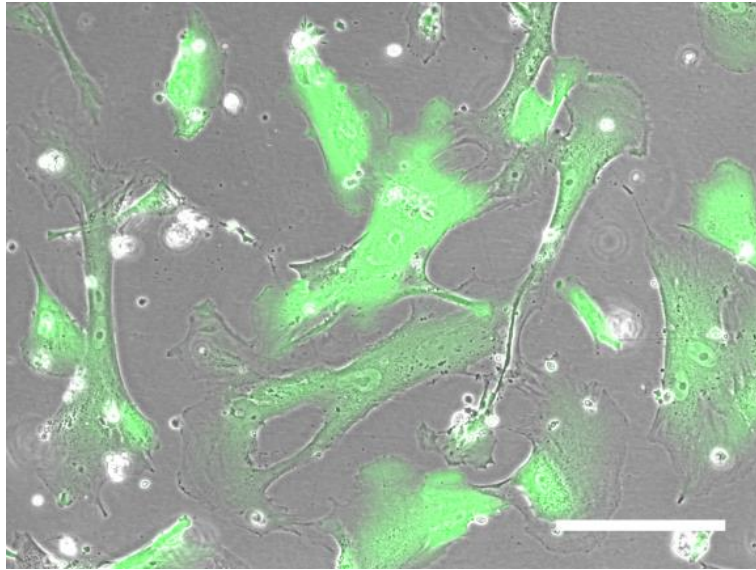


Figure 2-2 Phase contrast and immunofluorescence image of eGFP expressing pericytes following lentiviral transfection. >95% of cells express the GFP signal. Scale bar = 25 μ m.

2.19 Mice used in experiments

For the human cardiac perivascular cell transplantation study 65 NOD/SCID mice were used. 10 mice were used in the initial validation of the technique and these were obtained from Charles River Laboratories (UK). The remaining 53 mice used in the main study were obtained from Harlan laboratories (UK) as the Charles River Laboratories had insufficient stock. Animals were kept in individually ventilated cages to limit exposure to airborne pathogens.

For the investigation of the role of PDGFR- β^+ cells in cardiac fibrosis several different genetically modified mouse models were used. To track PDGFR- β^+ cells a mTmG;PDGFR- β -Cre mouse colony (mTmG reporter mice) was established by breeding PDGFR- β Cre male mice with mTmG (tdTomato-eGFP) homozygote females. The latter are double fluorescent Cre reporters in which the membrane targeted tomato fluorescence (mT) is expressed prior to Cre-mediated excision and the membrane bound green fluorescence (mG) after excision (Muzumdar et al., 2007). Cre positive offspring were used in experiments. Several mice from a second reporter line, Ai14;PDGFR- β -Cre (Rosa-CAG-LSL-tdTomato-WPRE), were also used (Madisen et al., 2010) to investigate the role PDGFR- β^+ cells play in fibrosis. These mice Ai14 reporter mice strongly express td tomato in the PDGFR- β^+ cell population. To investigate the role of α_v integrin (Itgav) expression by PDGFR- β^+ cells in the development of interstitial fibrosis an α_v integrin^{flox/flox}; PDGFR- β -Cre colony (Lacy-Hulbert et al., 2007) was established by breeding Itgav^{flox/flox} PDGFR- β Cre⁺ females with Itgav^{flox/flox} PDGFR- β Cre⁻ males. Both cre positive (α_v integrin Cre⁺) and cre negative (α_v integrin Cre⁻) offspring were used in experiments. All breeding animals and additional Ai14 reporter mice were supplied by Dr Neil Henderson. Female mice aged 9 to 12 weeks were used in *in-vitro* experiments. Male mice aged 9 to 12 weeks were used in *in-vivo* experiments.

2.20 Genotyping of genetically modified mice

Both mTmG;PDGFR- β -Cre reporter mice Itgav^{flox/flox};PDGFR- β -Cre mice were genotyped by PCR. Mice were ear clipped for identification at weaning by staff in the BRR facility and tissue was kept at -20°C. Individual ear clips were placed in 2ml

Eppendorf tubes containing 200µl DirectPCR lysis reagent (VIAGENBIOTECH) plus 4µl Proteinase K (QIAGEN) and incubated overnight at 55°C. The following morning samples were heated to 85°C for 45 minutes to inactivate the Proteinase K then centrifuged for 10 minutes at 1000rpm. A PCR mix containing the following components was made for each reaction (total 23.5µl): 5xQ solution 5µl; 10x buffer (red) 2.5µl; dNTP (10mM) 0.5µl; forward primer (25uM); Reverse primer (25uM); Taq polymerase 0.2µl; nuclease free water 14.5 µl (all reagents from the QIAGEN Taq PCR Core kit). Primers sequences were provided by the Henderson group and primers were constructed by Eurofins (Table 2-1). 1.5µl of sample supernatant was added to each reaction and a negative control containing 1.5µl of nuclease free water instead of sample was included. Reactions were run on a thermocycler using the following programme: 94°C for 5mins; 29 cycles of 94°C for 30 seconds, 57 °C for 45 seconds and 72°C for 2 minutes; 72°C for 10 minutes; 12 °C for infinity. The PCR product was then run on a 1.5% agarose gel with a 100bp DNA ladder for 80 minutes at 90V and imaged using a UV transilluminator.

Table 2-1 Conventional RT-PCR primer sequences used for genotyping.

Gene	Sequence	Product (bp)
α_v integrin	f CACAAATCAAGGATGACCAA r TTCAGGACGGCACAAAGACCG	400
Cre	f TGC CAC GAC CAA GTG ACA GCA r AGA GAC GGA AAT CCA TCG CTC	400

2.21 High frequency ultrasound assessment

Acquisition of ultrasound images was carried out by Adrian Thompson of the Edinburgh Preclinical Imaging department. Animals were anaesthetised with Isoflurane and secured in dorsal recumbency. Long axis B mode images and short axis M mode images of the left ventricle were acquired. Data was analysed using Visualsonics vevo software. In B mode both diastolic and systolic endocardial and epicardial outline were traced from the aortic valve annulus and the distance from the annulus to the endocardial and epicardial surface of the apex measured. In M mode the dorsal and ventral endocardial surfaces were traced over three contraction and relaxation cycles. Diastolic endocardial area, systolic endocardial area, endocardial area change, fractional area change, fractional shortening and ejection fraction were calculated. Endocardial area change (EAC) is the difference between the diastolic left ventricular areas in end diastole and end systole and is an indicator of the contractility of the left ventricle. Fractional area change (FAC) is EAC represented as a percentage of the left ventricular end diastolic area and again is a measure of contractility. Fractional shortening (FS) is the difference between the left ventricular diastolic and systolic diameters represented as a percentage of the diastolic diameter. Ejection fraction (EF) represents the volumetric fraction of blood ejected from the left ventricle with each heartbeat. $LV \text{ ejection fraction} = (LV \text{ stroke volume} / LV \text{ end diastolic volume}) \times 100\%$.

2.22 Histological assessment of cardiac collagen content in mouse hearts

Sections were stained with Masson's trichrome stain and their identities blinded before being analysed using a brightfield light microscope with an automated stage and Q Capture Pro imaging software was used to obtain an x 4 objective tiled image of each heart cross section. The size of the scar in the myocardium was measured in three ways. In technique 1 the outline of the collagen region was traced manually using Image J software from the National Institutes of Health (NIH) and represented as a fraction of the area of the left ventricle free wall and septum. In technique 2 the area occupied by the blue staining collagen was measured using a colour picker thresholding tool using Q Capture Pro software. A region of interest was first

selected to include the left ventricular free wall and interventricular septum. The colour of interest was selected and all pixels of an identical shade and intensity were counted. For technique 3 a thresholding macro on Image J was used. The right ventricular free wall was first erased from the image and the RGB image converted to a three colour stack which was split into its component images. The red image was discarded and the upper and lower threshold values were set for the blue image to determine the entire area of tissue in the image. The green image was chosen to assess the collagen content as this gave the best contrast between the blue staining areas and red staining intact myocardium seen in the original RGB image. The threshold values were set to determine the areas of collagen staining. The macro was recorded and run on all the images individually. All techniques were validated at first on sections exhibiting intense staining and sections exhibiting weaker staining.

2.23 Statistics

All statistical calculations were carried out using R software. Results of the pericyte versus adventitial cell characterisation studies and α_v integrin *in-vitro* studies in which there were two experimental groups were analysed using an independent t test. Results from the human perivascular progenitor cell injection study in which there were 3 groups but one variable (genotype) were analysed using one way ANOVA pairwise comparison of the means followed by Tukey's post hoc analysis. Results of the α_v integrin angiotensin II *in-vivo* study in which there were four groups and two variables (genotype and treatment) were analysed using two way ANOVA pairwise comparison of the means followed by Tukey's post hoc analysis. All results were graphically displayed using Excel (Microsoft).

Chapter 3: Human cardiac perivascular cells *in-vitro* express MSC and cardiomyocyte progenitor like phenotypes with limited cardiomyocyte differentiation potential

3.1 Introduction

Adult stem cell populations within the heart are poorly understood. Several cardiac candidate progenitor populations have been identified including c-kit⁺ cells, insulin gene enhancer protein Isl-1⁺ cells and fms-like tyrosine kinase Flt1/Flt4⁺ cells and some of these have shown promise in cell transplantation studies. Pericytes are now widely accepted as the precursors of MSCs in a wide variety of non-cardiac tissues and have been reported to differentiate into several distinct tissue types including adipose tissue, cartilage and skeletal muscle (Crisan, Yap et al. 2008). In many, if not all, tissues a second perivascular progenitor population exists, the CD34⁺ adventitial cell. Like pericytes these cells express MSC markers and are reported to acquire a pericyte-like phenotype in culture in response to growth factors involved in re-modelling (Corselli, Chen et al. 2012). The general aim of this part of the study was to identify and characterise the pericyte and adventitial populations in the heart with regard to their surface marker expression, anatomical location and differentiation capacity and in doing so gain an insight as to their suitability for use as a donor population in cardiac cell therapy.

3.2 Specific hypothesis

The hypothesis for this part of the study was: “Perivascular cells in the heart will express similar surface markers as in other tissues, will show mesenchymal stem cell like differentiation capacity and will be able to differentiate into cardiomyocytes.”

3.3 Specific aims

Specific aims for this part of the study were:

- To confirm the staining characteristics and determine the anatomical location of CD146⁺ CD34⁻ pericytes and CD146⁻ CD34⁺ adventitial cells within the foetal human heart.
- To isolate, purify and expand these cells from foetal human heart.
- To compare and contrast expression of pericyte, MSC and cardiomyocyte markers in cultured cell populations.
- To investigate the *in-vitro* MSC-like differentiation capacity of these cells.
- To investigate the *in-vitro* cardiomyocyte differentiation capacity of these cells.

3.4 Specific materials and methods

3.4.1 Preparation and preservation of tissue

As described in 2.1 and 2.4.

3.4.2 Immunohistochemistry

Seven foetal heart samples between gestational ages of 14 and 20 weeks were stained and the presented images are representative of the findings in the left ventricular myocardium. Staining was carried out as described in 2.6.1 using antibodies detailed in Table 3-1.

3.4.3 Fluorescence Activated Cell Sorting (FACS)

Nineteen individual foetal heart samples were sorted. The youngest donor was 14 weeks whilst the oldest was 20 weeks. Tissue from donors less than 14 weeks of age was not used for cell isolation due to the poor cell yields resulting from samples of this size. A five antibody panel comprising CD45, CD56, CD144, CD34, and CD146, was used to isolate pericyte (CD146⁺ CD34⁻) and adventitial cell (CD146⁻ CD34⁺) populations. Cell isolation and staining was carried out as described in 2.7. The details of the flow cytometry antibodies used are presented in Table 3-2.

3.4.4 *Ex-vivo* expansion of pericyte and adventitial populations

Pericytes and adventitial cells from donors of between 14 weeks and 20 weeks of age were cultured and cryopreserved as described in 2.10 before being used in

experiments between passages 2 and 6. Paired populations of perivascular cells from the same donor were used whenever possible.

3.4.5 Flow cytometry analysis

Flow cytometric analysis for pericyte and MSC markers was performed on three pericyte and adventitial cells populations after a minimum of two passages in culture. Approximately 300,000 cells were analysed as described in 2.7. The details of the antibodies used are presented in Table 3-3.

Table 3-1 Antibodies used for immunofluorescence staining and chromagen staining (Chr) of human tissue.

Antibody	Type	Raised in	Manufacturer	Catalogue No.	Dilution
CD146	Monoclonal	Mouse	BD Pharmingen	550314	1:100
CD34	Monoclonal	Mouse	BD Pharmingen	550390	1:50
NG2	Monoclonal	Mouse	BD Pharmingen	554275	1:100
PDGFR- β	Polyclonal	Rabbit	Abcam	ab21234	1:100
α -SMA-FITC	Monoclonal	Mouse	Sigma Aldrich	F3777	1:100
CD31	Polyclonal	Rabbit	Abcam	ab28364	1:100
c-kit/ CD117	Polyclonal	Rabbit	Abcam	ab5505	1:50
CD105	Monoclonal	Mouse	Invitrogen	mhcd10500	1:100
CD34	Monoclonal	Mouse	Abcam	ab8536	1:100 (Chr)
Alexa Fluor 647	IgG H&L	Goat anti mouse	Life Technologies	A-212422	1:600
Alexa Fluor 555	IgG H&L	Goat anti rabbit	Life Technologies	A-0212422	1:600
Alexa Fluor 488	IgG H&L	Goat anti mouse	Life Technologies	A-11029	1:600
Alexa Fluor 555	IgG H&L	Goat anti mouse	Life Technologies	A-21424	1:600
Alexa Fluor 488	IgG H&L	Goat anti rabbit	Life Technologies	A-11034	1:600

Table 3-2 Primary antibodies and isotypes used for FACS of human heart tissue. All antibodies were used at 1:100 dilution.

Antibody	Manufacturer	Raised in	Catalogue No.
CD146-AF647	AbD Serotec	Mouse	MCA2141A647
CD34-PE	BD Pharmingen	Mouse	555822
CD56-PE-Cy7	BD Pharmingen	Mouse	557747
CD45-APC-Cy7	BD Pharmingen	Mouse	557833
CD144-PerCP-Cy5.5	BD Pharmingen	Mouse	561566
IgG1κ-647	AbD Serotec	Mouse	MCA1209A647
IgG1κ-PE	BD Pharmingen	Mouse	559320
IgG1κ-PE-Cy7	BD Pharmingen	Mouse	557872
IgG1κ-APC-Cy7	BD Pharmingen	Mouse	557873
IgG1κ-PerCP-Cy5.5	BD Pharmingen	Mouse	550795

Table 3-3 Antibodies and isotypes used for cell flow cytometry of cultured human cells. All antibodies were used at 1:100 dilution.

Antibody	Manufacturer	Raised in	Catalogue No.
CD45-APC-Cy7	BD Pharmingen	Mouse	557833
CD56-PE-Cy7	BD Pharmingen	Mouse	557747
CD31-PE	BD Pharmingen	Mouse	555446
NG2-APC	R&D Systems	Mouse	FAB2585A
CD146-FITC	AbD Serotec	Mouse	MCA2141F
CD105-PE-CF594	BD Pharmingen	Mouse	562380
CD90-PE-CY5	BD Pharmingen	Mouse	555597
CD73-FITC	BD Pharmingen	Mouse	561254
CD271-V450	BD Pharmingen	Mouse	562123
CD44-AF-700	BD Pharmingen	Mouse	561289
IgG1κ-APC-Cy7	BD Pharmingen	Mouse	557873
IgG1κ-PE-Cy7	BD Pharmingen	Mouse	557872
IgG1κ-PE	BD Pharmingen	Mouse	555749
IgG1κ-APC	BD Pharmingen	Mouse	IC002A
IgG1κ-FITC	AbD Serotec	Mouse	MCA1209F
IgG1κ-PE-CF594	BD Pharmingen	Mouse	562292
IgG1κ-PE-CY5	BD Pharmingen	Mouse	555750
IgG1κ-FITC	BD Pharmingen	Mouse	554679
IgG1κ-V450	BD Pharmingen	Mouse	560373
IgG1κ-AF-700	BD Pharmingen	Mouse	560543

3.4.6 mRNA expression analysis by conventional PCR

RNA from pericytes and adventitial cells was preserved, extracted and reverse transcribed as described in 2.12, 2.13 and 2.14. The cDNA was examined via conventional RT-PCR as described in 2.15 for expression of pericyte, MSC and cardiomyocyte marker mRNA. The primer sequences used are detailed in Table 3-4.

3.4.7 mRNA expression analysis by qRT-PCR

Pericytes and adventitial cells were investigated for pericyte, MSC and cardiomyocyte marker mRNA expression by qRT-PCR as described in 2.16. The primer sequences used are detailed in Table 3-5.

Table 3-4 Primer sequences for conventional RT-PCR analysis of pericyte, MSC and cardiomyocyte associated marker genes in human cells.

Gene	Sequence	Product (bp)
CD34	f CAT CAC TGG CTA TTT CCT GAT G r AGC CGA ATG TGT AAA GGA CAG	419
CD146	f AAG GCA ACC TCA GCC ATG TCG r CTC GAC TCC ACA GTC TGG GAC	435
NG2	f GCT TTG ACC CTG ACT ATG TTG GC r TCC AGA GTA GAG CTG CAG CA	195
PDGFR- β	f CAGTAAGGAGGACTTCCTGGAG r CCTGAGAGATCTGTGGTTCCAG	178
CD56	f GTA TTT GCC TAT CCC AGT GCC r CAT ACT TCT TCA CCC ACT GCT C	331
β -actin	f CCT CGC CTT TGC CGA TCC r GGA ATC CTT CTG ACC CAT GC	204
GATA-4	f TGGGACGGGTCACTATCTGT r AAGGCTCTCACTGCCTGAAG	328
Mef2c	f TGTACAGCAACCCTGTCAGC r ACTGGGGTAGCCAATGACTG	418
Nkx2.5	f CGCCCTTCTCAGTCAAAGAC r TTGTCCGCCTCTGTCTTCTC	613
ACTC-1 (α -actinin)	f GGACTCTGGGGATGGTGTA r AGGGCTGGAAGAGTGTCTCA	335
MHC7	f AGTATGAGACGGACGCCATT r GATGTTCCAGGGACTCCTCA	363
MLC2	f CTGAGAGACACCTTTGCTGC r GTGGTCAGCATTTCCCGAAC	230
Troponin T	f TGCAGATTGCAAAGCAAGAG r CCTTCTTCACCTGCTTGAGG	375
ANP	f ATTTGCTGGACCATTGGA r TTGCTTTTATAGGAGGGCAGA	217
CD44	f AAGGTGGAGCAAACACAACC r AGCTTTTTCTTCTGCCACA	151
CD105	f CACTAGCCAGGTCTCGAAGG r CTGAGGACCAGAAGCACCTC	165
CD73	f ACGCAACAATGGCACAATTA r CTCGACACTTGGTGCAAAGA	245
Isl1	f GTTACCAGCCACCTTGGA r GGACTGGCTACCATGCTGTT	181
c-kit	f CCACACCCTGTTCACTCCTT r TTCTGGGAACTCCCATTG	206

Table 3-5 Primer sequence and UPL probe details for qRT-PCR analysis of pericyte and cardiomyocyte associated marker genes in human cells.

Gene	Sequence	UPL Probe
β -actin	CCAACCGCGAGAAGATGA CCAGAGGCGTACAGGGATAG	64
GAPDH	AGCCACATCGCTCAGACAC GCCCAATACGACCAAATCC	60
GATA-4	TTCCAGCAACTCCAGCAA TCGCACTGACTGAGAACGTC	77
Mef2c	TGATCAGCAGGCAAAGATTG GGACACTGGGATGGAGACTG	10
Nkx2.5	AGACAGAGGCGGACAACG CCGCTCCAGCTCATAGACC	69
ACTC1 (α -actinin)	GCTTCCGCTGTCCTGAGA ATGCCAGCAGATTCCATACC	11
Troponin T	AAGAAGGAGGACACCGAGAAG CAGTGCATCGATGTTCTTGC	10
MYH7	ACACCCTGACTAAGGCCAAA TCCAGGGATCCTTCCAGAT	78
CD146	GGGTACCCCATTCCTCAAGT CAGTCTGGGACGACTGAATG	63
NG2	GAGAGGCAGCTGAGATCAGAA TGAGAATACGATGTCTGCAGGT	78
PDGFR- β	CCCTTATCATCCTCATCATGC CCTTCCATCGGATCTCGTAA	29

3.4.8 Immunocytochemistry

Immunocytochemistry of cultured cells was carried out as described in 2.17. Cells were seeded at approximately 40,000 cells per cm² in 20% FBS/DMEM and reached 80% confluence within 24 to 48hr. The same primary antibodies were used as detailed previously for the tissue stains (Table 3-1) with the addition of a rabbit monoclonal PDGFR- β antibody (Abcam ab32570), a rabbit polyclonal GATA-4 antibody (Abcam ab61767) and a mouse monoclonal MEF2c antibody (Abcam ab118406). All primary antibodies were used at 1:100 dilutions in 5% goat serum in PBS and incubated overnight at 4°C.

3.4.9 Adipogenic and osteogenic differentiation

For adipogenic and osteogenic differentiation studies cardiac pericytes and adventitial cells suspended in 20% FBS/DMEM growth medium were seeded at 10,000 cells per cm² in triplicate wells of 6 well plates and allowed to reach 70% confluence over 24 to 48hr. This was considered time point 0 and the cells in one plate were fixed in 4% PFA for 10 min then washed twice with PBS before being stored at 4°C. In the remaining wells growth medium was aspirated and replaced with fresh osteogenic or adipogenic differentiation medium every 72 hours and cells were fixed at 7, 14 and 21 days. A negative control population of cells were included and maintained in 20% FBS/DMEM growth medium throughout. A positive control population of adipose pericytes known to show both adipogenic and osteogenic differentiation potential were also included. Osteogenic differentiation promoting medium was composed of DMEM, 10% foetal bovine serum, 0.1 μ M dexamethasone, 0.3mM L-ascorbic acid & 10mM β -glycerophosphate. Adipogenic differentiation promoting medium was composed of DMEM, 10% foetal bovine serum, 1 μ M dexamethasone, 0.5 μ M isobutylmethylxanthine, 60 μ M indomethacine & 170 μ M insulin. At the end of the experiment stored cells from each time point were stained together to eliminate variability in staining technique. Cells in adipogenic culture were washed with de-ionized water before being incubated in 60% isopropanol (diluted in de-ionized water) for five min at room temperature. The liquid was then aspirated and the cells were allowed to dry before Oil red O solution was added and incubated at room temperature for 10 min. Osteogenic differentiation samples were

washed twice with de-ionized water before incubation for 30 min with Alizarin Red solution. In both cases the stain was removed with 4 washes of de-ionized water and the wells imaged on a trans-illuminator. Oil Red O stock solution was made by dissolving 0.7g of Oil Red O dye (Sigma Aldrich) in 200ml of isopropanol (VWR). This solution was stirred on a magnetic stirrer overnight before being filtered using a 0.2µm syringe filter. Working solution was made by diluting 6 parts Oil Red O stock in 4 parts de-ionized water. Alizarin Red stain solution was made by dissolving 2g of Alizarin Red S (Sigma Aldrich) in 100ml of de-ionized water then adjusting the pH to between 4.1 and 4.3 with 10% ammonium hydroxide.

3.4.10 Neonatal rat cardiomyocyte co-culture

3.4.10.1 Neonatal rat cardiomyocyte (NRCM) isolation

Cardiomyocytes were isolated from the hearts of three day old rat pups using enzymatic digestion. Initial digestion techniques used serial digestion in a pancreatin (Sigma Aldrich, 0.8mg/ml) and collagenase II (Worthington, 80 units/ml) enzyme solution followed by Percoll (Sigma Aldrich) gradient separation. This technique was found to give highly variable and often disappointingly low yields of viable cardiomyocytes with excess contamination with fibroblasts.

To improve yields a commercial neonatal rat cardiomyocyte isolation system (Worthington Biomedical Corporation) was used and digestion and isolation carried out according to the detail protocol supplied. Briefly neonatal rat pups of 3 days of age were anaesthetised by inhalation of isoflurane (Merial) and then decapitated with scissors (schedule 1 euthanasia). The thorax was wiped with 100% ethanol, opened and the beating heart excised and washed in sterile calcium and magnesium-free Hank's Balanced Salt Solution before being minced with a sterile razor blade and incubated overnight at 4°C in a trypsin solution (concentration not specified). The following morning the trypsin activity was quenched by adding a trypsin inhibitor whilst warming to 37°C in a waterbath. At this point purified collagenase (type and concentration not specified) was added and the solution continuously agitated for 45 minutes. Following enzyme incubation the sample was triturated by repeated passage through a 10ml serological pipette (Corning). The solution was allowed to settle and the supernatant was removed and passed through a 70µm cell strainer into a separate

tube. Leibovitz L-15 medium was added to the tissue residue and the titration step repeated before again filtering the supernatant. Filtered cells were left for a further 20 min to allow complete digestion of collagen before being sedimented at 50 x g for 5 min. The supernatant was removed and the pellet re-suspended in cardiomyocyte induction media (DMEM/M199 (4:1), 10% horse serum, 5% foetal bovine serum and 1% penicillin streptomycin) before cell numbers and viability were estimated using a haemocytometer. Cells were then seeded onto 0.2% gelatin coated T25 flasks at a density of 50,000 cells per cm². After 24hr the supernatant was aspirated removing dead or dying cells and the adherent cells were incubated with induction medium containing 3µg/ml of Mitomycin C (Sigma Aldrich) for 2 hr. This medium was then removed and the cells washed with sterile PBS twice and replaced with induction media for a further 24 hr before being used in experiments. This technique yielded much higher numbers of viable cells and contaminating fibroblast numbers were significantly reduced.

3.4.10.2 Neonatal rat cardiomyocyte co-culture

Mitomycin C treated rat cardiomyocytes were seeded in triplicate in the wells of six well plates containing sterile 10mm coverslips and T25 flasks at a density of 24,000 cells per cm² and allowed to settle for 24 hr. Pericytes and adventitial cells were labelled with CM-Dil or eGFP as described in 2.18 and then seeded at a density of 6,000 cells per cm² giving a ratio of 4:1 and a total density of 30,000 cells cm². Plates and flasks were coated with 0.1% gelatin prior to seeding. Cells were initially seeded in cardiomyocyte induction medium and this was exchanged after 48 hr for maintenance medium (DMEM/M199 (Life Technologies) 4:1, 10% foetal bovine serum, 1% penicillin streptomycin) and then at 72 hourly intervals. At 10 and 21 days time-lapse images of live cell co-cultures in flasks were recorded using an inverted fluorescence microscope (Zeiss Axio Observer) and AxioVision LE software (Zeiss). Images were captured both under phase contrast and fluorescent light and the movement of filters between these settings resulted in a slight delay (milliseconds) in the capture of the subsequent image. As a result cell contraction in phase contrast and the movement of dye granules/fluorescent cytoplasm appeared slightly asynchronous. Following time lapse imaging coverslips were removed,

placed in the wells of a 24 well plate and fixed with 4% paraformaldehyde (Sigma Aldrich) for 10 min. Following fixation coverslips were washed twice with PBS before being stored at 4°C for later staining. Cells from wells not containing coverslips were washed with PBS twice, trypsinised, washed again in PBS and placed in TRIzol (Life Technologies) before being frozen at -80°C for later RNA extraction. Staining of cells on coverslips was carried out as described in 2.17 using an anti- α -actinin (sarcomeric) antibody (monoclonal raised in mouse, Sigma Aldrich A7811) at 1:800 dilution and an anti-cardiac Troponin-T antibody (monoclonal raised in mouse, Abcam Ab8295) at 1:200 dilution with an overnight incubation. Secondary antibodies of Alexa Fluor goat anti-mouse 555 and 488 (Life Technologies) were applied at a concentration of 1:600.

3.4.10.3 5-Aza-2'-deoxycytidine treatment and neonatal rat cardiomyocyte co-culture

Mitomycin C treated neonatal rat cardiomyocytes were seeded as described in 3.4.10.2. Wells were left empty or contained 18mm or 10mm round sterile glass coverslips and were coated with 0.2% gelatin before seeding. Cardiomyocytes were allowed to settle for 24 hr then de-methylated pericytes or adventitial cells were added at 6000 cells cm². Demethylation was achieved by treating cells grown in 20% FBS DMEM 1% P/S with 5 μ M of 5-aza-2'-deoxycytidine (5-azacytidine) (Sigma Aldrich) for three consecutive days. Co-culture studies lasted 14 days in total. The first 24 hours of culture was carried out in cardiomyocyte induction medium with the remainder of the period in maintenance medium (as described in 3.4.10.2). Medium was exchanged every 72 hours. At the end of the experiment the 10mm coverslips were removed and fixed in 4% PFA before being stained for sarcomeric α -actinin and troponin-T as described in 3.4.10.2. Two wells without coverslips were washed with PBS, the cells trypsinised and pelleted before being re-suspended in TRIzol and frozen at -80°C for later RNA extraction. The cells in the remaining culture wells were loaded with the acetoxymethyl ester form of fluo-4 (Life Technologies, 5 μ mol/L) in order to monitor changes in cytoplasmic free calcium (Ca). The cells were incubated in fluo-4 for 20 min at room temperature (23 °C) then a modified tyrode solution (Diaz et al., 2002) was added to excess, to stop the loading process and the cells were then let to de-esterify in an incubator at 37°C for 30 min. Wells

were then examined using a fluorescence microscope (Zeiss Axio Observer) and images captured using AxioVision LE software.

3.5 Results

3.5.1 Foetal cardiac perivascular cell populations express pericyte and adventitial cell markers

Both autolysis and freeze associated artefact were commonly encountered on screening with H&E staining (Figure 3-1). Changing from a protocol of freezing with dry ice to liquid nitrogen reduced the incidence of the latter.

Anti-CD31 (PECAM-1) labelling of endothelial cells revealed the ventricular myocardium to be highly vascularised with large numbers of capillaries and fewer arterioles and small to medium size arteries (Figure 3-2). Anti-CD146 labelling labelled large numbers of perivascular cells closely associated with capillaries and small arteries. These cells wrap around endothelial cells and in some instances there was apparent overlap in expression of CD31 and CD146 suggesting that CD146 also labelled a subset of the endothelial cell population (Figure 3-3).

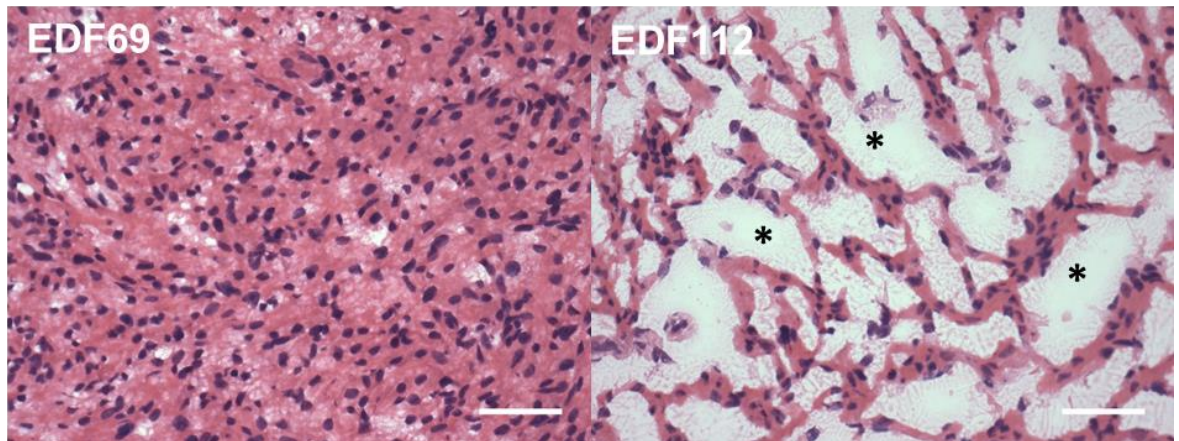


Figure 3-1 Representative brightfield images of H&E stained frozen foetal human myocardium. EDF69 illustrates the compact, highly cellular myocardium of well frozen tissue whilst in EDF112 the poorly frozen tissue contains multiple clefts and fissures (*) as a result of ice crystal formation (n=10). Scale bars = 50µm.

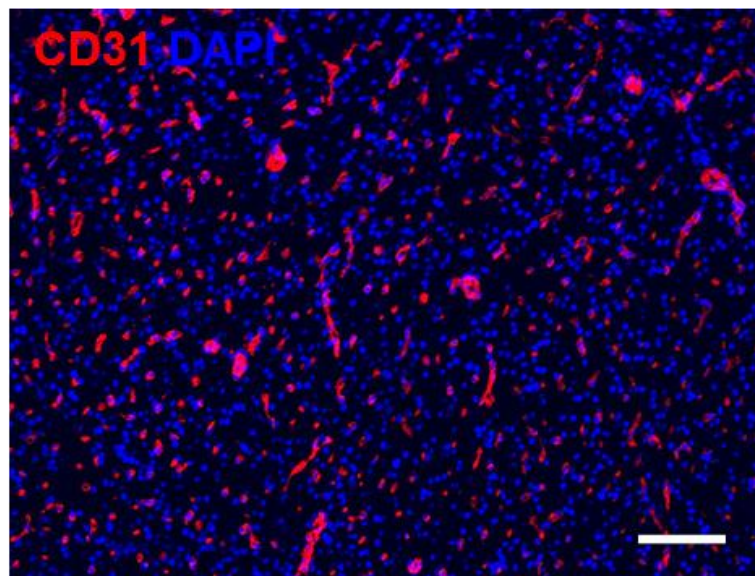


Figure 3-2 Representative immunofluorescence image of foetal human myocardium stained with anti-CD31 (red) to label endothelial cells. A high density of capillaries is evident (n=4). DAPI nuclear staining in blue. Scale bar = 100µm.

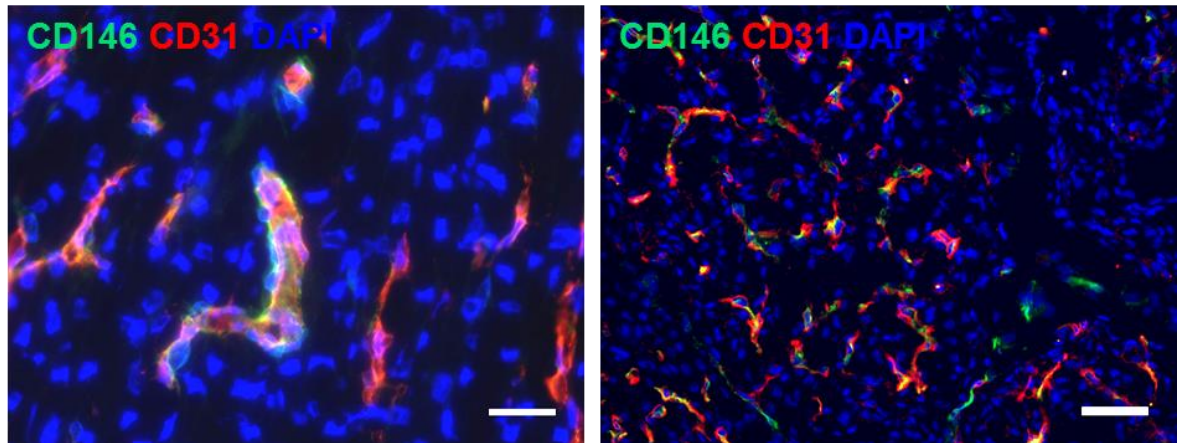


Figure 3-3 Representative immunofluorescence image of foetal human myocardium stained with endothelial cell marker CD31 and perivascular cell marker CD146. CD146⁺ perivascular cells are abundant and closely associated with the endothelium of capillaries (n=4). DAPI nuclear staining in blue. Scale bars = 25µm & 50µm.

Anti- α -SMA and anti-CD31 co-staining revealed α -SMA⁺ smooth muscle cells associated with small and large arteries but not microvessels. Co-staining with anti-CD146 and anti- α -SMA showed CD146⁺/ α -SMA⁺ smooth muscle cells present in the arterial wall but CD146⁺/ α -SMA⁻ cells associated with capillaries (Figure 3-4).

Anti-NG2 staining frequently overlapped with anti-CD146 labelling particularly in cells within the walls of arterioles and small arteries. CD146⁺/NG2⁻ cells and lesser numbers of CD146⁻/NG2⁺ cells however were also present. The majority of anti-CD34 staining cells were CD146⁻ with expression overlapping on only a minor subset of the CD34⁺ population (Figure 3-5). Chromagen staining with anti-CD34 antibody confirmed CD34 expression in the endothelium of vessels of all sizes but also expression in large numbers of spindle shaped cells within the adventitia of muscular arteries (Figure 3-6). No CD34 expression was seen in the smooth muscle cells of the media.

Staining for c-kit demonstrated low numbers of scattered positive cells throughout the myocardium in relatively close proximity to CD146⁺ cells. A marker to identify mast cells, such as CD25, was not used so it is possible some of these c-kit⁺ cells were mast cells rather than cardiomyocyte precursors. Staining of the MSC associated marker CD105 was noted in approximately 50% of CD146⁺ cells (Figure 3-7).

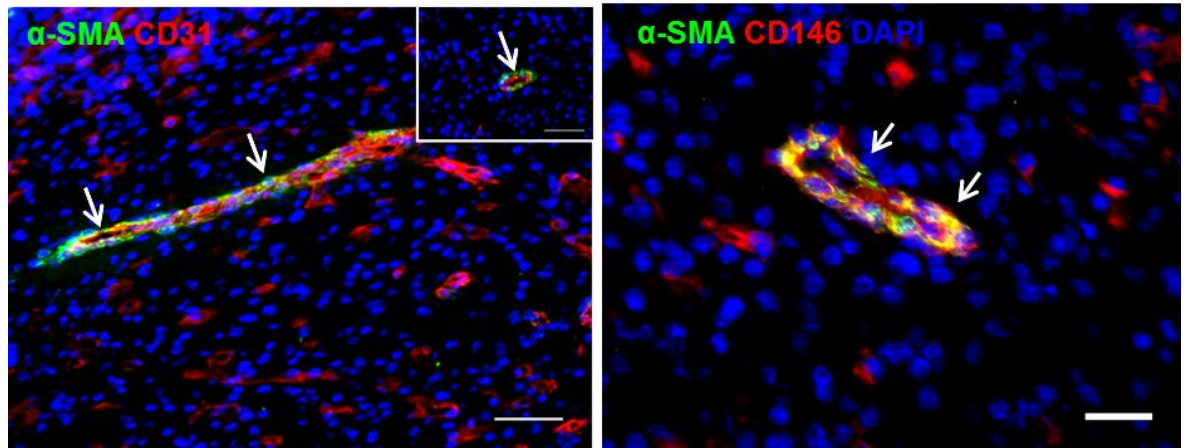


Figure 3-4 Representative immunofluorescence image of anti-CD31, anti- α -SMA and anti-CD146 stained human foetal myocardium. α -SMA expression is restricted to smooth muscle cells in the wall of muscular arteries where there is co-expression (white arrows) of the pericyte marker CD146 (n=4). DAPI nuclear staining in blue. Scale bars = 50 μ m & 25 μ m.

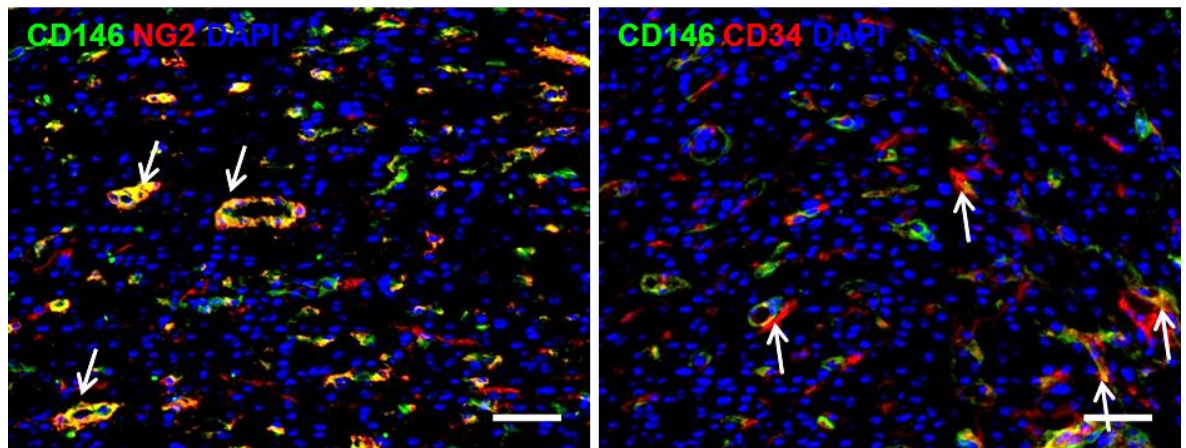


Figure 3-5 Representative immunofluorescence image of anti-CD146, anti-NG2 and anti-CD34 stained human foetal myocardium. Broad overlap in expression of CD146 and NG2 is present in perivascular cells (white arrows). CD34 and CD146 co-express in a minor subpopulation of cells (white arrows) (n=4). DAPI nuclear staining in blue. Scale bars = 50 μ m.

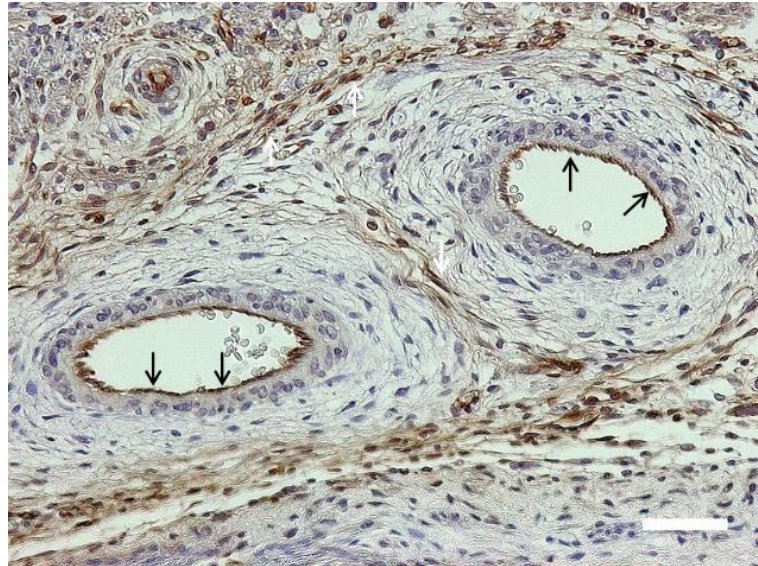


Figure 3-6 Representative brightfield image of anti-CD34 chromagen stained human foetal myocardium. CD34 is widely expressed by spindle shaped cells in the adventitia of the coronary artery (white arrows) and also by endothelial cells (black arrows) (n=3). Scale bar = 100µm.

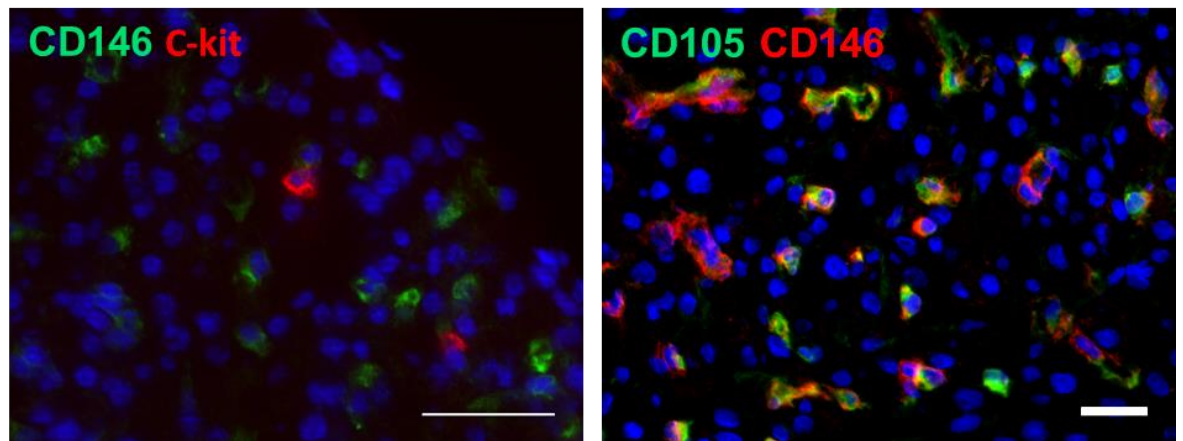


Figure 3-7 Representative immunofluorescence image of anti-CD146, anti-C-kit and anti-CD105 stained human foetal myocardium. C-kit expression (red) does not overlap with CD146 (green). CD105 expression (green) overlaps in a subset of CD146 (red) positive cells (n=4). DAPI nuclear staining in blue. Scale bars = 25µm.

3.5.2 Cardiac perivascular cells can be isolated from collagenase digested foetal cardiac tissue using FACS

Forward scatter versus side scatter area gating was used to distinguish cells from the abundant cellular debris in enzymatically digested preparations of myocardium. From this singlets were identified on forward scatter height and area. Next live, DAPI negative, CD45 negative cells were selected and the CD56 negative subpopulation of these was identified. CD144 positive mature endothelial cells were excluded and the final live, CD54⁻, CD56⁻, CD144⁻ subpopulation was examined for CD34 and CD146 expression. Expression of Pericytes were identified as CD34⁻ CD146⁺ and represented 2.0% (+/- 1.8) of the live cell population whilst adventitial cells were CD34⁺ CD146⁻ and represented 3.7% (+/- 2.7) (Figure 3-8). Post-sort purity checks revealed consistent continued expression or absence of CD146 and CD34 confirming reliable and accurate isolation of the starting populations. The numbers of total viable cells and cells collected per sort was highly sample dependent and varied between 10,000 and 417,000 pericytes and 15,000 and 683,000 adventitial cells. This variability was considered to be a result of both differences in sample size and the autolytic state of the tissue. In all, seven separate paired and viable populations of pericytes and adventitial cells were obtained for expansion in culture.

Following seeding in EGM2 medium adherence of cells frequently took 4 to 7 days and subsequent growth was slow with visible expansion of the population only detectable after 10 to 14 days. In low density culture conditions both cell populations adopted a triangular morphology becoming progressively more fusiform as cell numbers increased (Figure 3-9). Pericytes were found to be slower growing and more sensitive to poor culture technique such as over-trypsinisation than adventitial cells.

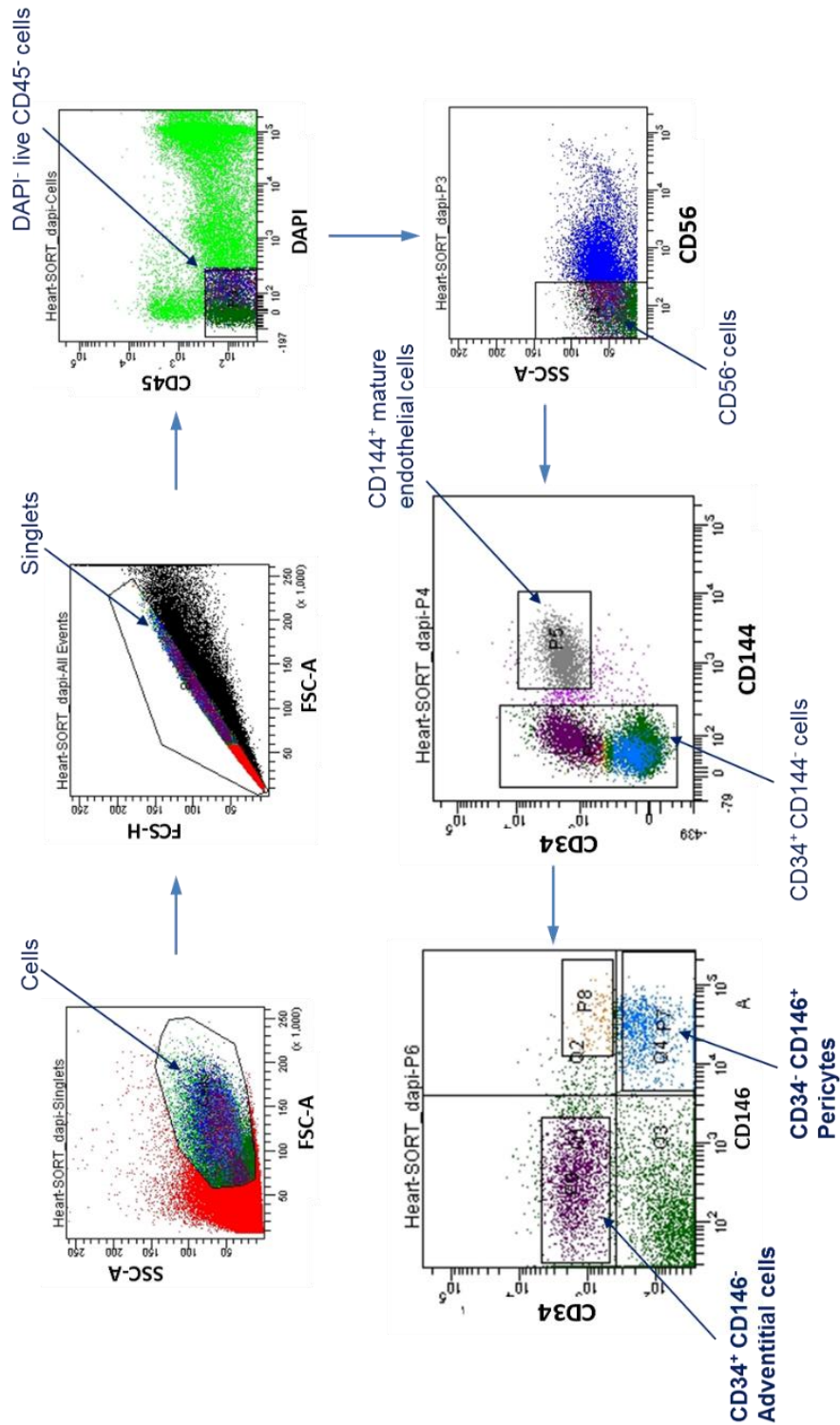


Figure 3-8 Representative fluorescence activated cell sorting gating strategy. Live DAPI⁻, CD45⁻, CD56⁻, CD144⁻, singlets were identified. From this population CD34⁻ CD146⁺ pericytes and CD34⁺ CD146⁻ adventitial cells were selected (n=7). SSC-A = side scatter area. SSC-H = side scatter height. FSC-A = forward scatter area. FSC-H = forward scatter height.

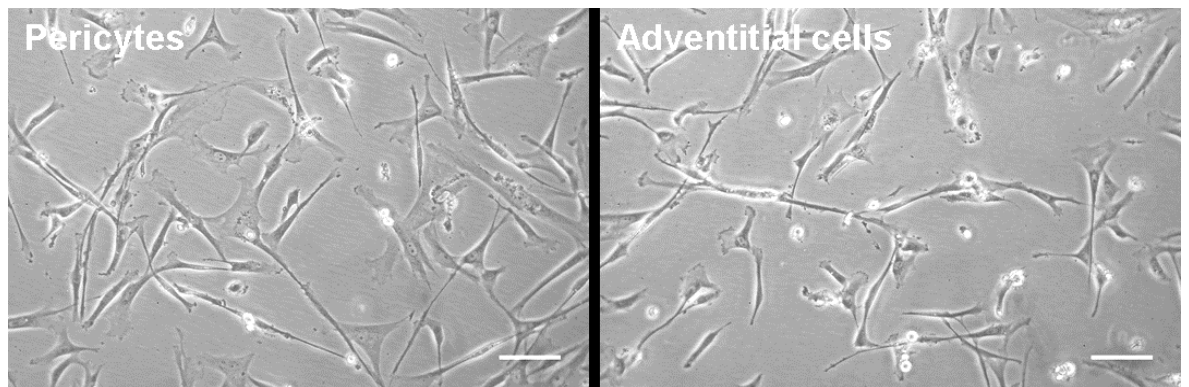


Figure 3-9 Representative brightfield images of pericytes and adventitial cells in culture. Cells of both populations adopt a similar morphology in 2D culture (n=7). Scale bars = 50μm.

3.5.3 Isolated cardiac perivascular cells maintain pericyte marker expression in culture

3.5.3.1 Gene expression – conventional RT-PCR

Three paired populations (minimum of 2 passages in culture) of pericytes and adventitial cells were examined for mRNA expression. Homology of pericyte marker mRNA expression profiles was present in both pericytes and adventitial cells with consistent expression of CD146 and NG2. PDGFR- β and CD34 expression were not detected (Figure 3-10).

3.5.3.2 mRNA expression analysis by qRT-PCR

Expression levels of the pericyte markers CD146, NG2 and PDGFR- β mRNA were compared for 5 populations of pericytes and 6 populations of adventitial cells. Several fold greater statistically significant expression of CD146 ($p < 0.05$) and NG2 ($p < 0.05$) was seen in cultured pericytes compared to adventitial cells. No statistically significant ($p > 0.05$) difference in expression was seen in PDGFR- β between groups (Figure 3-11).

3.5.3.3 Surface protein analysis by flow cytometry

Flow cytometry analysis of cultured pericytes and adventitial cells between passages 3 and 5 showed high expression levels of pericyte markers CD146 and NG2 in both populations (Figure 3-12).

3.5.3.4 Surface protein analysis by immunostaining

Immunostaining of four populations of cultured pericytes and adventitial cells between passages 3 and 5 revealed uniform expression of the pericyte markers CD146, NG2, PDGFR- β and α -SMA in both populations (Figure 3-13).

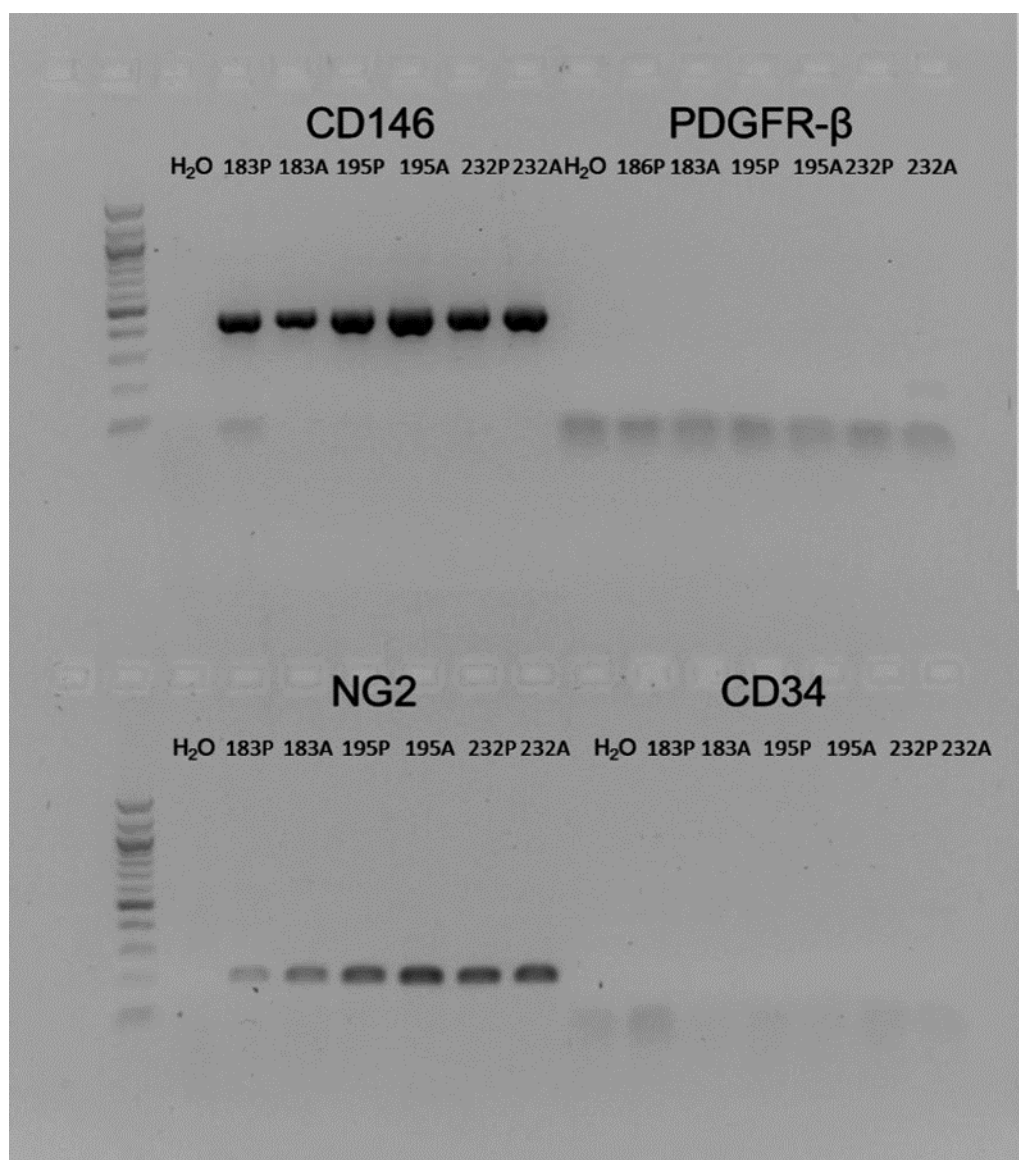


Figure 3-10 Representative conventional RT-PCR electrophoresis gel for pericyte marker genes. Both pericytes (P) and adventitial cells (A) populations from donors EDF183, 195 and 232 expressed pericyte markers CD146 and NG2 but not PDGFR- β or CD34 in culture (n=3). DNA ladder bands represent 100 base pairs. H₂O represents negative control.

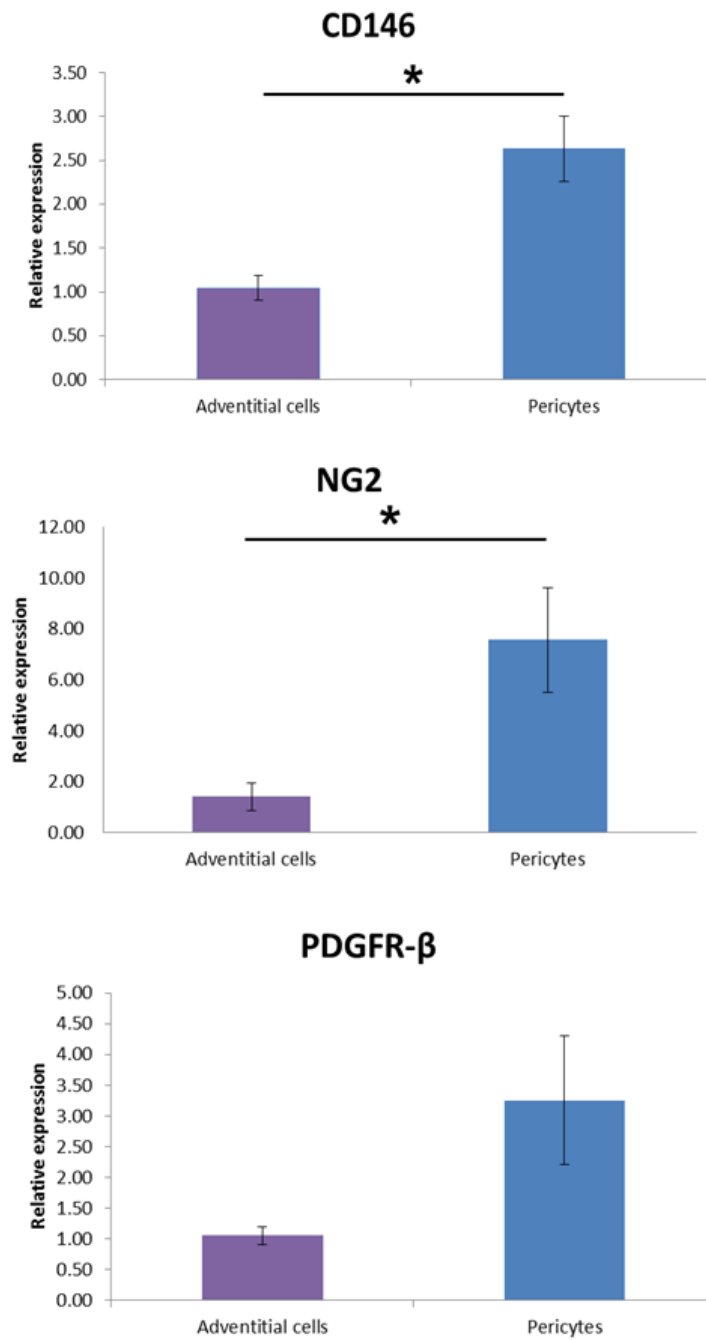


Figure 3-11 Relative mRNA expression of pericyte markers CD146 and NG2 is significantly increased in pericytes compared to adventitial cells. No difference in PDGFR- β expression was noted (n= 5 to 6). Columns represent mean $\pm$ SEM. * $p < 0.05$, independent t-test.

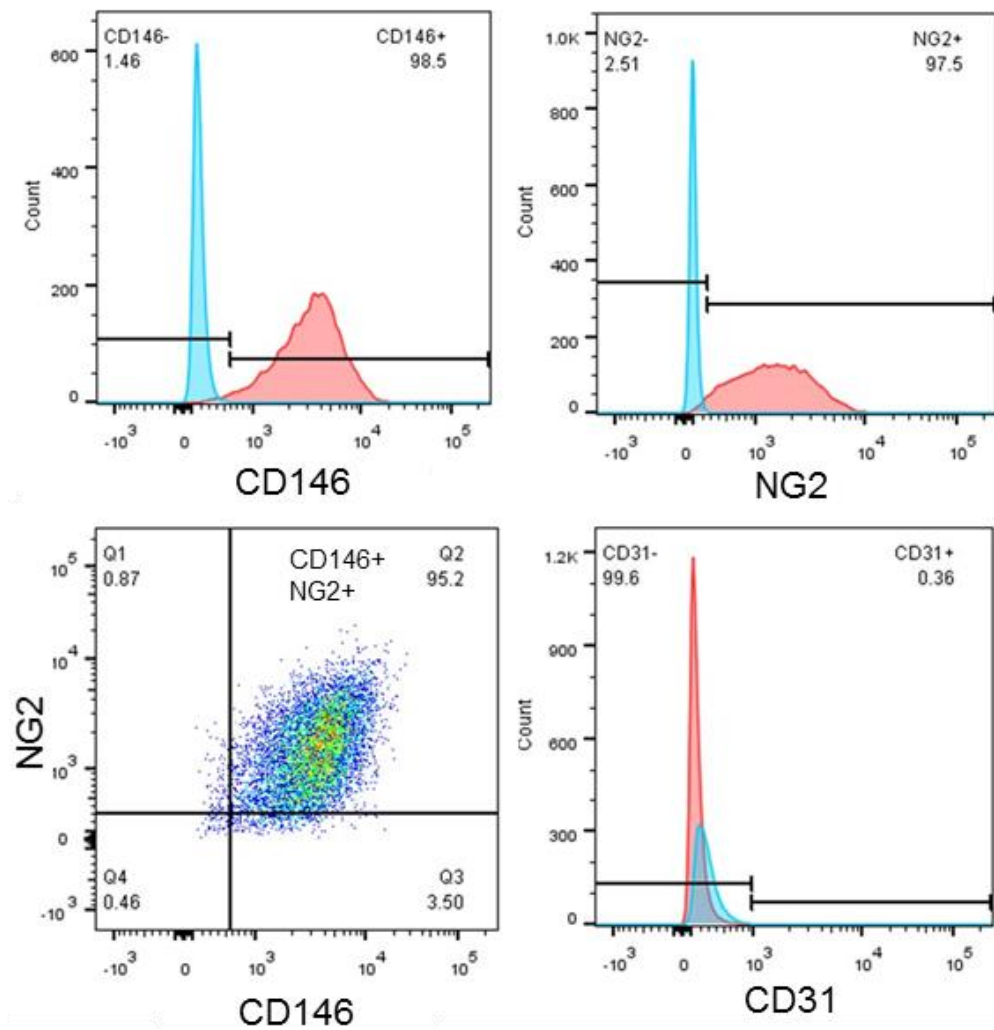
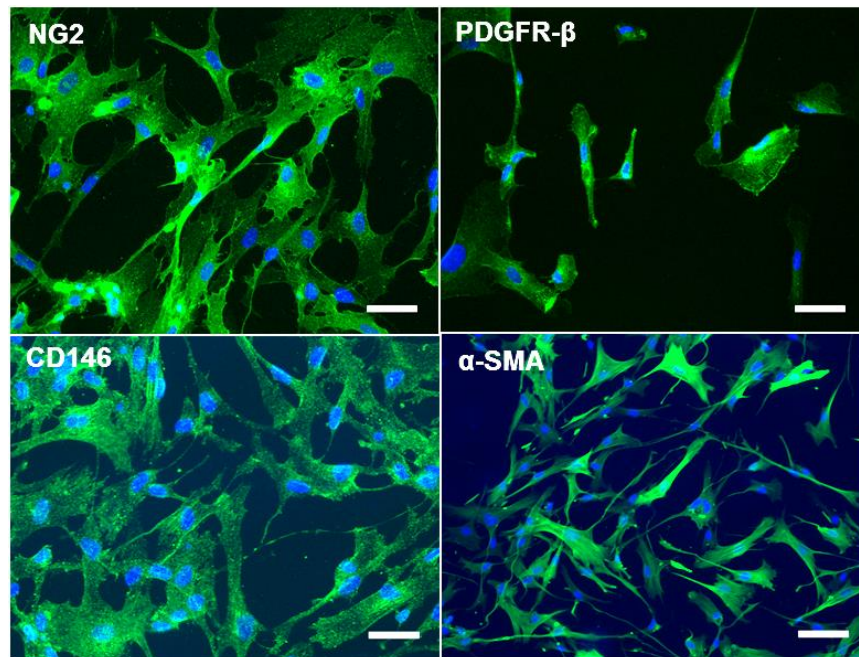


Figure 3-12 Representative flow cytometry histograms and dot plot of pericyte and endothelial cells marker expression in cultured pericytes. High levels of CD146 and NG2 expression are maintained whilst CD31 expression is negligible (n=7).

Pericytes



Adventitial Cells

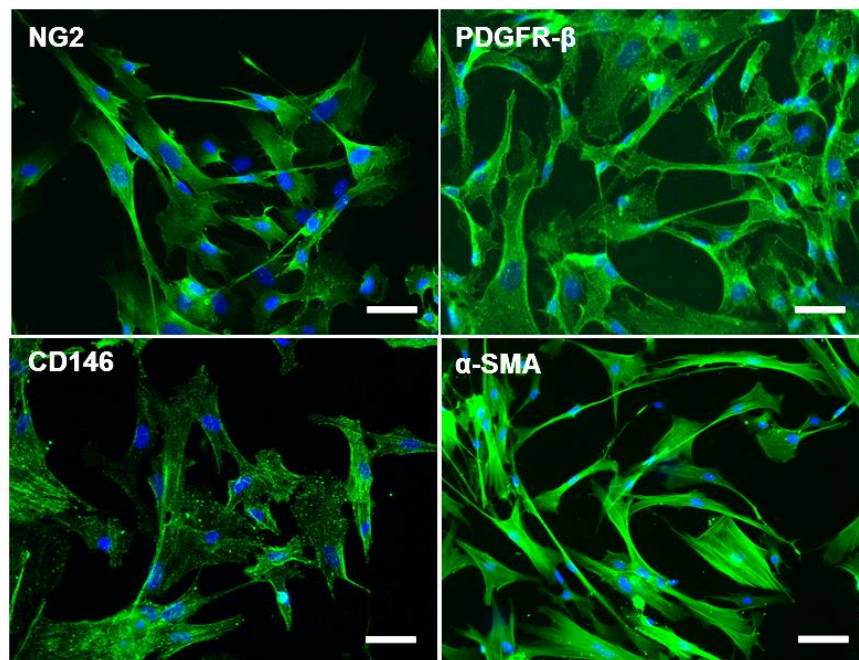


Figure 3-13 Representative immunofluorescence images of cultured pericytes and adventitial cells stained for pericyte markers. Both cultured pericytes and adventitial cells uniformly express NG2, PDGFR-β, CD146 and α-SMA (n=4). Specific pericyte markers in green. DAPI nuclear staining in blue. Scale bars = 50μm.

3.5.1 Isolated cardiac perivascular cells express mesenchymal stem cell markers in culture

3.5.1.1 Gene expression - conventional RT-PCR

Three paired populations of pericytes and adventitial cells were examined for mRNA expression. MSC associated markers CD90, CD44, CD105 and CD73 were consistently expressed in both pericyte and adventitial cell populations (Figure 3-14).

3.5.1.2 Surface protein analysis by flow cytometry

Three paired populations of pericytes and adventitial cells were examined by flow cytometry for expression of MSC surface markers. Very high percentages of cells expressed CD44, CD90 and CD105 in both pericyte and adventitial cell groups. CD73 expression was less common and much more variable between donors. No significant differences ($p>0.05$) were evident in gene expression between pericyte and adventitial cell groups however, in general, more variability in marker expression levels was seen in the pericyte group compared with the adventitial cell group (Figure 3-15).

3.5.1.3 Surface protein analysis by immunostaining

Immunostaining of four populations of cultured pericytes and adventitial cells revealed expression of the MSC marker CD90 in both populations. Subjectively a greater proportion of adventitial cells expressed CD90 than pericytes supporting the findings of the flow cytometry analysis (Figure 3-16).

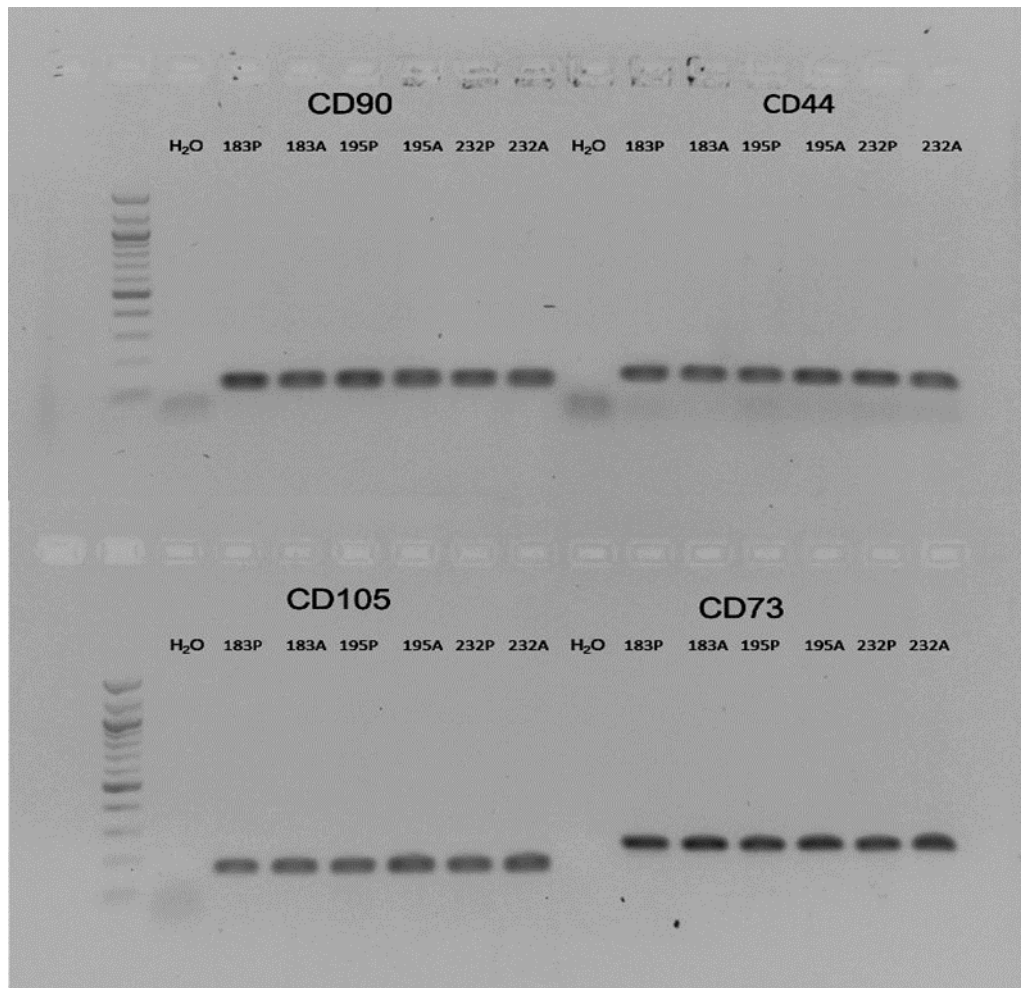


Figure 3-14 Representative conventional RT-PCR electrophoresis gel for MSC marker genes. Both pericytes (P) and adventitial cells (A) populations from three donors EDF183, 195 and 232 express MSC markers CD90, CD44, CD105 and CD73 in culture (n=3). DNA ladder - bands represent 100 base pairs. H₂O represents negative control.

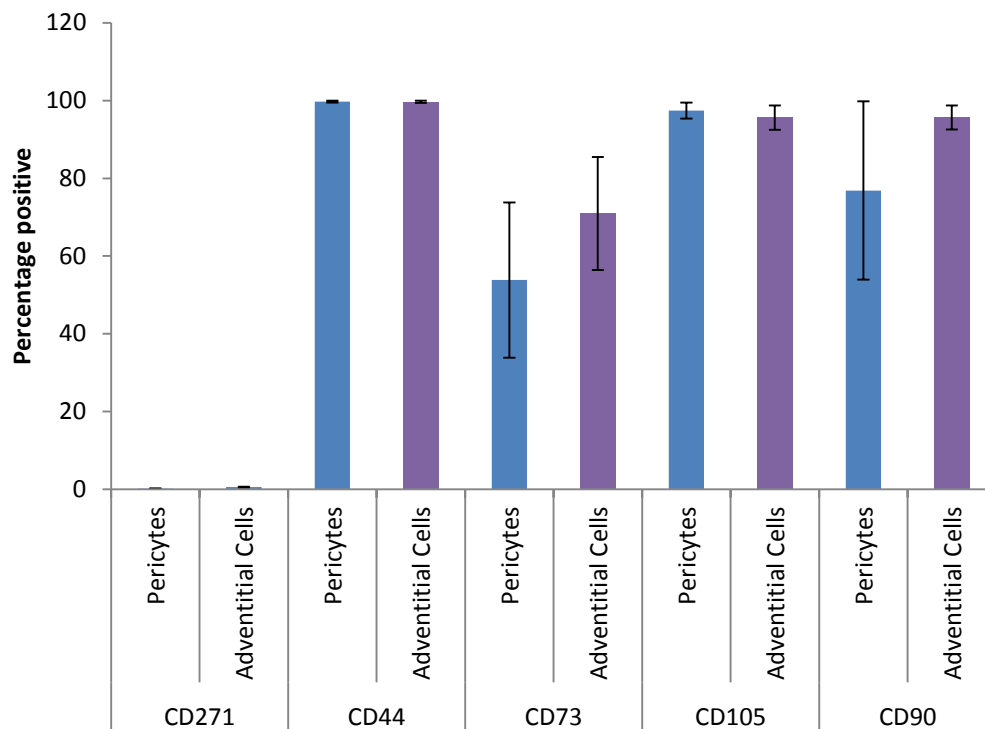


Figure 3-15 Flow cytometric analysis of MSC marker expression in cultured pericytes and adventitial cells. High levels of expression of CD44, CD105 and CD90 were seen in both groups. Moderate levels of CD73 were expressed by both groups and CD271 was not expressed by either (n=3). Columns represent mean $\pm$ SEM. No statistical significance detected, independent t-test.

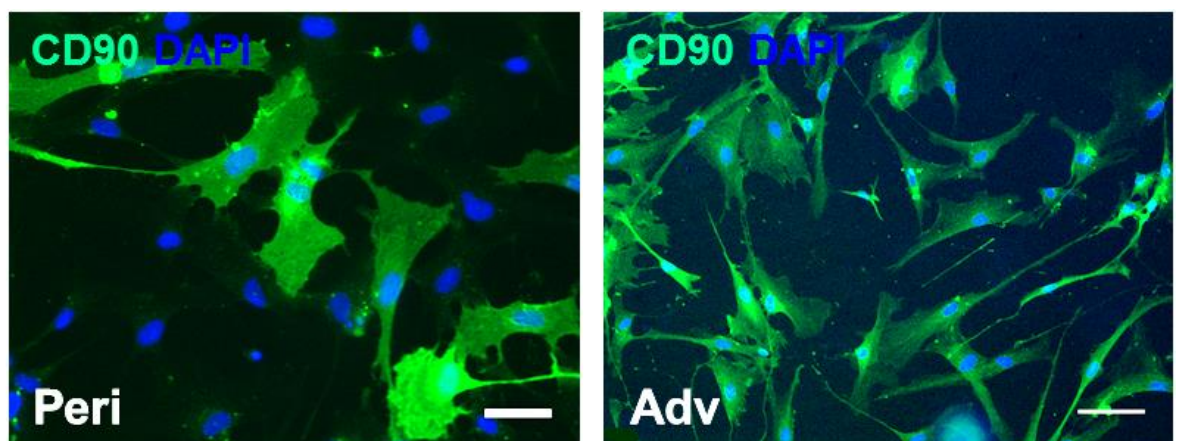


Figure 3-16 Representative immunofluorescence image of cultured pericytes and adventitial cells stained for MSC associated marker CD90. Both pericytes (Peri) and adventitial cells (Adv) label for CD90 however expression is greater in the latter (n=4). DAPI nuclear staining in blue. Scale bar = 50 μ m.

3.5.2 Isolated cardiac perivascular cells express early cardiomyocyte markers in culture

3.5.2.1 Cardiomyocyte marker gene expression analysis by conventional RT-PCR

Three paired populations of pericytes and adventitial cells were examined for cardiomyocyte marker mRNA expression. Three early cardiomyocyte transcription factors were investigated: Mef2c; Nkx2.5; and GATA4. The majority of the pericyte and adventitial cell populations demonstrated weak expression of Mef2c mRNA. GATA4 expression was seen in both cell types but was more common and more strongly expressed in adventitial cells. Nkx2.5 was not expressed by either cell type (Figure 3-17).

Late sarcomeric protein expression was more limited with weak expression of α -actinin seen in the three adventitial cell populations and only faint bands present in two of the pericyte populations. Myosin heavy chain II (MYH2) was weakly expressed in two of the three adventitial populations but not in pericytes. No atrial natriuretic peptide (ANP) or troponin T was expressed in either population (Figure 3-18). Weak expression of c-kit (CD117) was detected in all of the pericyte and adventitial cell populations whilst Isl-1 was not detected in any of the samples. Wilm's tumour 1 (WT1) expression was weakly present in all three adventitial cell populations and one of the pericyte populations (Figure 3-19).

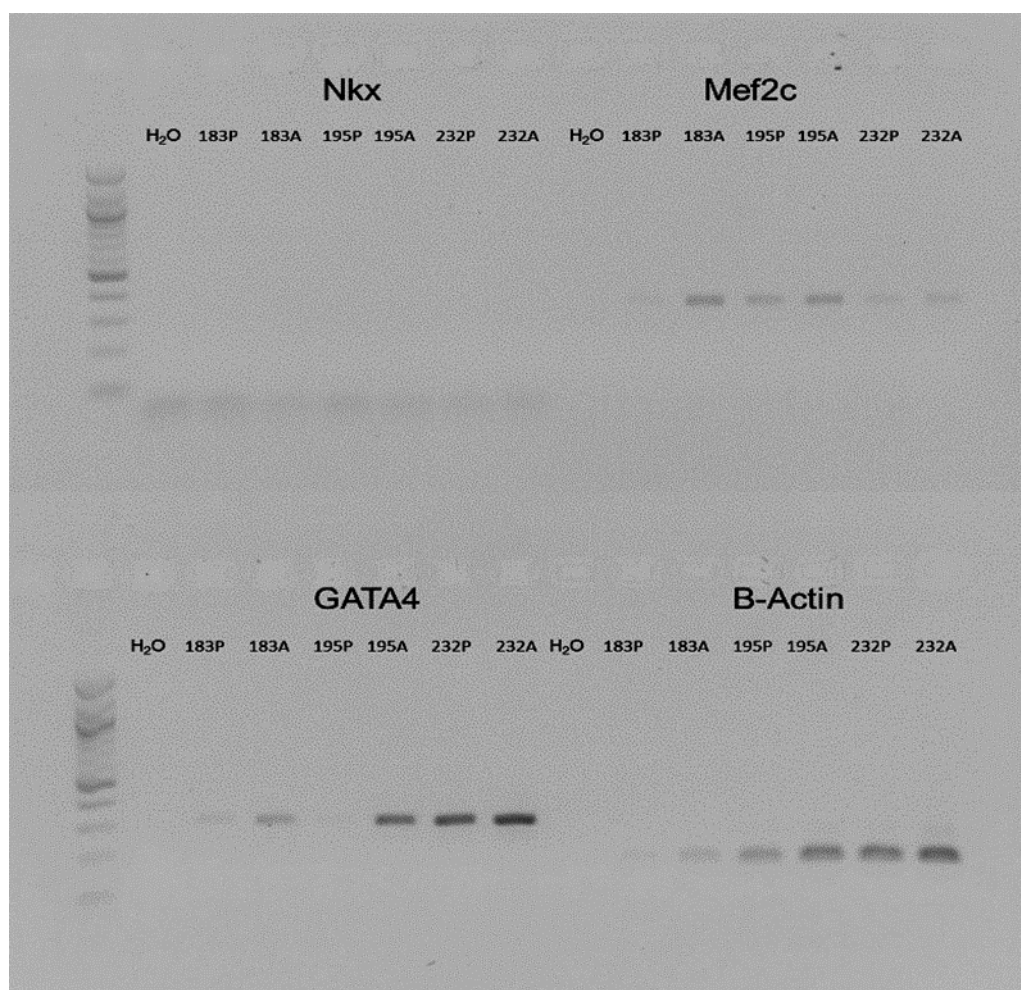


Figure 3-17 Representative conventional RT-PCR electrophoresis gel for early cardiomyocyte marker genes. The majority of both pericytes (P) and adventitial cells (A) populations from donors EDF183, 195 and 232 express GATA4 and Mef2c. Nkx2.5 is not expressed by either population. House keeper gene β -actin is expressed by all populations (n=3). DNA ladder bands represent 100 base pairs. H₂O represents negative control.

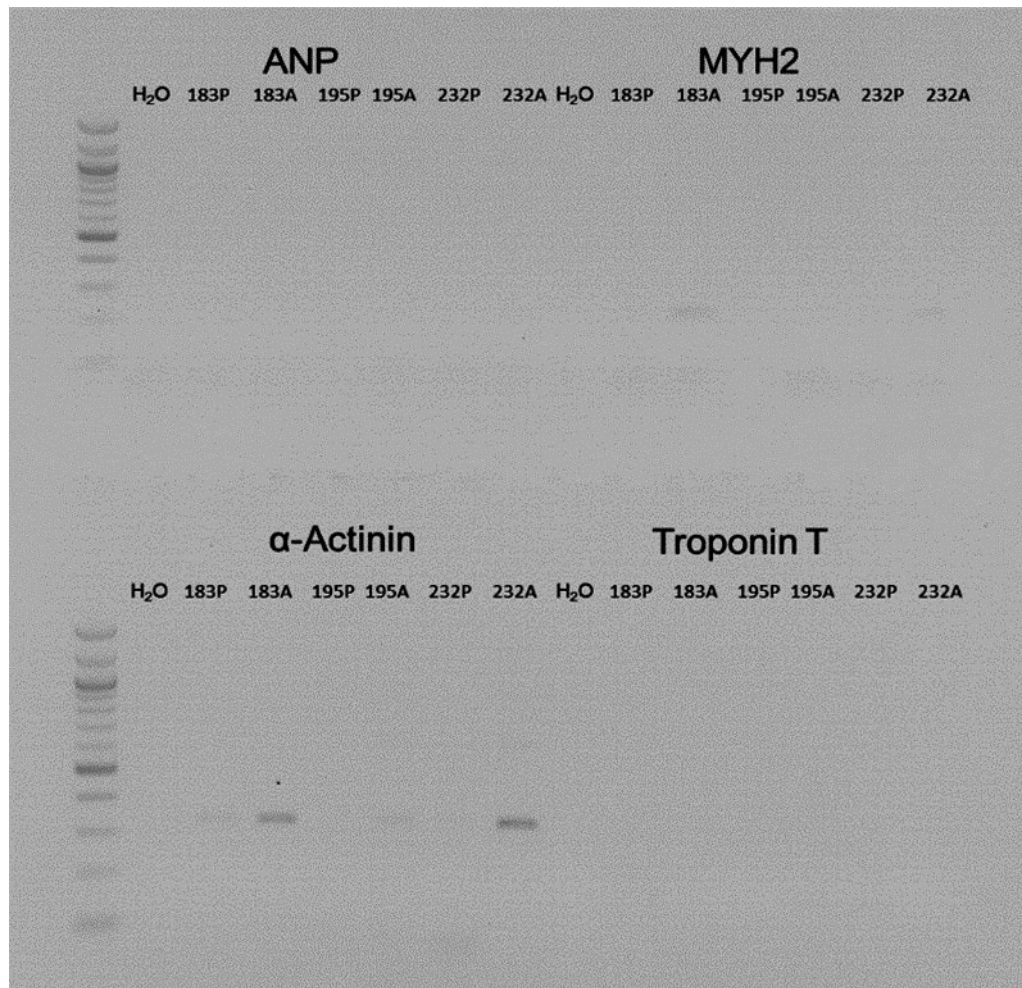


Figure 3-18 Representative conventional RT-PCR electrophoresis gel for late cardiomyocyte marker genes. Pericytes (P) and adventitial cells (A) populations from donors EDF183, 195 and 232 show limited expression of late cardiomyocyte markers in culture. MYH2 was weakly expressed by two of the adventitial populations. α-actinin was expressed by the adventitial populations and weakly by one pericyte population. ANP and troponin T were not expressed by either population. DNA ladder bands represent 100 base pairs (n=3). H₂O represents negative control.

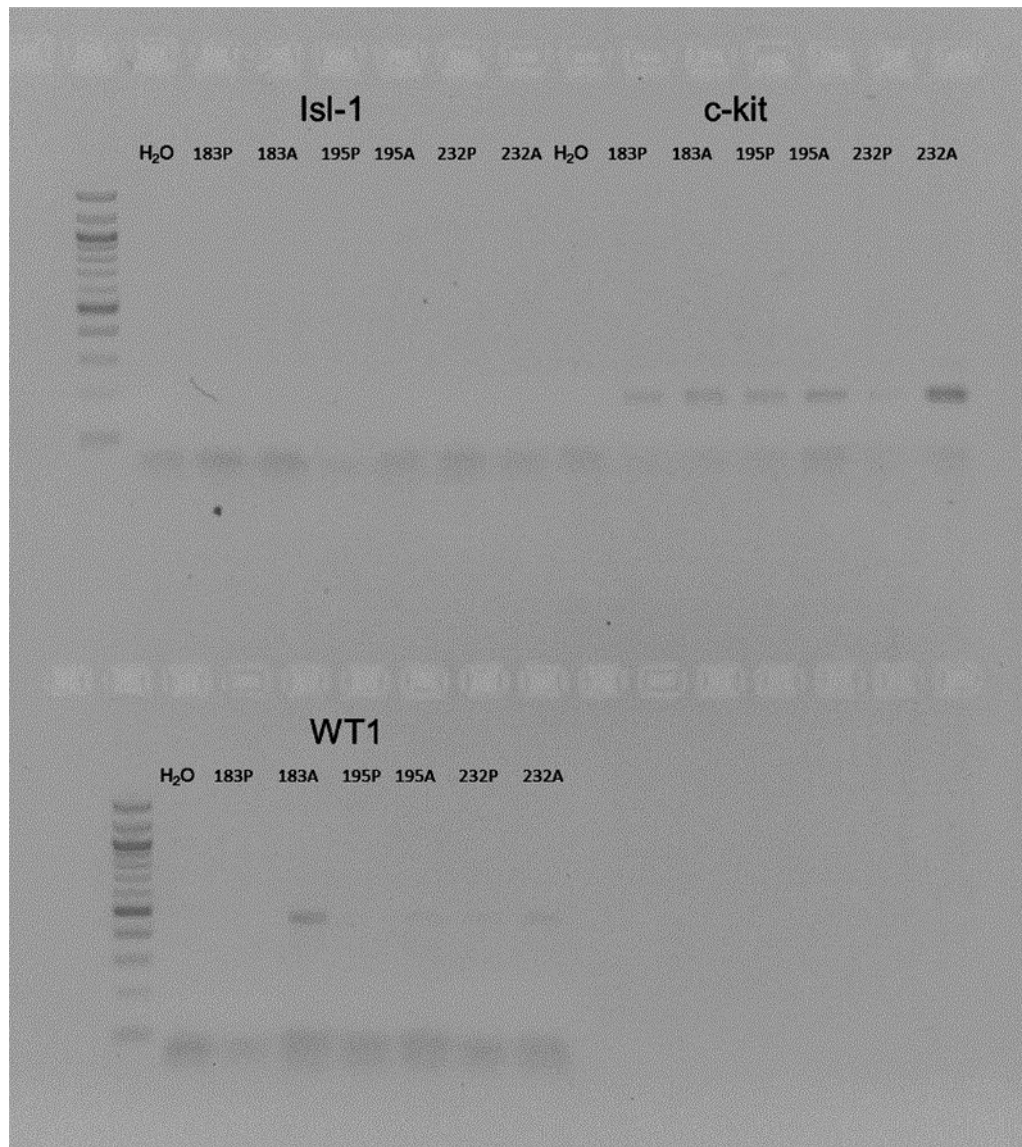


Figure 3-19 Representative conventional RT-PCR electrophoresis gel for cardiac progenitor associated genes. Weak expression of c-kit is present in all pericyte (P) and adventitial cell (A) populations from donors EDF183, 195 and 232. Weak WT1 expression is seen in all adventitial populations and one pericyte population (n=3). DNA ladder bands represent 100 base pairs. H₂O represents negative control.

3.5.2.2 Cardiomyocyte marker protein analysis by immunostaining

Immunostaining of four populations of cultured cells revealed wide spread expression of the early cardiomyocyte associated transcription factors GATA4 and Mef2c and limited expression of cardiac progenitor cell marker c-kit (CD117) in both pericytes and adventitial cells. Cardiac transcription factor Nkx2.5 and mature cardiomyocyte marker sarcomeric specific α -actinin were absent in both cell types (Figure 3-20, Figure 3-21).

Wilm's tumour 1 (WT1) expression was detected in a subpopulation of both pericytes and adventitial cells (Figure 3-22).

Pericytes

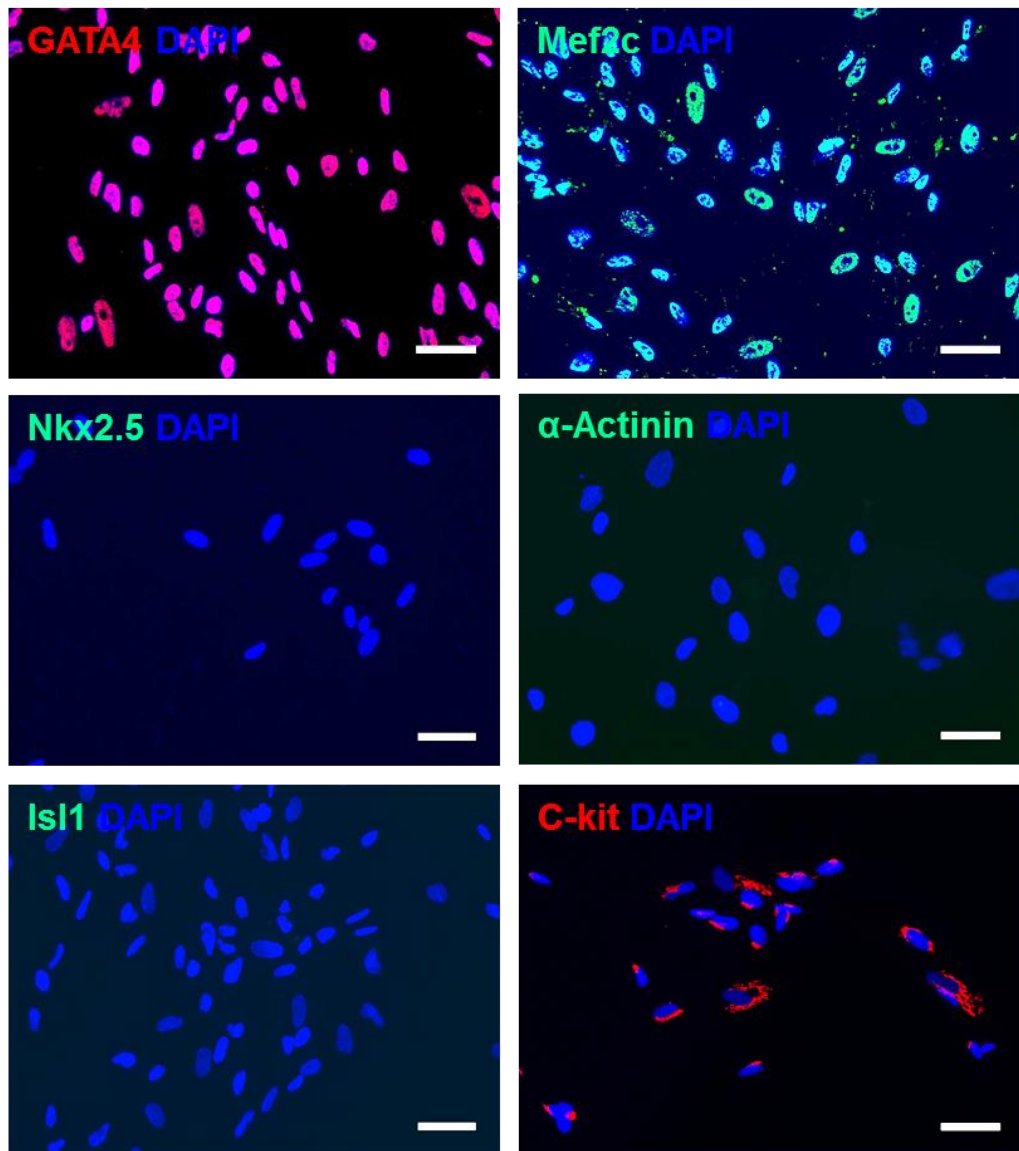


Figure 3-20 Representative immunofluorescence images of cultured pericytes stained for early and late cardiomyocyte markers and cardiac progenitor associated markers. Cultured pericytes label for GATA4, Mef2c and c-kit but not Nkx2.5, Isl-1 or α -actinin (n=4). DAPI nuclear staining in blue. Scale bars = 50μm.

Adventitial Cells

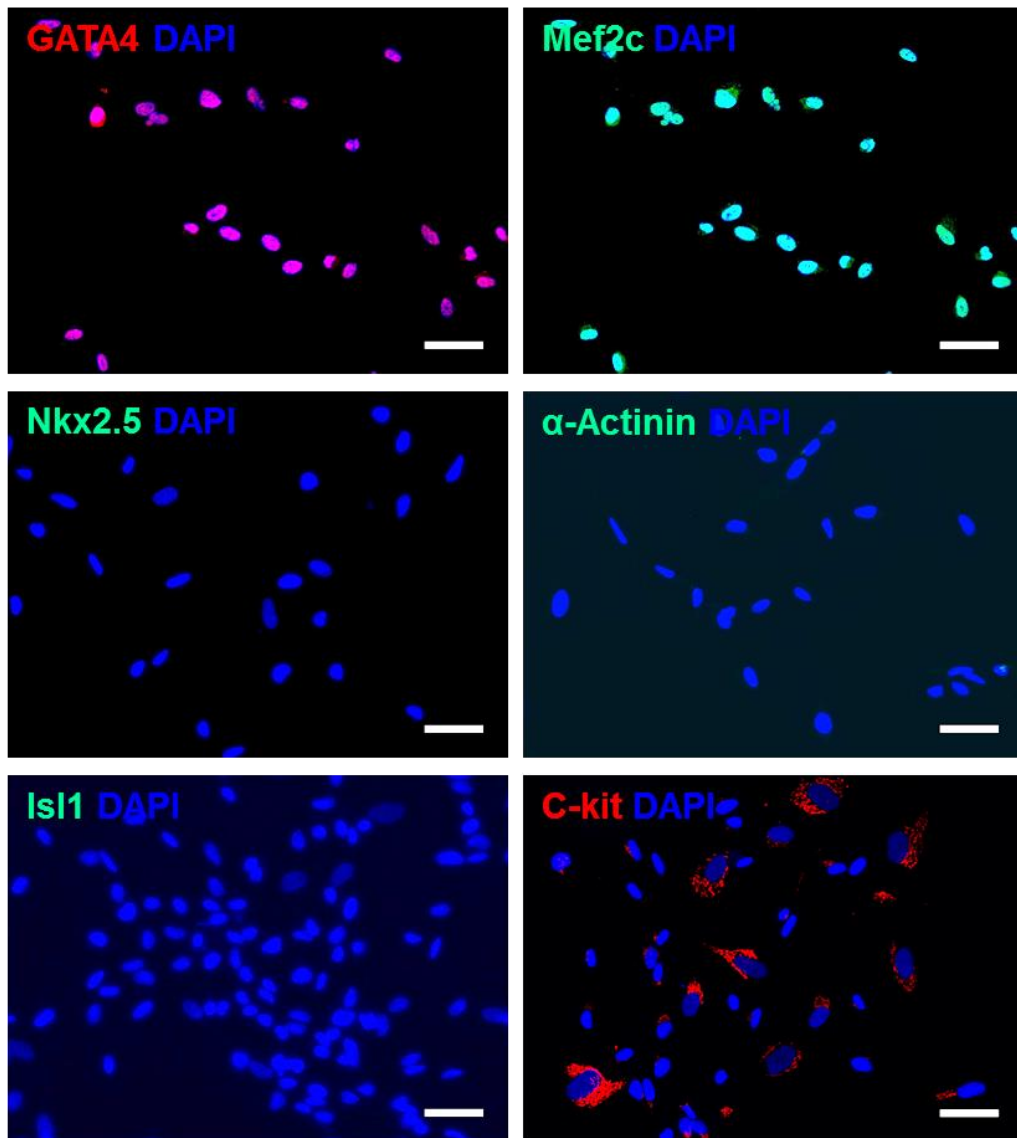


Figure 3-21 Representative immunofluorescence images of cultured adventitial cells stained for early and late cardiomyocyte markers and cardiac progenitor associated markers. Cultured adventitial cells label for GATA4 and Mef2c. A subpopulation also labels for c-kit but not Nkx2.5, Isl-1 or α -actinin (n=4). DAPI nuclear staining in blue. Scale bars = 50μm.

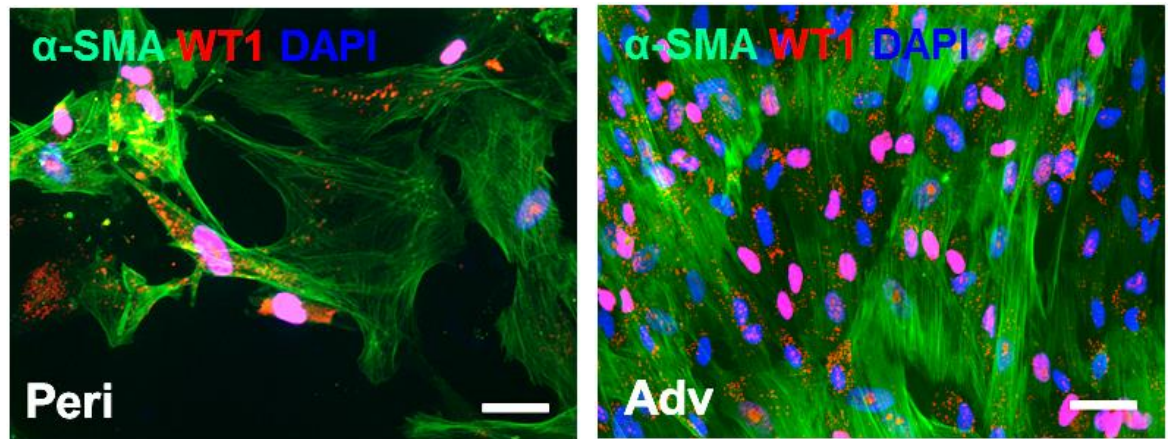


Figure 3-22 Representative immunofluorescence images of cultured pericytes (Peri) and adventitial cells (Adv) stained for α -SMA and cardiac progenitor cell marker WT1. A subpopulation of both pericytes (Peri) and adventitial cells (Adv) label for WT1 (n=4). DAPI nuclear staining in blue. Scale bar = 50 μ m.

3.5.3 Cultured cardiac perivascular cells possess mesenchymal differentiation potential

Following 21 days of osteogenic differentiation culture both pericytes and adventitial cells from 2 donors revealed similar differentiation potentials with clear evidence of extracellular mineral deposition by 7 days with Alizarin Red staining (Figure 3-23). Following 21 days of adipogenic differentiation culture using the same donor cells the results were considerably less pronounced in both populations with the formation of weakly Oil Red O staining cytoplasmic lipid droplets visible in most cells only by day 14 (Figure 3-24). Subjectively adventitial cells appeared to exhibit an enhanced adipogenic differentiation capacity compared with pericytes.

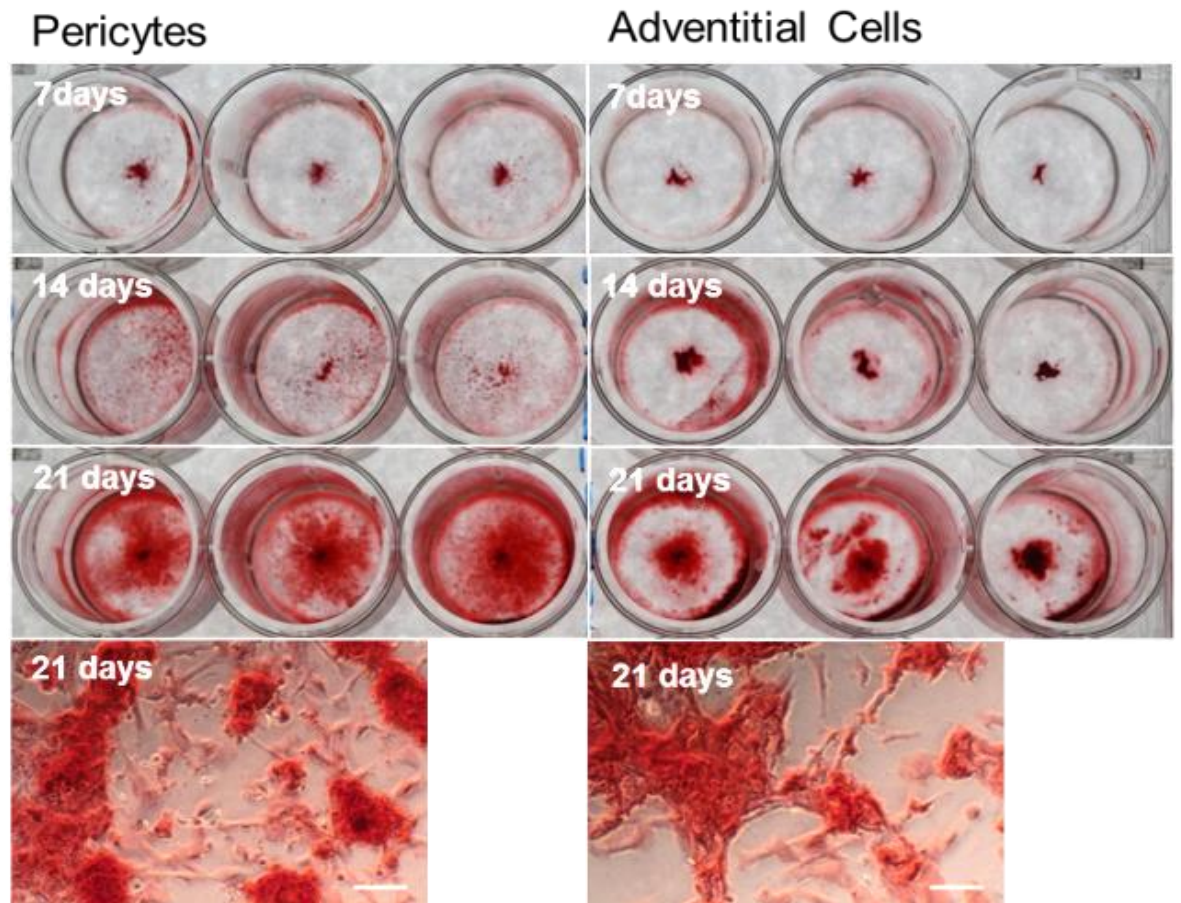


Figure 3-23 Representative images of alizarin red stained culture wells in triplicate containing perivascular cells subjected to 21 days of osteogenic differentiation. Extracellular calcium deposits (red) are evident in both the pericyte and adventitial cell populations and increase with time (n=2). Scale bar = 50 μ m

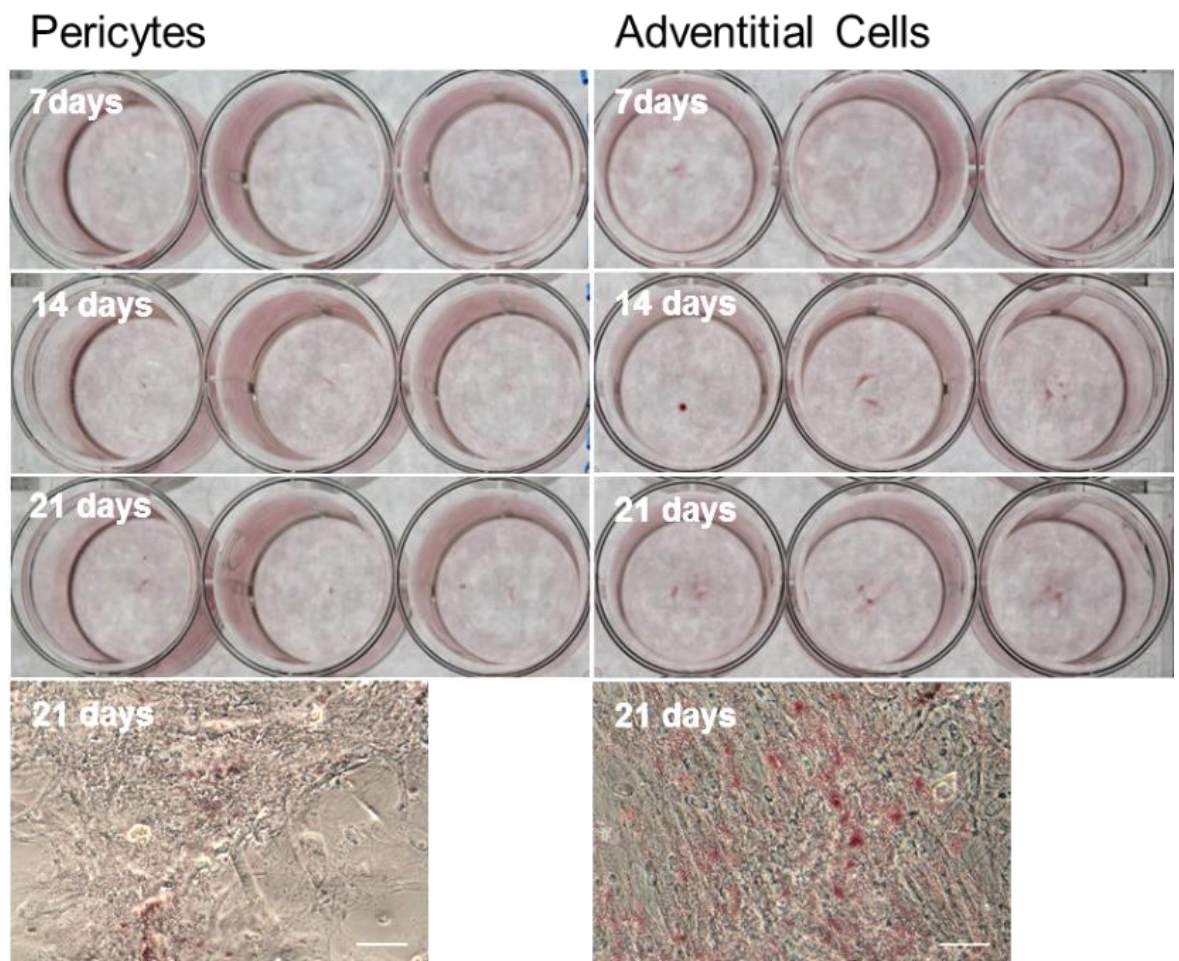


Figure 3-24 Representative images of Oil red O stained culture wells in triplicate containing perivascular cells subjected to 21 days of adipogenic differentiation. Lipid (red) is weakly present in both pericyte and adventitial cell populations (n=2). Scale bar = 50 μ m.

3.5.4 A minor subset of perivascular cells exhibit early cardiomyocyte differentiation in culture

3.5.4.1 Neonatal rat cardiomyocyte (NRCM) co-culture differentiation experiments

3.5.4.1.1 Validation of human specific conventional RT-PCR primers

Conventional RT-PCR primer sequences were tested on human and rat cDNA to ensure they did not cross react. The final panel of genes selected to be investigated included the pericyte and adventitial cell markers: CD146, PDGFR- β , NG2 and CD34; cardiac progenitor cell associated markers c-kit and ISL-1; early cardiomyocyte associated markers GATA-4, Nkx2.5 and Mef2c; and mature cardiomyocyte associated markers troponin-T and ANP (Figure 3-25).

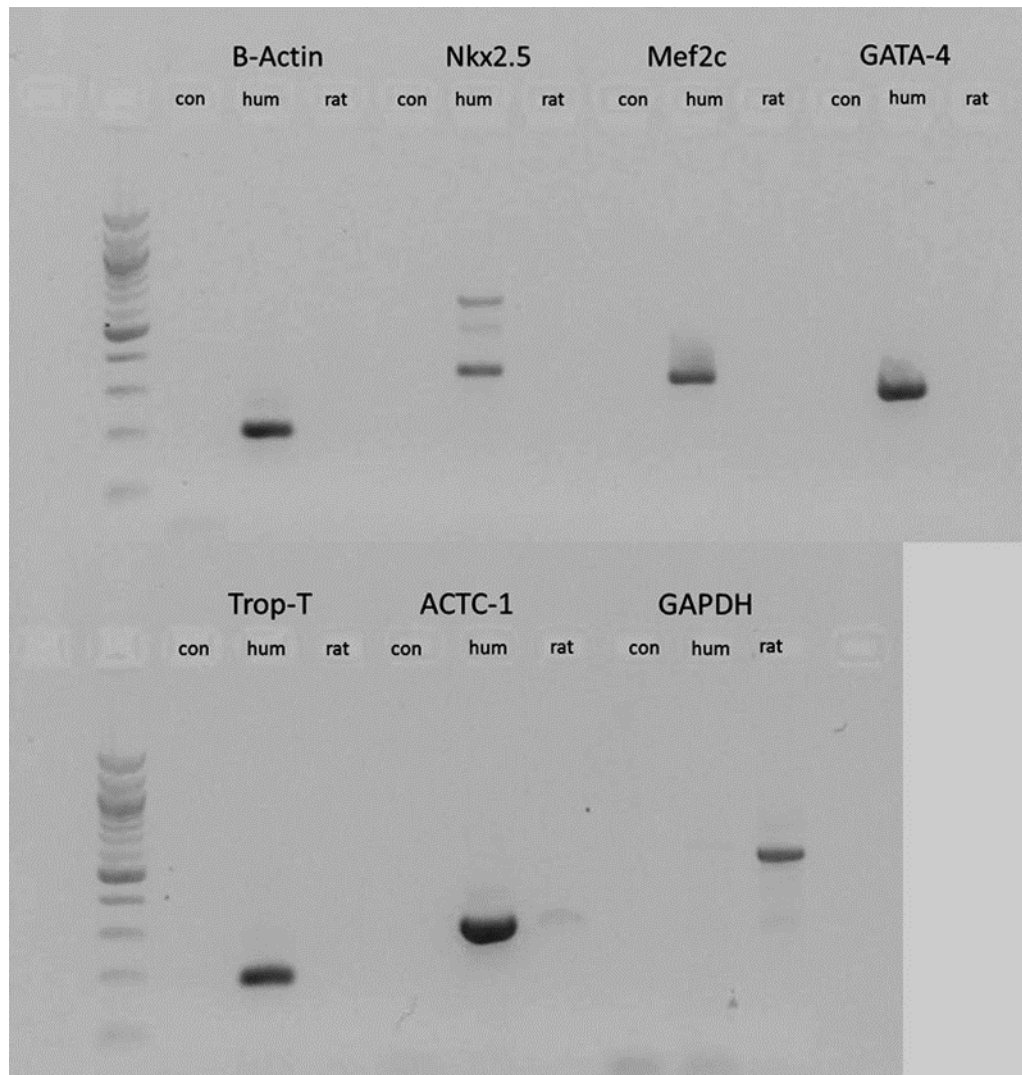


Figure 3-25 Conventional RT-PCR electrophoresis gel for validation of human primers with rat cDNA. α -actinin (ACTC-1) was excluded from final analysis due to production of product of a weak product with rat cDNA. β -actinin reference gene primers were human specific whilst GAPDH were rat specific (n=1). DNA ladder bands represent 100 base pairs. Con = water control, hum = human heart cDNA and rat = rat heart cDNA.

3.5.4.1.2 NRCM co-culture induces mild cardiomyocyte gene up-regulation in adventitial cells but not pericytes

Human pericytes and adventitial cells from four different donors were cultured with neonatal rat cells and expression of pericyte and cardiomyocytes genes was examined by RT-PCR at day 0 (non co-cultured perivascular cell population only), day 10 and day 21.

Pericyte markers CD146, NG2 were expressed uniformly at all-time points. Day 0 adventitial cells did not express PDGFR- β . CD34 gene expression was not detected in either population at any of the time points (Figure 3-26, Figure 3-27).

Of the early cardiomyocyte transcription factors GATA4 was present in both populations throughout culture (Figure 3-27). Mef2c was detectable throughout culture with the exception of day 0 adventitial cells. Nkx2.5 was not detectable in either population at any time point (Figure 3-28). Cardiac progenitor cell marker Isl-1 was seen in adventitial cells after co-culture (10 and 21d). C-kit was seen in both pericytes and adventitial cells after 21 days co-culture. Of the two mature sarcomeric proteins investigated, ANP and troponin T, only expression of ANP was detected. Low level constant expression was present in day 0 control and co-cultured pericytes however greater expression was seen in adventitial cells after co-culture with no day 0 expression detected.

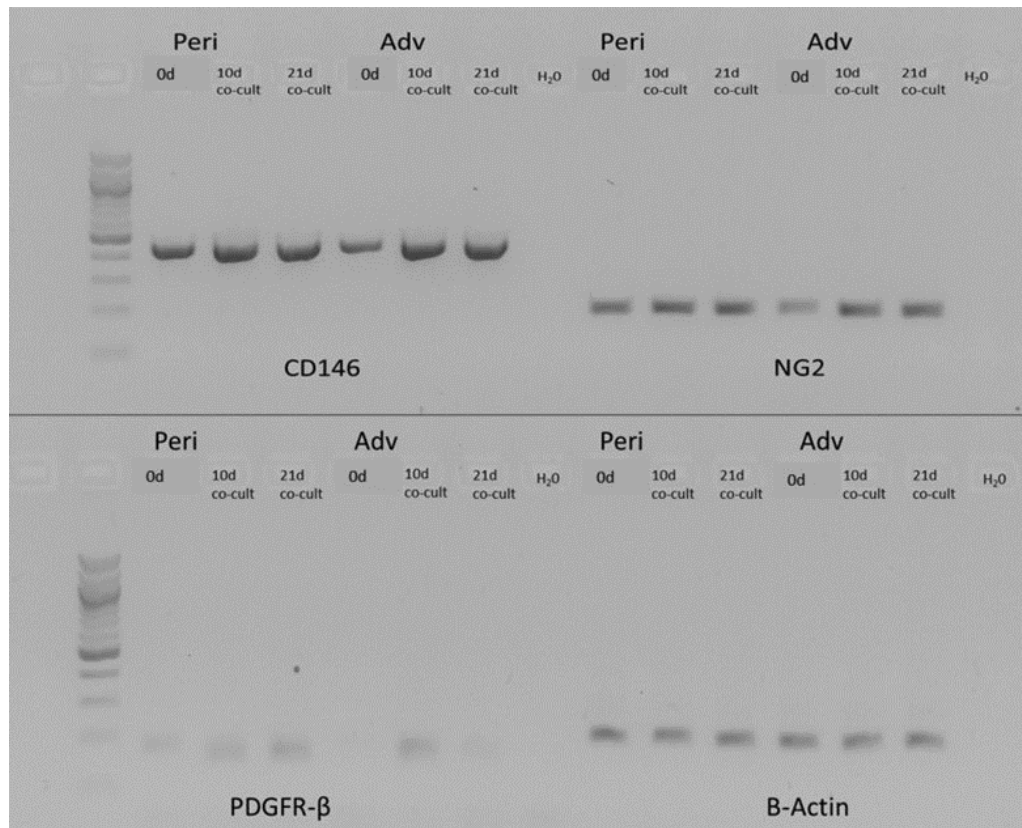


Figure 3-26 Representative conventional RT-PCR electrophoresis gel for pericyte marker genes in pericytes and adventitial cells co-cultured for 10 and 21 days with NRCMs. Pericyte associated genes were uniformly expressed in co-cultured pericytes (Peri) and adventitial cells (Adv) at day 0, day 10 and day 21 with the exception of PDGFR- β which was absent in adventitial cells at day 0 (n=4). DNA ladder bands represent 100 base pairs. H₂O represents negative control.

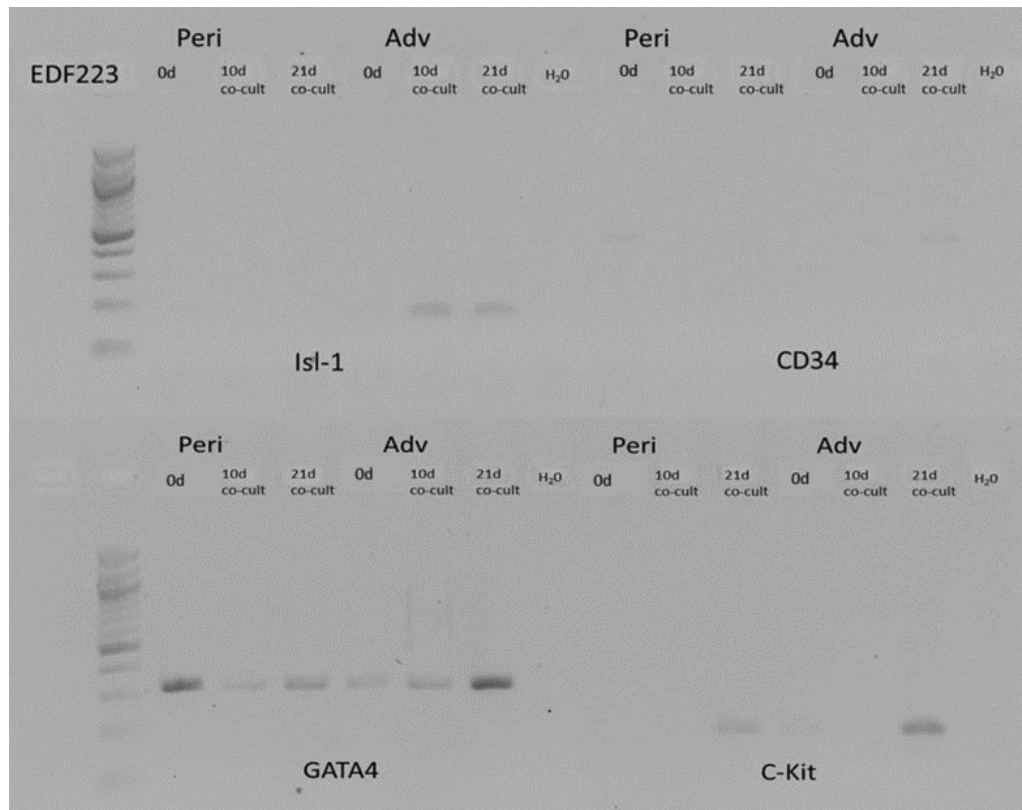


Figure 3-27 Representative conventional RT-PCR electrophoresis gel for early cardiomyocyte marker genes and cardiac progenitor cell marker genes in pericytes and adventitial cells co-cultured for 10 and 21 days with NRCMs. GATA4 was expressed in both pericytes (Peri) and adventitial cells (Adv) at day 0, day 10 and day 21. Isl-1 expression was seen only in adventitial cells after 10 and 21 days in co-culture. c-kit expression was present after 21 days in both populations. No CD34 expression was noted in either population (n=4). DNA ladder - bands represent 100 base pairs. H₂O represents negative control.

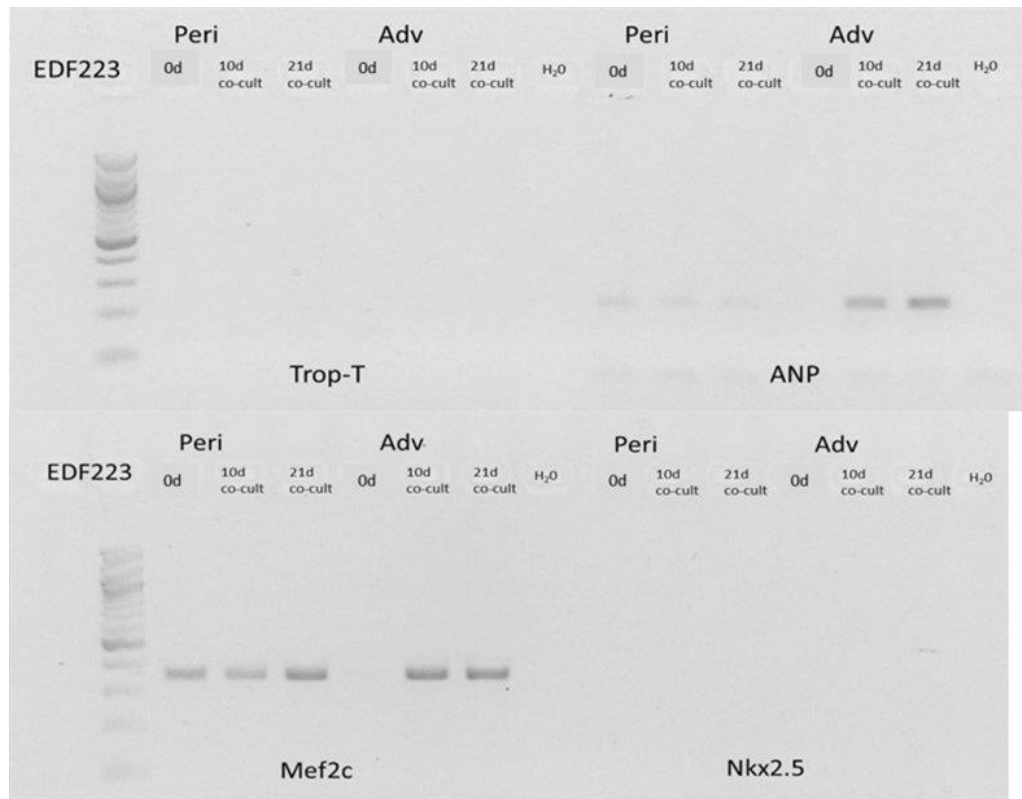


Figure 3-28 Representative conventional RT-PCR electrophoresis gel for early and late cardiomyocyte marker genes in pericytes and adventitial cells co-cultured for 10 and 21 days with NRCMs. ANP was weakly expressed in pericytes at all-time points but more strongly in adventitial cells after 10 and 21 days in co-culture. Troponin T (Trop-T) and Nkx2.5 were not expressed in either population at any time point. Mef2c was expressed at all-time points in pericytes and only after 10 and 21 days co-culture in adventitial cells (n=4). DNA ladder - bands represent 100 base pairs. H₂O represents negative control.

3.5.4.1.3 NRCM co-culture results in adhesion and tugging of perivascular cells but not independent contraction

Time-lapse imaging of cells in co-culture revealed that after several days beating rat cardiomyocytes adopt a stellate morphology with multiple radiating cell processes. Pericytes and adventitial cells were by contrast generally polygonal to spindle shape with only occasional cells taking on a stellate morphology. Both cell labelling techniques worked well with CM-Dil labelling perinuclear vesicles within the cytoplasm and emitting fluorescence signal in the red channel whilst eGFP lentiviral transduction labelled the entire cell cytoplasm and emitted fluorescence in the green channel upon excitation (Figure 3-29).

There was close association between pericyte/adventitial cells populations and rat cardiomyocytes with apparent “beating” of the former on initial observation of CM-Dil labelled cells. Closer examination revealed that in the majority of these cells the dye laden vesicles were moving in a single direction before returning to their original position (Video 3.1). This was considered to represent fusion of the labelled cell to an adjacent rat cardiomyocyte with pulling of the attached cell in a single plane as the former contracted. In some pericyte co-cultures rare labelled cells were seen with a stellate morphology and dye within their cytoplasmic projections. These cells demonstrated more convincing beating behaviour with movement of the dye centripetally before returning to their original positions (Video 3.2). These rare contracting cells were however not detected in the GFP labelled cell co-cultures in which the attached and tugged cell type was common (Video 3.3).

3.5.4.1.4 NRCM co-culture alone does not stimulate production of sarcomeric proteins in non-demethylated perivascular cells

At the end of the 21 day co-culture cells were stained for sarcomeric proteins troponin-T and α -actinin. The antibodies used react with both human and rodent proteins and the presence of rat neonatal cardiomyocytes provided an internal positive control. Staining of the rat cells with both antibodies was strong and consistent but no positive staining of sarcomeric filaments was detected in either the labelled human pericytes or adventitial cells (Figure 3-30).

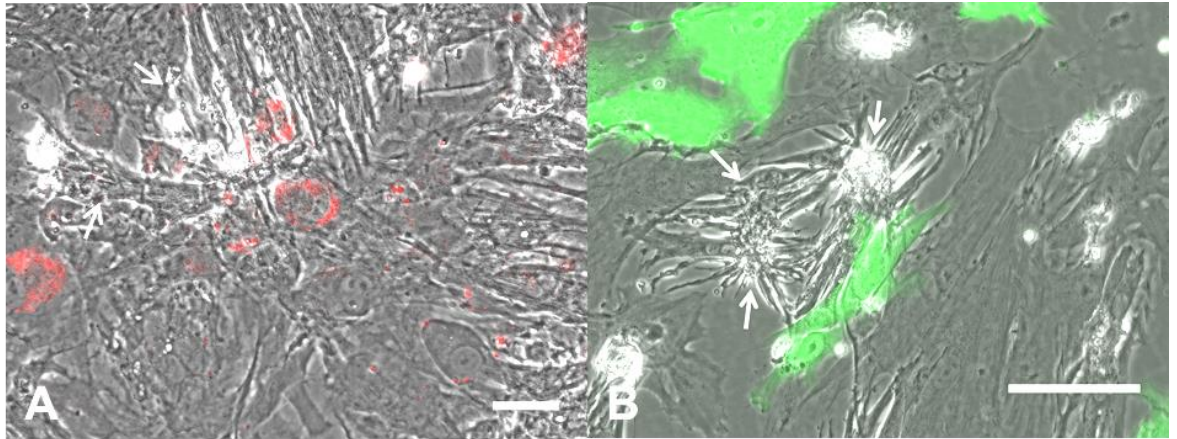


Figure 3-29 Representative combined immunofluorescence and phase contrast images of co-cultured human foetal pericytes labelled via membrane dye staining and viral transduction. Comparison of CM-Dil (red) labelled cells (A) and eGFP (green) labelled cells. White arrows indicate neonatal rat cardiomyocytes. Scale bars = 50µm (A) and 25µm (B).

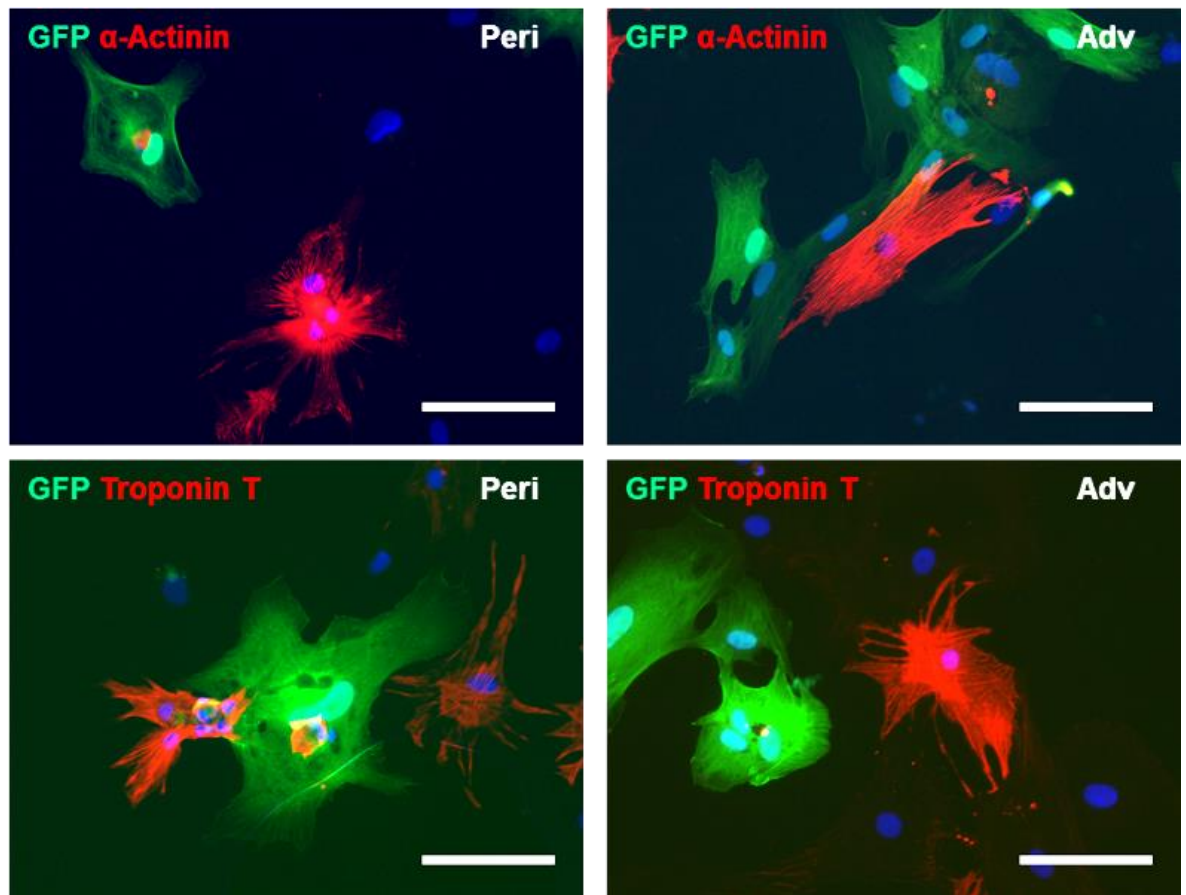


Figure 3-30 Representative immunofluorescence images of virally transduced, non-demethylated, human foetal pericytes and adventitial cells co-cultured with neonatal rat cardiomyocytes and stained for mature cardiomyocyte markers. Troponin T and α -Actinin (red) are expressed by rat cardiomyocytes but not eGFP (green) labelled pericytes (Peri) or adventitial cells (Adv) (n=4). DAPI nuclear staining in blue. Scale bars = 50 μ m.

3.5.4.1.5 Demethylation of perivascular cells before NRCM co-culture enhances cardiomyocyte differentiation

Three populations of pericytes and four populations of adventitial cells were demethylated prior to co-culture with NRCMs. Following 14 days co-culture no convincing independent contraction was seen in either population of perivascular cells. However examination of cells for spontaneous cytoplasmic calcium oscillations using fluo-4 dye revealed regular and strong calcium fluxes not only in numerous neonatal rat cells but also in occasional pericytes (Figure 3-31). These cells were present in the pericyte populations from all three donors but not in every culture well examined. By comparison spontaneous calcium fluxes were uncommon in neonatal rat cells in the adventitial co-cultured populations and seen only very rarely in labelled human cells. Addition of solution containing additional free calcium resulted in an increase in the rate of contraction and strength of the fluorescent signal in both populations but no apparent recruitment of human cells in either population.

Unlike in the earlier non-demethylated co-cultures staining for sarcomeric α -actinin and cardiac troponin-t revealed expression of both proteins in rare CM-Dil labelled pericytes and adventitial cells populations (Figure 3-32, Figure 3-33). These double labelled cells were more common in the pericyte populations than the adventitial ones where they were seen in only one of the four donor populations examined.

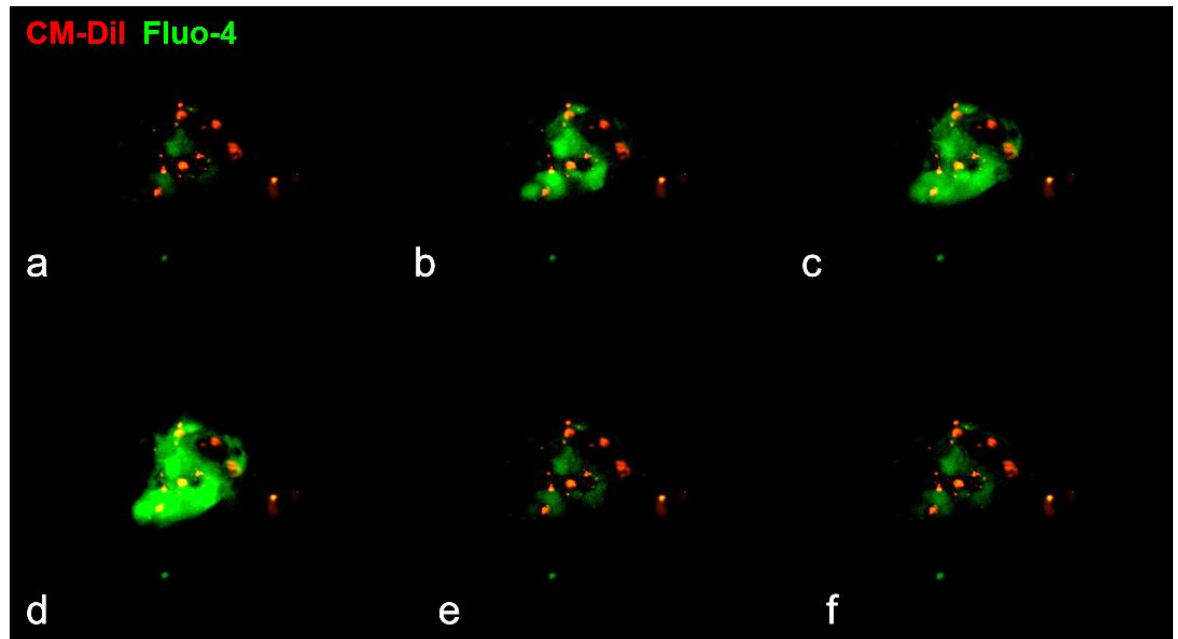


Figure 3-31 Representative chronologically (a to f, 2.4 seconds total) immunofluorescence images of a CM-Dil labelled (red granules) demethylated human foetal pericyte after co-culture with NRCMs for 14 days. Rise and fall of cytoplasmic calcium is indicated by the expression of a fluo-4 fluorescent signal (green) within the cytoplasm (n=3). x 400 magnification.

Pericytes

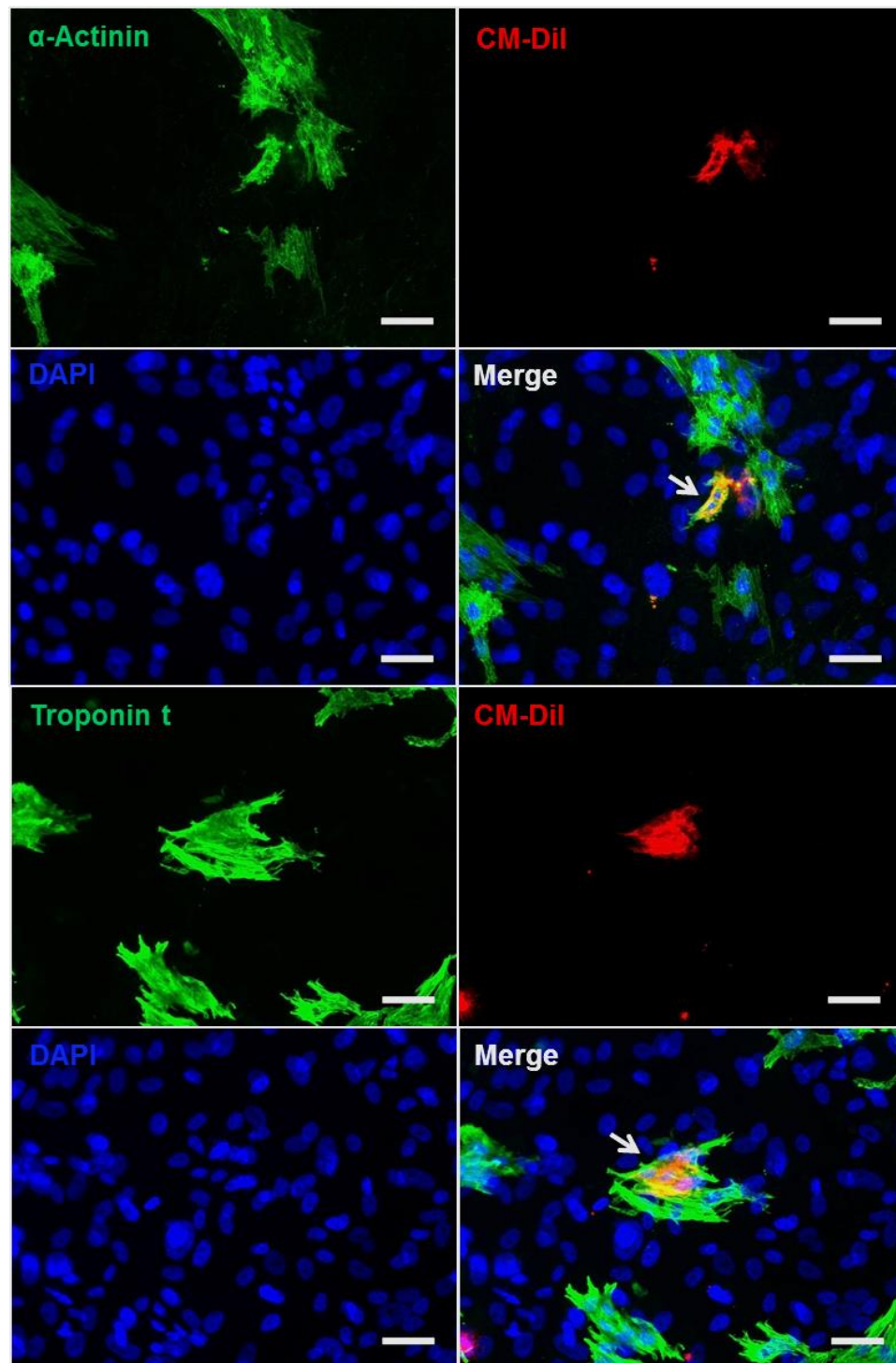


Figure 3-32 Representative immunofluorescence images of CM-Dil labelled (red) demethylated human foetal pericytes co-cultured with NRCMs for 14 days and stained for mature cardiomyocyte markers (green). Rare pericytes also express α -actinin and troponin t (white arrows) (n=3). Scale bars = 50 μ m.

Adventitial cells

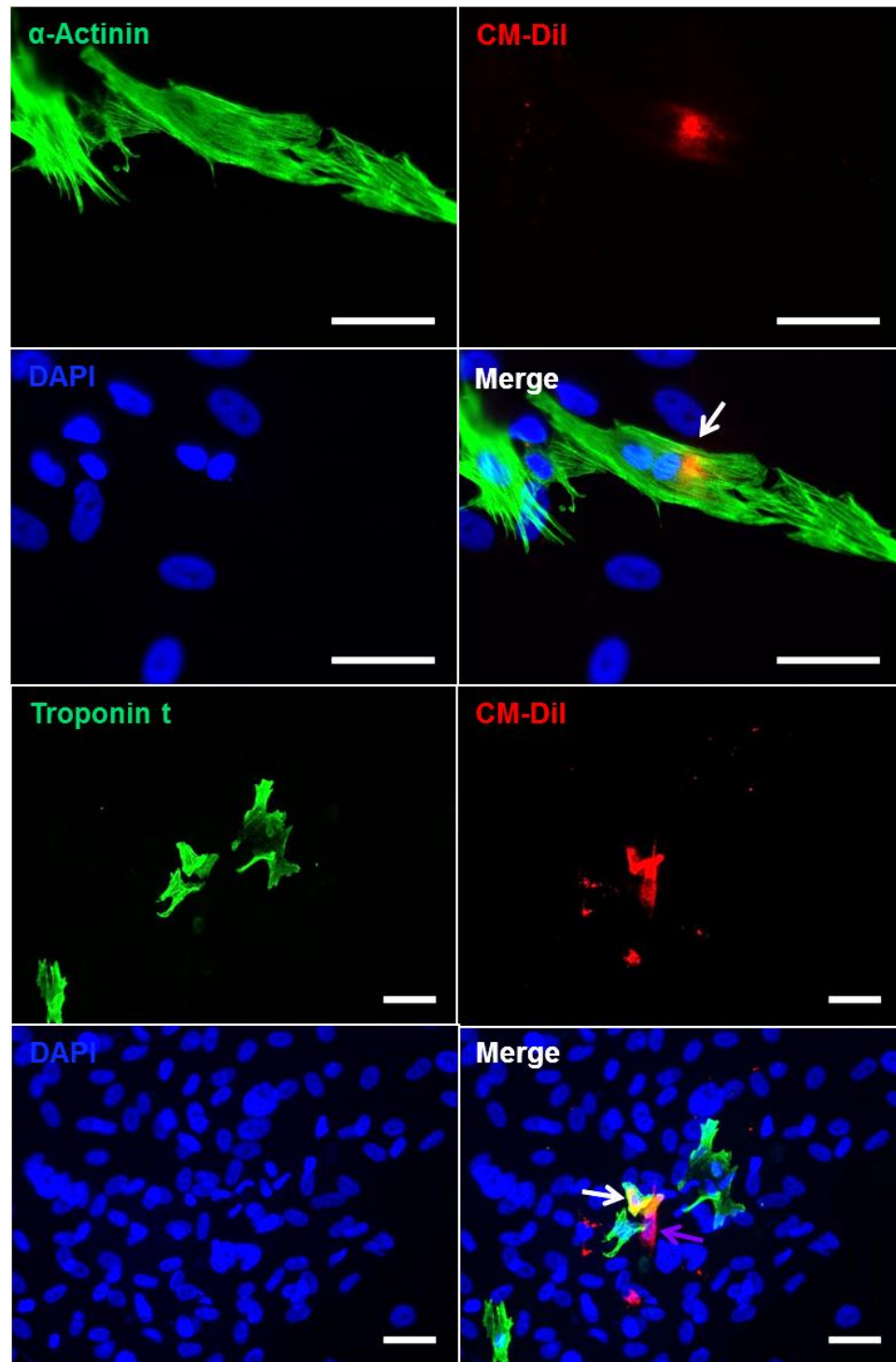


Figure 3-33 Representative immunofluorescence images of CM-Dil labelled (red) demethylated human foetal adventitial cells co-cultured with NRCMs for 14 days and stained for mature cardiomyocyte markers (green). Rare adventitial cells also express α -actinin and troponin t (white arrows) (n=4). Scale bars = 50 μ m.

3.6 Summary and discussion

This initial part of the study characterises two distinct populations of human foetal cardiac perivascular cells, pericytes and adventitial cells, both *in-vivo* and *in-vitro* with regard their anatomic location, surface marker expression and differentiation potential.

3.6.1 Cardiac perivascular cells express the same markers and reside in a similar location as in other organs

CD31 labelling confirms the high vascularity of the foetal myocardium consistent with a vascular density of approximately 1 capillary per myocyte as previously reported (Cotran et al., 1993). CD146⁺ pericytes are restricted predominantly to a perivascular location in the heart as noted in other tissues (Crisan et al., 2008b) with only a minor subset of endothelial cells also expressing CD146. CD34⁺ adventitial cells are a harder to define population given that CD34 is more widely expressed in endothelial cells. Adventitial cells have a less close association with microvessels and staining of the myocardium reveals that they are mainly located within the adventitia of coronary arteries, a similar location as reported in other tissues (Corselli et al., 2012). The expression of both of CD146 and CD34 in the vascular endothelium emphasises the importance of having a mature endothelial marker in the FACS antibody panel to exclude these cells from the purified population. The incomplete overlap of CD146⁺ staining with other established pericyte markers NG2 and α -SMA illustrates the heterogeneity in the cardiac pericyte population, as has been reported in the mesentery and retina (Nehls and Drenckhahn, 1991). NG2 and CD146 co-expressing perivascular cells form the majority population but there are also subpopulations positive for only one marker suggesting that these accepted pericyte markers label separate perivascular cells subsets in the foetal heart.

3.6.2 FACS enables sufficient cardiac perivascular cells to be obtained for use in experiments

The combination of antibodies used here to isolate pericytes and adventitial cells has been successfully applied to skeletal muscle, placenta, adipose tissue and kidney by

the Peault group (Crisan et al., 2008a). CD56, also known as neural cell adhesion molecule, is a surface glycoprotein involved in cell-to-cell adhesion and expressed on skeletal muscle myoblasts, natural killer (NK) cells, neurons and glia (Poli et al., 2009). Exclusion of CD56 positive cells in the original skeletal muscle study was to remove myoblasts (Stewart et al., 2003). These are not present in cardiac tissue but this antibody step was retained to exclude NK cells and nerve cells. Exclusion of the common leukocyte antigen CD45, a transmembrane glycoprotein of the PTP family involved in JAK kinase and Src signalling was to remove tissue or circulating leukocytes retained within vessels (Lorenz et al., 1994). Gating out of CD144⁺ cells crucially removes mature endothelial cells, a subset of which, as mentioned earlier, are also positive for CD146 and CD34 (Vestweber, 2008). On the final gate two discrete populations of cells were selected for collection: CD146⁺ CD34⁻ pericytes; and CD146⁻ CD34⁺ adventitial cells. Both CD146⁺ and CD34⁺ cells are present in high numbers on tissue stains of foetal heart and on FACS CD34⁺ CD146⁻ adventitial cells were found to be approximately twice as abundant as CD34⁻ CD146⁺ pericytes making up around 2% and 3.7% of the total cell population respectively. These yields of pericytes were similar to those reported from other tissues (Crisan et al., 2008a) and sufficient cells were generally obtained from hearts of fetuses greater than 16 weeks of age to enable successful expansion in culture, thus achieving one of the key aims of this study.

3.6.3 Expanded populations converge to a stable pericyte phenotype in culture

Following isolation from tissue and *in-vitro* culture over several passages the presence of pericyte markers, CD146, NG2 and PDGFR- β was consistently detected in both pericytes and adventitial cells on gene expression, cell surface marker staining and flow cytometry, suggesting that a stable pericyte phenotype is maintained in culture. Adventitial cells rapidly lost expression of CD34, the key surface receptor on which they were originally isolated, and up-regulated expression of CD146. This shift of surface marker expression suggests that cardiac adventitial cells are able to adopt a “pericyte phenotype” in culture. This phenomenon has been reported in adipose adventitial cells (Corselli et al., 2012) but required stimulation

with angiopoietin 2. In the cardiac adventitial cell population no angiopoietin 2 treatment was required suggesting a spontaneous convergence to a pericyte culture phenotype. Adventitial cells also expressed NG2 and PDGFR- β surface proteins (absence of detectable PDGFR- β mRNA was considered a result of primer or PCR reaction failure) however it remains to be elucidated as to whether they natively express these pericyte markers *in-vivo*. Expression levels in cultured pericytes of CD146 and NG2 mRNA were approximately 2.5 fold and 7.5 fold higher respectively than in cultured adventitial cells suggesting that although convergence to a pericyte phenotype occurs genuine differences remain between populations. PDGFR- β expression levels did not discriminate between populations with no statistically significant difference in expression evident.

3.6.4 Cardiac perivascular cells adopt an MSC-like phenotype in culture

Several criteria have been proposed in defining the mesenchymal stem cell phenotype by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. These include plastic-adherence when maintained in standard culture conditions, expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules. Finally MSCs should also differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006). Both pericyte and adventitial cell populations were isolated in part on negative expression of CD45 and both populations expressed CD90, CD73 and CD105 in culture. CD105 co-staining was also noted on CD146⁺ cells in tissue sections. Mesenchymal stem cell-like properties were also suggested by differentiation to adipogenic and osteogenic lineages in culture, a well-recognised feature of MSCs (Caplan, 2007). Pericytes are now widely accepted as the source of tissue MSCs and the expression of MSC associated markers by pericytes *in situ* has been shown in multiple organs (da Silva Meirelles et al., 2006, Crisan et al., 2008b). Interestingly the *in-vivo* expression of CD34 by adventitial cells should exclude them from consideration as MSCs since conventional MSCs are CD34⁻. However in culture this marker is rapidly lost and cells express the

standard MSC associated markers suggesting again that they rapidly and spontaneously transition into a pericyte/MSC phenotype in culture conditions.

3.6.5 Cardiac perivascular cells express markers synonymous with early cardiomyocyte phenotype in culture

Whereas pericyte and MSC genes were uniformly expressed by the pericyte and adventitial cell populations there was, by comparison, a marked variation in cardiomyocyte associated gene expression. The majority of pericyte and adventitial cell populations expressed mRNA of the early cardiac transcription factors GATA4 and Mef2c but not Nkx2.5. This was confirmed on staining of cultured cells. These transcription factors are considered critical in the development of the cardiac phenotype and interact in the translation of inductive signals to cardiomyogenesis (Figure 3-34). GATA4 is a member of the GATA zinc-finger transcription factor family which regulate the expression of cardiac contractile proteins genes and other regulatory genes such as Mef2c and Nkx2.5. Mef2c activates a subset of cardiac contractile protein genes and is involved in the development of cardiac structures from the secondary heart field. Nkx2.5 is expressed on cardiac progenitor cells and is a target for signals inducing cardiogenesis (Olson, 2006). Together they play a key role in the development, maturation and homeostasis of cardiomyocytes and genetic deletion of each individually results in embryonic lethality due to abnormal cardiac morphogenesis or failure of cardiomyocytes differentiation (Schlesinger et al., 2011). Expression of GATA4 and Mef2c by pericytes and adventitial cells therefore implies an early cardiomyocyte commitment by these cells. It is important however to recognise that expression of these transcription factors does not automatically make cells cardiac progenitors as some are also expressed by non-cardiac cells. In addition to developing cardiomyocytes Mef2c is also expressed within developing skeletal myofibres (Edmondson et al., 1994). Nkx2.5 is expressed in the spleen, liver, stomach tongue, larynx and skeletal muscle during foetal development (Kasahara et al., 1998). GATA4 expression however, by contrast, is tightly restricted to cardiac tissues (Olson, 2006). Weak up regulation of ANP expression was seen in both pericytes and adventitial cells. ANP is a specific and early marker for differentiation of the myocardium of the atria and ventricles in the developing heart that is down

regulated in the ventricle at birth (Houweling et al., 2005) and this finding supports the development of a cardiomyocyte phenotype in isolated cardiac pericytes and adventitial cells.

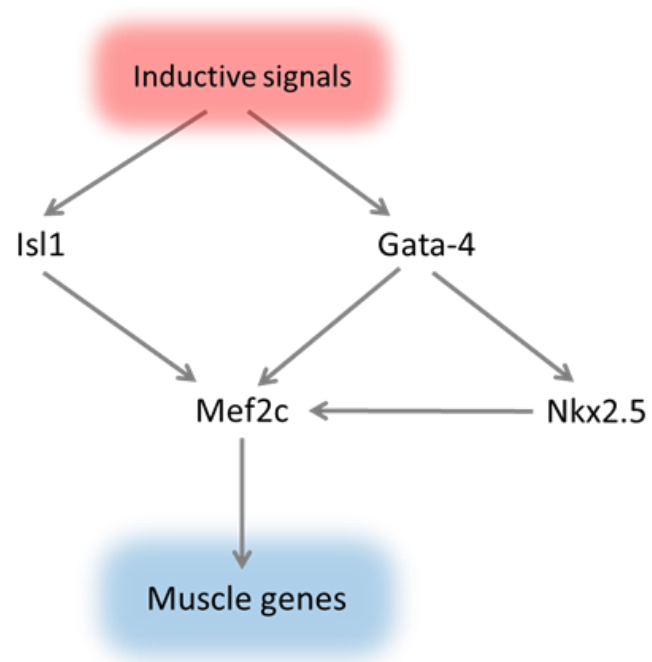


Figure 3-34 Regulatory interactions among key cardiac transcription factors, Isl-1, Gata-4, Mef2c and Nkx2.5, in the secondary heart field during cardiac development. Adapted from Olson 2006.

3.6.6 Cardiac perivascular cells express markers synonymous with a cardiac progenitor cell phenotype in culture

Several putative cardiomyocyte progenitor cells, including c-kit⁺ cells (Tallini et al., 2009, Ellison et al., 2013), WT1⁺ cells (Smart et al., 2011) and Isl-1⁺ cells (Laugwitz et al., 2008) have been identified in the mouse and human hearts on the basis of their surface marker expression and their ability to contribute to cardiac repair through the generation of new cardiomyocytes. Intriguingly conventional RT-PCR and immunostaining revealed that both pericyte and adventitial cell populations expressed c-kit and WT1 with wider expression of the latter seen in adventitial cells. Immunostaining results suggested positivity in a subset of both cell types rather than the entire population. Gating out of CD45⁺ cells on the initial sort should have excluded all leukocytes including mast cells and thus the c-kit⁺ population is unlikely to comprise the latter. It is possible however that endogenous foetal cardiac perivascular cells are c-kit⁻ and only switch on this marker in culture. This suggestion is supported by the apparent absence of co-expression by cells of c-kit and CD146 in tissue sections but needs to be confirmed on FACS. WT1 is expressed in the mesothelium and has been reported in α -SMA and PDGFR- β positive perivascular cells in the developing lung (Que et al., 2008). It has also been reported in cardiomyocytes, vascular smooth muscle and endothelial cells in the heart where it is associated with epithelial to mesenchymal transition of the mesothelium (Zhou et al., 2008). It is perhaps therefore not surprising if a subpopulation of cardiac perivascular cells express this marker after isolation and culture. Taken together the expression of c-kit and WT-1 by a subpopulation of perivascular cells hints at the presence of a subset with cardiac progenitor cell potential, particularly when the expression of early cardiac transcription factors is also considered.

3.6.7 Cardiac perivascular cells do not spontaneously adopt a mature cardiomyocyte phenotype in culture

As cardiomyocytes mature they express contractile sarcomeric proteins such as cardiac troponin-t, myosin heavy chain II (MYH II), atrial natriuretic peptide (ANP) and cardiac specific α -actinin (Ehler and Perriard, 2000, Smits et al., 2009).

Expression of mRNA of these proteins in cultured pericytes and adventitial cells was limited with weak expression of α -actinin mRNA noted in pericytes and adventitial cells and weak expression of MYH II in adventitial cells. No ANP or troponin-t expression was evident. α -actinin expression was not detected on immunostaining in either pericytes or adventitial cells which suggests these cells do not develop a mature cardiomyocyte phenotype once isolated and excludes contamination of the sorted population by mature cardiomyocytes.

3.6.8 A rare subset of cardiac pericytes exhibit early cardiomyocyte differentiation after co-culture with NRCMs but only after demethylation

In-vitro cardiomyocyte differentiation has been achieved in pluripotent embryonic and induced pluripotent stem cells with relative ease (Boheler et al., 2002, Gherghiceanu et al., 2011). Differentiation of more developmentally restricted adult stem cell populations to a cardiac phenotype is less routinely reported and recognised as more of a challenge (Heng et al., 2004). The current study used co-culture with neonatal rat cardiomyocytes as this appears to have been the most successfully applied technique (Zaruba et al., 2010). Several authors have reported success with this method using bone marrow or adipose derived MSCs although direct cell to cell contact would appear to be a key requirement with none or only limited differentiation occurring in cultures in which the two cell types are separated by a membrane (Xu et al., 2004). The extent of cardiomyocyte differentiation in these studies is generally limited with expression of early transcription factors but failure of development of cross striated sarcomeric proteins or beating (Condorelli et al., 2001, Choi et al., 2010). The initial co-culture results obtained in the current study were generally disappointing with only rare potentially contractile cells seen in the CM-Dil labelled pericyte populations after 21 days of co-culture but with no

expression of crucial contractile proteins detected on staining. Therefore demethylation of the cells was undertaken to increase the likelihood of greater differentiation occurring. DNA methylation is the addition of a methyl group to the cytosine or adenine nucleotides resulting in the stable suppression of expression of the gene affected. It was discovered that in embryonic stem cells the pluripotency state is conferred by certain transcription factors such as oct4, nanog and sox2, the expression of which is controlled by promoter methylation and that differentiation is accompanied by down regulation of these genes by methylation (Fouse et al., 2008). Conversely re-programming of fibroblasts into induced pluripotent stem cells was associated with unmethylated active promoters of these genes (Takahashi et al., 2007). In contrast to embryonic and induced pluripotent stem cells little is known about the epigenetic control of genes associated with self-renewal or differentiation in adult stem cells. Oct4 is silenced by hypermethylation in adipose derived stem cells and bone marrow MSCs whereas nanog and sox2 are unmethylated but possibly silenced by other mechanisms such as histone modification (Barrand and Collas, 2010). Adult multipotent stem cells tend to differentiate into closely related cells such as chondrocytes, myocytes or adipocytes (Pittenger et al., 1999) and it has been suggested that differentiation potential is regulated by DNA methylation of specific lineage promoters (Berdasco and Esteller, 2011). Circumstantial evidence exists to support this hypothesis such as strong methylation of the endothelial markers CD31 and CD144 in adipose stem cells that have a very limited endothelial differentiation capacity (Boquest et al., 2007). In addition, treatment of stem cells with demethylating agents has been reported to result in spontaneous differentiation such as osteogenic differentiation in bone marrow MSCs treated with 5-aza-2'-deoxycytidine. This treatment has also been used to enhance cardiomyogenic differentiation in cardiac progenitor cells (Oh et al., 2003). The results of NRCM co-culture following demethylation of perivascular cells were considerably more encouraging with the expression of sarcomeric proteins α -actinin and troponin t in rare cells and the development of spontaneous cytoplasmic calcium oscillations. This suggest that a minor subset of perivascular cells is able to differentiate some way to a cardiomyocyte phenotype in culture but stop short of developing fully mature

independently beating cells. Interestingly evidence of differentiation, although still rare, was more commonly seen in the pericyte than adventitial cell populations.

3.6.9 Limitations of *in-vitro* cardiomyocyte differentiation studies

The *in-vitro* neonatal rat cardiomyocyte co-culture model allows direct cell to cell contact and attempts to replicate the neonatal myocardial environment in which cellular signals promoting cardiomyocyte differentiation might be expected to be present (Rangappa et al., 2003). This model however has several potentially important limitations that are both conceptual and technical. Firstly, the cells are from two different species raising the possibility of failure of effective communication between cells. Very little work appears to have been done on cellular communication between species. An early study examined electrical coupling between cells from a variety of organs and a variety of mammalian species including rabbit, hamster, rat and mouse and found that in all combinations membrane communication junctions were formed (Michalke and Loewenstein, 1971). This suggests that some degree of communication is possible at least yet it is unclear whether human perivascular cells are likely to respond to paracrine factors produced by rat cells. Equally important is the fact that cells in culture are not subject to a hypoxic and inflammatory environment as they would be in the infarcted myocardium (Heng et al., 2004). On a technical level co-cultures require a relatively high density of cells to ensure contact between NRCMs and the cells being investigated. This frequently results in the establishment of multi-layered cultures that can make discerning true differentiation of labelled cells versus overlapping of two different cells types a challenge (Gruh et al., 2006). Consequently co-cultures wells and coverslips must be examined critically. Labelling of the cells can also prove problematic. The uptake of the CM-Dil varies between individual cells and is retained in peri-nuclear vesicles rather than outlining the entire cytoplasm making individual cell processes difficult to determine. GFP transduction on the other hand may result in inadvertent labelling of the non-target co-culture population through viral transfer in media (Ramkisoensing et al., 2012). However given that no GFP⁺

cardiomyocytes were seen in the co-cultures it is unlikely that this was an issue in this study.

3.6.10 Conclusion

The findings presented here suggest that there are two distinct populations of abundant progenitor cells within the cardiac peri-vascular compartment. Once isolated and expanded in culture the phenotype of these populations converges with very similar expression patterns of pericyte, MSC, cardiac progenitor and early cardiomyocyte markers. Both perivascular populations express MSC like differentiation potential forming extracellular lipid and osteoid. Despite this, differences in gene/protein expression levels and behaviour in culture conditions suggest an underlying difference between the populations. This is supported by NRCM co-culture studies in which a minor subset of predominantly pericytes appears able to partially differentiate towards cardiomyocytes. The specific aims of this section of the study were achieved and the initial hypothesis was largely confirmed albeit with the caveat that cardiomyogenic differentiation was a rare event and did not reach development of mature cardiomyocytes. *In-vitro* cardiomyocyte differentiation is however notably inefficient and it was hoped that the cell transplantation studies of the next chapter would stimulate a greater differentiation response.

Chapter 4: Transplantation of human cardiac perivascular cells into the injured murine heart has a deleterious rather than beneficial effect

4.1 Introduction

In the previous chapter definitive *in-vitro* cardiomyocyte differentiation of cardiac perivascular populations was not demonstrated however these populations natively express markers associated with early cardiomyocyte commitment and a cardiac progenitor phenotype. It is therefore possible that the *in-vitro* conditions used were simply inadequate to trigger the differentiation process. The inflammatory cytokine environment in infarcted myocardium *in-vivo* has been shown to be important in controlling the remodelling process through promotion of myocyte survival or death, influencing collagen deposition and dissolution and angiogenesis (Nian et al., 2004). These chemical mediators are likely to play a role in the stimulation or inhibition of cardiomyocyte differentiation of cardiac progenitors and will not be present in the standard co-culture conditions. It is not surprising then that the majority of cardiac progenitor cell investigations demonstrate *in-vivo* differentiation capacity through cell transplantation (Jackson et al., 2001). This part of the study examines the cardiac functional effect and fate of foetal derived cardiac pericytes and adventitial cells following transplantation into the injured myocardium.

4.2 Specific hypothesis

The work in this section was based on the hypothesis that “Foetal human cardiac perivascular cells will differentiate into cardiomyocytes when transplanted into ischaemically injured myocardium and by doing so will regenerate heart muscle, reduce scarring and improve cardiac function.”

4.3 Specific aims

The specific aims of this part of the study were:

- To validate the coronary artery ligation surgical technique using NOD/SCID mice.

- To select a suitable population of human cardiac perivascular cells for transplantation.
- To effectively induce myocardial infarctions and transplant cells into the infarct border zone.
- To assess and compare the functional impacts of pericyte and adventitial cell therapy on the heart after 21 days.
- to reliably identify retained transplanted cells of both populations and to assess and compare their fate.

4.4 Specific material and methods

4.4.1 Expansion of candidate populations prior to transplantation

Pericyte (peri) and adventitial cell (adv) populations from donors EDF183 and EDF197 were selected for expansion and potential injection. These were chosen due to their similar gestational age (18 and 19 weeks of gestation respectively) and their robust growth characteristics which had enabled suitable numbers of cells to be cryopreserved. The donor cells were cryo-preserved, as described in 2.10, between passages P2 and P4 (EDF183 peri P4, adv P3/EDF197 peri P3, adv P2). Cells were thawed as described in 2.11 and expanded in culture as described in 2.9. Surgery was planned over 4 weeks and cell expansion times, based on prior experience, were coordinated to generate a surplus of cells for injection at the right time whilst minimizing passage number and overall time in culture. Adventitial cells are more robust than pericytes, survive cryopreservation/thawing in higher numbers and grow faster (3.5.2) thus expansion periods were staggered accordingly.

4.4.2 Assessment of candidate populations for injection by gene expression

For each population of cells the expression profiles of pericyte, MSC and cardiomyocyte genes were compared by conventional RT-PCR at the time of cryopreservation and after expansion prior to use for injection (EDF183 peri P4+2, adv P3+3/EDF197 peri P3+4, adv P2+3). RNA was extracted and reverse-transcribed as described in 2.13 and 2.14. Conventional RT-PCR was performed as described in 2.15 using primers for human pericyte marker genes and MSC marker genes as listed in Table 3-4.

4.4.3 Assessment of injection technique on cell survival

In order to determine whether prolonged storage in PBS (simulating a delay between syringe loading and administration) and ejection through a 30 gauge needle had a detrimental effect on cell viability a pilot study was carried out. A population of pericytes and adventitial cells was trypsinised to a single cell suspension, then assessed for viability via Trypan blue staining and counting using a haemocytometer, before being loaded into syringes as per the injection study. These were stored on ice for 4hr before the cells were ejected through the needle and re-examined for viability.

4.4.4 eGFP viral transduction and loading of cells into syringes

Forty eight hours prior to injection cells were virally transduced as described in 2.18. On the morning of surgery cells were washed twice with PBS before being dissociated with trypsin, quenched with a 3ml of 20% DMEM and counted using a haemocytometer. The cell suspension was then centrifuged at 1000rpm for 5 min and the resulting pellet was re-suspended in sterile PBS to give a concentration of 100,000 cells per 10 μ l. The plunger of a 30 gauge 0.3ml insulin syringe was removed and 40 μ l of cell suspension pipetted into the lumen. The plunger was replaced and air and excess suspension were expressed to leave 30 μ l loaded. The vehicle control comprised sterile PBS with no cells. Tape was affixed to the syringe identifying its contents and the syringe were then placed on ice for transportation to the surgical facility. Excess cells surplus to requirements for the injection study were preserved in TRIzol for later RNA extraction and conventional RT-PCR as described in 2.13, 2.14 and 2.15 or grown on sterile coverslips for immunocytochemistry as described in 2.17.

4.4.5 Infarct Model

Left ventricular infarcts were induced surgically via thoracotomy and ligation of the left anterior descending (LAD) coronary artery. Initial surgeries were performed on C57/Bl6 mice to obtain control tissue. Prior to surgery the procedure room was thoroughly cleaned. Hard surfaces were disinfected with 70% alcohol and bactericidal disinfectant wipes (Premier Healthcare & Hygiene Ltd) were attached to

contact points of the surgical microscope. Between animals all surgical instruments were sterilised in cold sterilising solution (Novasapa cold sterilise, Pfizer). Mice were anaesthetised in an induction chamber via exposure to 3% isoflurane (Abbott) before being given a subcutaneous (SC) injection of 0.02mg/kg of buprenorphine analgesic (Vetergesic, Alstoe Animal Health) and having their left lateral thorax shaved. They were then suspended in dorsal recumbency by their upper incisors on a 45° angled rig whilst anaesthetic gas was supplied via a silicon tube. The distance between the mid thoracic trachea and the level of the point of the mandible was marked on a 20 gauge catheter (SURFLO ETFE I.V., Terumo). Using an illuminated surgical microscope focused at the level of the caudal pharynx the tongue was retracted and the vocal cords visualised so that the lubricated catheter could be gently passed into the trachea up to the mark. Placement of the catheter an excessive distance into the trachea risks entry into a main stem bronchus and inflation of a single lung field or puncturing of the tracheal bifurcation with the development of a pneumothorax. Once in place the catheter was connected to a mechanical ventilator providing 120 breaths per minute at a stroke volume of 240µl to 270µl for mice 25g to 36g in weight. The chest was carefully observed for regular expansion and contraction. Misplacement of the catheter into the stomach was recognised by limited chest movement and rhythmic movement of the abdomen, gradual expansion of the abdomen and loss of pink colour of the tongue, toes and tail tip. Once stable the animal was moved to a heat pad set at 37°C and placed in right lateral recumbency. The catheter hub was taped carefully to the animals muzzle to prevent accidental extubation.

All surgery was carried out using a surgical microscope providing 1.6 x magnification. The surgical site was disinfected and a 2cm² window was cut in a cling film drape. A forward slanting skin incision of approximately 1cm length at the approximate midpoint dorso-ventrally was made and the underlying superficial muscle layers bluntly dissected to expose the ribcage. Stay sutures were placed within the musculature to maintain exposure. Ribs 4 and 5 were identified by counting caudally from the axillary region and careful blunt dissection of the intercostal muscle was carried out with the tips of curved forceps. A pair of rat rib

retractors was used to open the thorax and expose the heart, the pericardium overlying the left ventricle was carefully opened and the left anterior descending coronary artery was identified. The artery initially lies within the superficial myocardium but rapidly passes deeper into the muscle meaning identification is easiest where it emerges from under the left atria. A ligature was placed approximately 1/3 of the distance between the tip of the left atrium and the apex. Ligation was performed using an 8/0 ethilon swaged suture on a round bodied needle. A small bite of myocardium, approximately 3mm long, was taken to avoid mechanical compromise from a larger ligature. Care was taken not to penetrate the myocardium too deeply resulting in cardiac puncture and haemorrhage. Blanching of the dependent myocardium was usually evident within a few minutes. If blanching was not confirmed a second or very rarely third ligature was placed at a similar level to the first to try and achieve definitive ischemia.

4.4.6 Validation of surgical technique in NOD/SCID mice

Ten 10 to 12 week old NOD/SCID mice from Harlan Laboratories were used in a pilot study to determine how these genetically altered mice respond to coronary ligation surgery. These animals did not receive cell transplantations. Serum was sampled at 24hrs post-surgery for troponin I assay and the animals were sacrificed at 14 days and necropsied

4.4.7 Transplantation of cells

Mice were randomly allocated to a treatment group to receive pericytes, adventitial cells or PBS control. The surgeon (J E Baily) was blinded to the contents of each syringe. Following infarction, cells or vehicle control were injected at two sites either side of the ligature and in the ischaemic border zone as identified by the blanched region (Figure 4-1). The needle was inserted obliquely to the surface of the heart and advanced to a distance of approximately 3 mm in an attempt to minimise loss of injected cells. Once successful injections had been made, the end of a sterile narrow gauge silicon tube attached to a 21 gauge needle and 1ml syringe was placed within the thoracic cavity and the thorax was closed with simple interrupted 6/0 ethilon sutures encircling the ribs. The overlying muscle layers were closed with a single simple interrupted suture; air remaining in the thorax was gently aspirated via the chest drain and the skin closed with two surgical staples. 500µl of sterile saline warmed to 37°C was injected (SC) and the isoflurane supply turned off. Animals were placed under a heat lamp and were allowed to recover on oxygen until a gagging or righting reflex was evident at which point they were extubated and returned to their cages. The entire surgical time from induction of anaesthesia to recovery varied from 45 min to 90 min depending on surgical complications and individual recovery times. The morning after surgery the animals were checked and weighed and a second 0.02mg/kg dose of buprenorphine was administered. 50µl of tail tip blood was obtained and mixed with 50µl of 3.2% sodium citrate buffer in a 500µl Eppendorf tube. This was stored at 4°C for no longer than 4 hr before being centrifuged at 5000rpm at 4°C for 5 min. The plasma was then aspirated and placed in a fresh Eppendorf tube before being immediately frozen at -80°C. 24 hour post-surgery serum troponin I levels were assayed in order to assess the size of the initial myocardial injury and exclude animals with extremely large or small infarcts. Serum troponin I concentrations were measured using an ELISA kit (Life Diagnostics) as per the manufacturer's instructions. Following surgery mice were checked daily by the animal house technicians and weighed weekly.

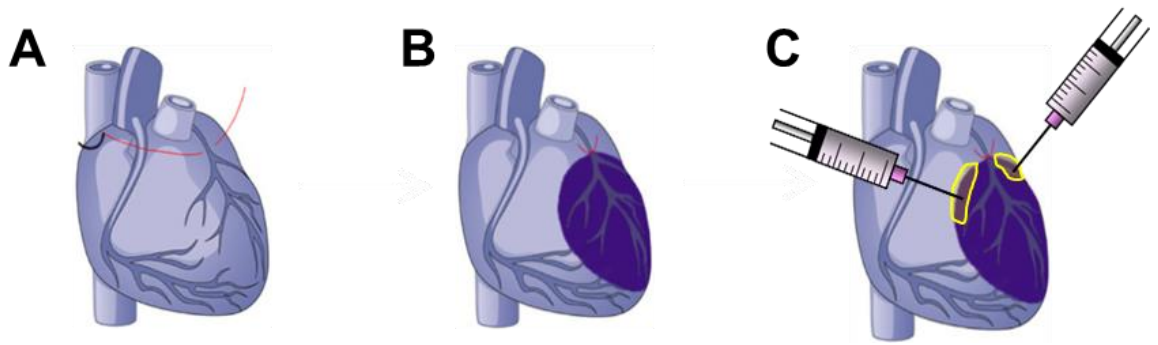


Figure 4-1 Schematic illustrating the surgical procedure to induce myocardial infarction and transplant cells. A – Following surgical exposure of the heart the LAD coronary artery is ligated. B – Occlusion of the blood supply induces regional blanching in the ischaemic myocardium. C – Once myocardial blanching is confirmed cells are injected into the border zone of the infarct in two sites either side of the ligature.

4.4.8 21 day ultrasound analysis

This was performed as described in 2.21.

4.4.9 Necropsy and tissue collection

This was performed as described in 2.2. Infarcts were macroscopically quantified at necropsy as absent, small, medium or large based on the area of myocardial pallor being absent, approximately less than 20%, 20 to 40% or greater than 40% of the total area of the left ventricle.

4.4.10 Measurement of apex to ligature distance

Following fixation in formalin, hearts were blotted dry and the distance between the apex and the ligatures measured in mm using a micrometre. Where two or more ligatures had been placed, the distance from the apex to the midpoint between the highest and lowest ligature was measured. Measurement of apex to ligature distance was performed blinded without knowledge of the animal treatment group.

4.4.11 Processing of samples for histologic assessment

Fixed hearts were embedded in a 1% agarose gel in a mouse brain matrix slicer (Zivic instruments). The gel held the tissue in position and allowed multiple transverse slices of the heart to be taken using a double side razor blade. Slices began at the ligature and were taken at 2mm intervals up to the apex resulting in 5 to 6 slices per heart (Figure 4-2). These were then embedded in 1% agarose gel in a cryo mold (Tissue Tek, VWR) to prevent the slice from curling before being processed and cut as detailed in 2.5. Sections were stained with Masson's trichrome for collagen or left unstained for later immunostaining. Masson's trichrome stained slides were reviewed and those in which a complete left ventricle (free wall and septum) was present were selected. From these a single comparable section at the level of the papillary muscles was chosen for histological assessment.

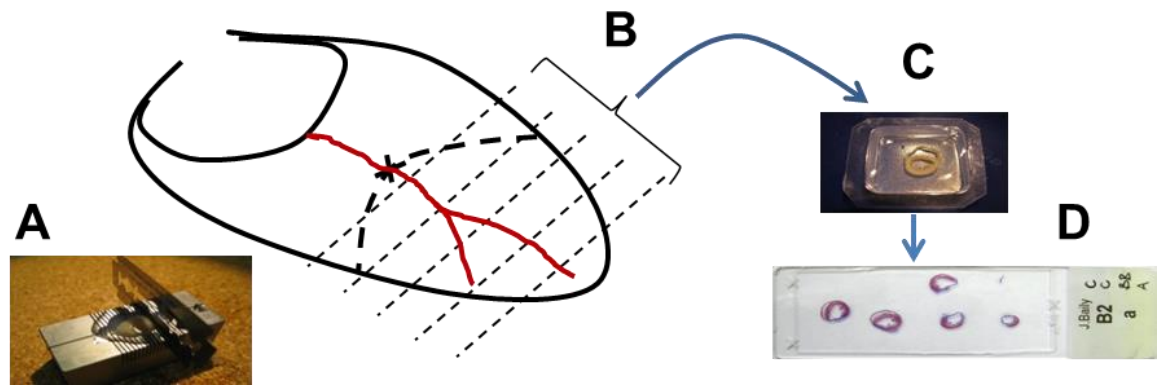


Figure 4-2 Schematic illustrating the processing of hearts to histology sections. **A** – hearts were embedded in 1% agarose gel in a matrix slicer. **B** – transverse slices were taken at approximately 2mm intervals. **C** – slices were then re-embedded in 1% agarose in a cryo mold. **D** – single Masson’s trichrome stained sections were cut from each level.

4.4.12 Histological assessment of collagen content

The collagen component of the scar was assessed histologically using the three techniques described in 2.22.

4.4.13 Measurement of wall thickness

Wall thickness of the left ventricular free wall was measured in mm at five points; one point at each of the two junctions with the inter-ventricular septum and three at equidistance between these two sites, separating the ventricular free wall into four equally size portions (Figure 4-3). The mean of these values was calculated.

4.4.14 Measurement of scar length

Infarct length was measured in mm by tracing a line at the mid depth of the infarct from either border. The limits of the infarct were determined as the points at which the infarct occupied 50% of the thickness of the myocardium (Figure 4-4).

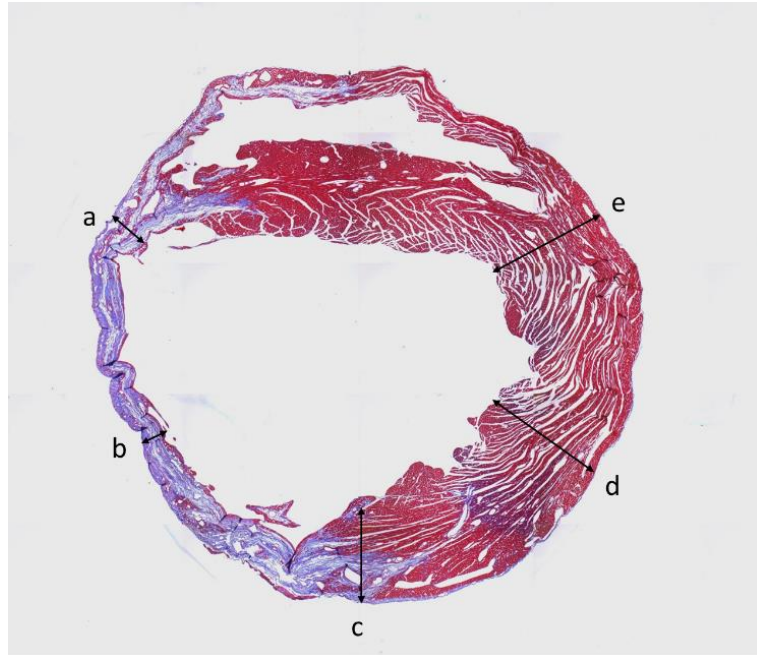


Figure 4-3 Measurement of wall thickness. Tiled brightfield image of a Masson's trichrome stained transverse heart section from an infarcted NOD/SCID mouse heart 21 days after coronary artery ligation. Thickness was measured at five locations in the left ventricular free wall (a to e) using Image J morphometric analysis software (NIH).

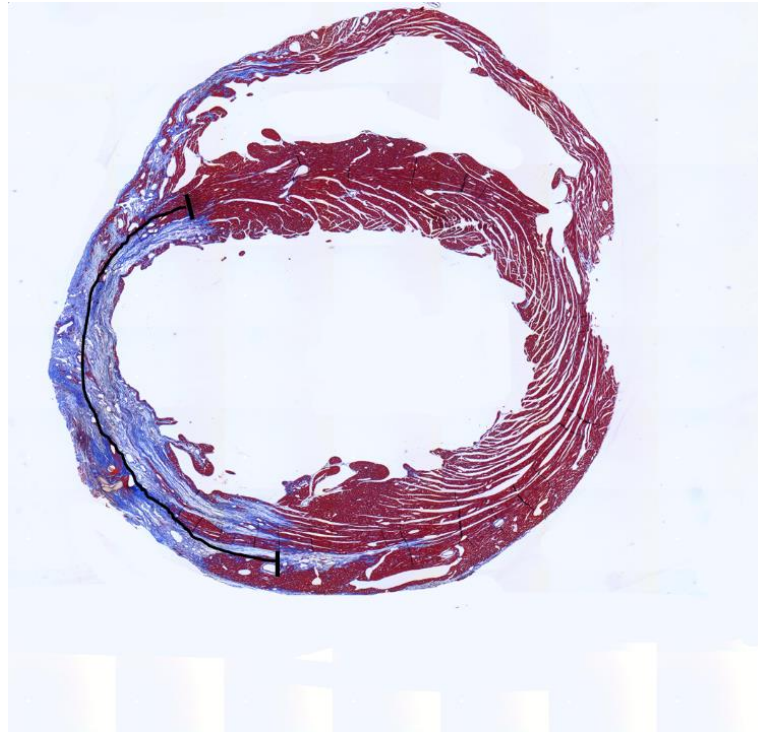


Figure 4-4 Measurement of infarct length. Tiled brightfield images of a Masson's trichrome stained transverse heart section from an infarcted NOD/SCID mouse heart 21 days after coronary artery ligation. Length was measured at mid depth of the left ventricular free wall using Image J morphometric analysis software (NIH). Measurements started and ended where collagen (blue) occupied approximately 50% of the wall thickness.

4.4.15 Staining and counting of microvessels

Sections were initially immunostained with anti-CD31 antibody (Abcam) using the DAB based protocols described in 2.6.2. Despite several modifications of protocol, including longer endogenous peroxidase quenching and greater primary antibody dilution, the level of background staining remained excessively high. Instead isolectin B4 was used to label vessels. Antibody details are provided in Table 4-1. Following antigen retrieval no peroxidase quenching was carried out before non-specific binding sites were blocked with 10% goat serum/PBS for 1 hour. Avidin block was applied for 15 min followed by washing and biotin block (Thermo Scientific) for 15 min. After washing sections were incubated with the isolectin antibody diluted in 5% goat serum/PBS for 2 hr at room temperature. After further washing Vectastain RTU ABC solution (Vector Labs) was incubated on the section for 30 min at room temperature. Sections were washed and DAB substrate incubation, haematoxylin staining, washing steps and coverslip application were carried out as described. Slides were examined using BX61 upright brightfield microscope and images were captured using a Q imaging Micropublisher 3.3RTV camera and Q Capture Pro imaging software (Figure 4-5). Three fields of view were randomly selected within the border zone on either side of the infarct giving a total of 6 fields of view captured. Fields were rejected if they contained greater than approximately 10% scar tissue and if greater than approximately 20% of the blood vessels were in oblique or longitudinal orientation. Microvessels less than 20µm diameter with a visible lumen were counted. All histological analysis of tissue sections was performed in a blinded manner.

4.4.16 Immunostaining and counting of injected cells

Virally transduced injected cells were identified in tissue sections via labelling for GFP with an anti-GFP antibody diluted in 1% bovine serum antigen or a specific anti-human nuclear antigen (ANA) antibody diluted in PBS. Antibody details are provided in Table 4-2. Both antibodies were incubated for 3 hr at room temperature. Nine pericyte injected hearts and 9 adventitial cell injected hearts were examined. Three transverse sections approximately 1000µm apart starting at the level of the ligature (and hence cell injection) were stained and examined for each heart.

Table 4-1 Details of the antibodies used in microvessel counting in mouse myocardium

Antibody	Type	Raised in	Company	Catalogue No.	Dilution
anti-CD31	polyclonal	rabbit	Abcam	Ab28364	1:100
Isolectin-B4	Biotin-XXconjugate	-	Life Technologies	I21411	1:100

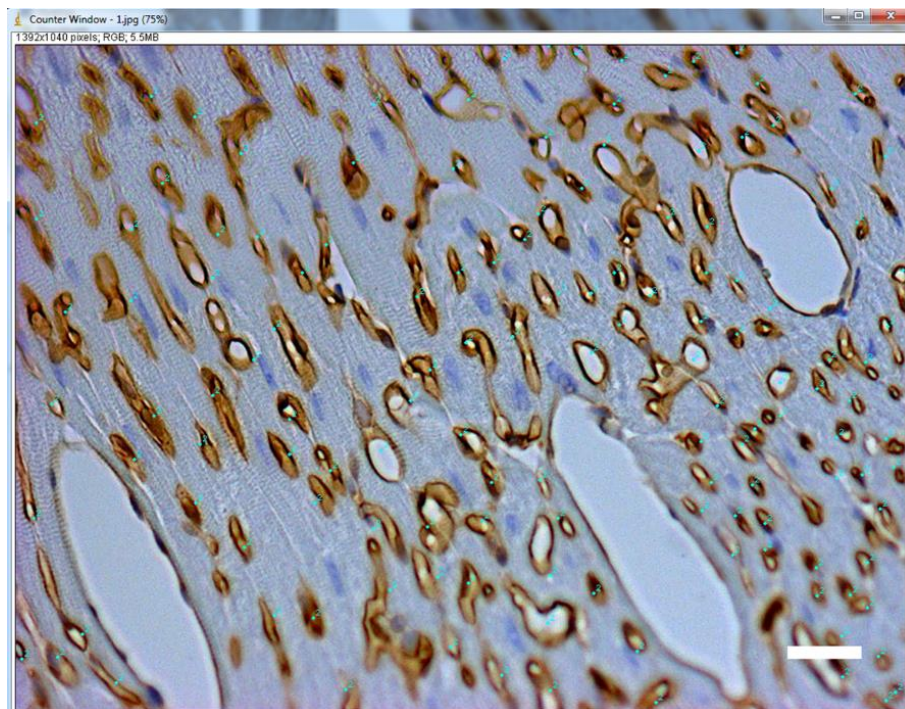


Figure 4-5 Screen grab of a representative image used for microvessel counting in the border zone myocardium of an infarcted NOD/SCID mouse heart 21 days after coronary artery ligation and cell transplantation. DAB staining with isolectin B4 antibody for endothelial cells highlights the endothelium of vessels in brown. Image J morphometric analysis software (NIH) was used to manually count vessels less than 20 μm diameter. Counted vessels were marked with a green dot. Scale bar = 20 μm .

Table 4-2 Details of the antibodies used in human transplanted cell tracking in mouse myocardium.

Antibody	Type	Raised in	Manufacturer	Catalogue No.	Dilution
anti-GFP	polyclonal	rabbit	Abcam	ab290	1:2000-1:2500
Anti-GFP	Monoclonal	Mouse	Abcam	ab1218	1:300
anti-HNA	monoclonal	mouse	Millipore	MAB1281	1:200
anti-Vimentin	monoclonal	rabbit	Abcam	Ab92547	1:100
AF 488	anti-rabbit	goat	Life Technologies	A11008	1:600
AF 555	anti-mouse	goat	Life Technologies	A21422	1:600

4.4.17 Pro-fibrotic marker analysis in cultured cells

The expression of fibroblast markers was investigated in three paired populations of pericytes and adventitial cells including both populations of cells expanded as potential candidates for transplantation (Table 4-3). Markers assessed were collagen 1 alpha 1 (Col1 α 1); Fibroblast Activated Protein alpha; Discoid Domain Receptor 2 (DDR2); alpha Smooth Muscle Actin (α -SMA). Gene expression was investigated via conventional RT-PCR as described in 2.12, 2.13 and 2.14. Primer details are provided in Table 4-4. Protein expression was investigated by immunostaining of cells as described in 2.17. Commercial human dermal fibroblasts (Life Technologies) from primary culture of neonatal foreskin were used as positive controls to validate fibroblast marker staining. Details of the antibodies used are provided in Table 4-5.

4.4.18 MSC and pericyte marker analysis in dermal fibroblast controls

The expression of MSC and pericyte markers in the dermal fibroblast control population was also investigated via conventional RT-PCR and immunostaining. MSC markers investigated were CD44, CD73, CD90 and CD105. Pericyte markers investigated were CD146, NG2, PDGFR- β and CD34. Primer details are provided in Table 3-4. Antibody details are provided in Table 3-1.

Table 4-3 Details of pericyte and adventitial cell populations investigated for expression of pro-fibrotic markers. Candidate populations for cell therapy study are in italics.

Population I.D.	Gestational age	Pericyte passage	Adventitial passage
<i>EDF 183</i>	<i>19 wks</i>	<i>P4+2</i>	<i>P3+3</i>
<i>EDF 195</i>	<i>20 wks</i>	<i>P3+4</i>	<i>P2+3</i>
EDF 232	18 wks	P4	P2

Table 4-4 Primer sequences used in conventional RT-PCR analysis of fibroblast associated marker genes.

Gene	Sequence	Product (bp)
Col1a1	f AGCCAGCAGATCGAGAACAT r TCTTGTCTTGGGGTTCTTG	250
FAP1 α	f GGTCTCCCAACAAAGGATGA r CATTGCCTGGAAATCCACTT	186
DDR2	f CAGCTTCCAGTCAGTGGTCA r AAATGGAGGGTGTGCAAGTC	148
α -SMA	f TGGCTATTCCTTCGTTACTA r CGATCCAGACAGAGTATTTGC	437
β -Actin	f CCT CGC CTT TGC CGA TCC r GGA ATC CTT CTG ACC CAT GC	204

Table 4-5 Details of the antibodies used to investigate fibroblast differentiation in human perivascular cells.

Antibody	Type	Raised in	Manufacturer	Catalogue No.	Dilution
Col1 α 1	Polyclonal	Rabbit	Abcam	ab122793	1:100
FAP α	Polyclonal	Rabbit	Abcam	ab28246	1:100
DDR2	Monoclonal	Mouse	R&D systems	MAB1438 1	1:100
α -SMA-FITC	Monoclonal	Mouse	Sigma Aldrich	F3777	1:100
Isotype IgG	-	Rabbit	Dako	X0903	1:10000 (Col1) 1:2000 (FAP)
Isotype IgG1	-	Mouse	Sigma Aldrich	M5284	1:25
Isotype IgG2a-FITC	-	Mouse	AbD Serotec	DC013	1:100

4.5 Results

4.5.1 Successful surgery can be carried out on NOD/SCID mice

In the pilot study 100% survival was observed and at post-mortem examination moderate to large left ventricular infarcts were present in 8 of the 10 animals.

4.5.2 Donor cell populations maintained pericyte and MSC marker expression throughout expansion

Both pericytes and adventitial cells from both donors were expanded in culture without problem. At cryopreservation pericytes and adventitial cells from both donor populations expressed mRNA of pericyte markers CD146, NG2 and PDGFR- β . Weak CD34 gene expression was also observed. MSC associated markers CD44, CD73, CD90 and CD105 were also consistently expressed (Figure 4-6). After 2 to 3 passages of expansion, just prior to injection, both populations from both donors continued to express pericyte markers CD146 and NG2 but lost PDGFR- β and CD34 expression. MSC associated markers continued to be consistently expressed and c-kit (not examined at cryopreservation) was expressed in both pericyte and adventitial populations from donor EDF197 but only in adventitial cells from EDF183 (Figure 4-7).

Given the greater expression of c-kit by EDF197 this population was selected as the donor for the injection study.

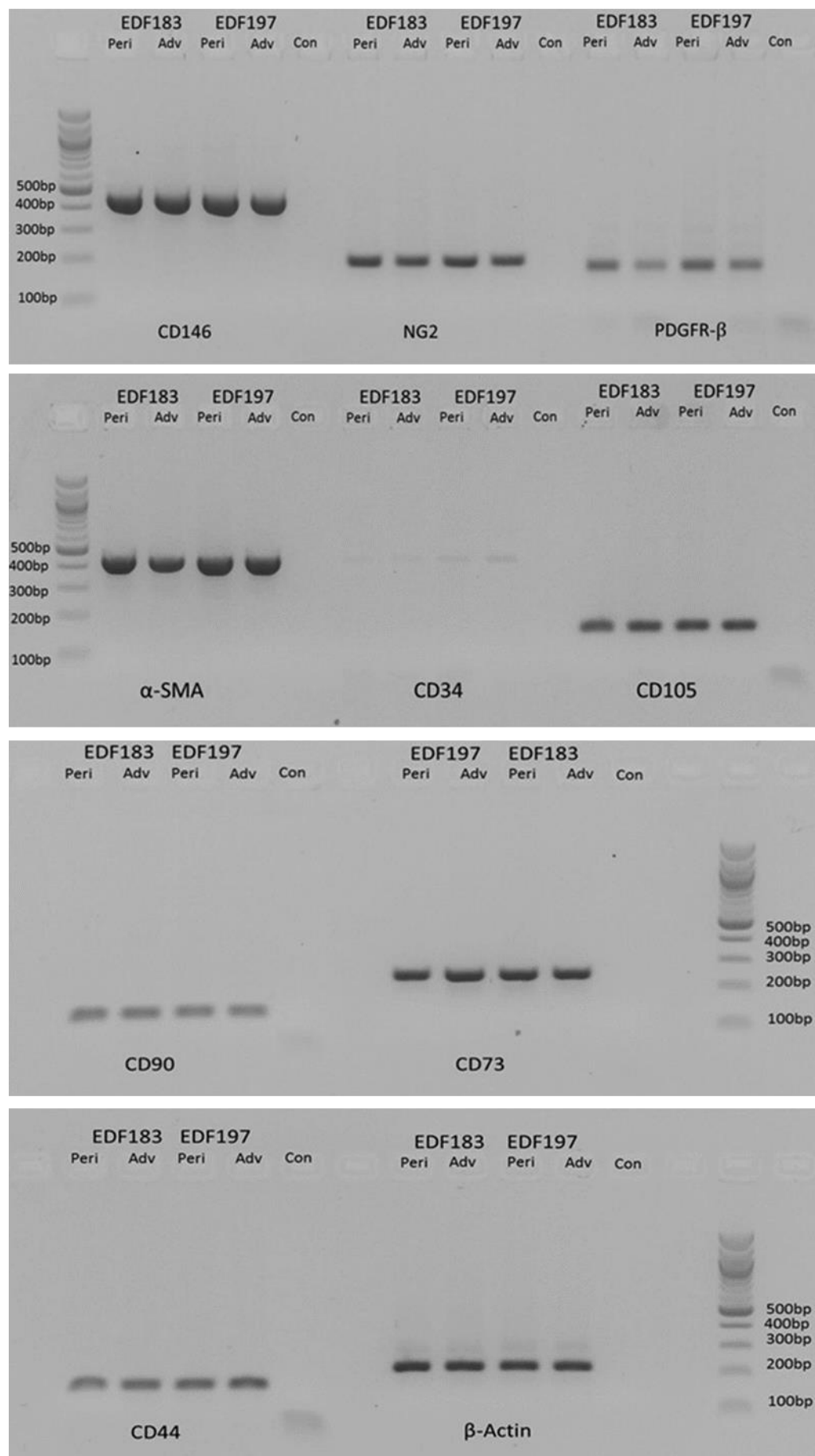


Figure 4-6 Conventional RT-PCR electrophoresis gel for pericyte and MSC marker genes in cell transplantation candidate populations of human foetal cardiac pericytes (peri) and adventitial cells (adv) from donors EDF183 and EDF197 at cryopreservation. Pericyte markers CD146, NG2, PDGFR- β and α -SMA were uniformly expressed by all populations. CD34 was weakly expressed by all populations. MSC markers CD44, CD73, CD90 and CD90 were expressed by all populations. Ladder bands are 100bp apart. Con = negative control.

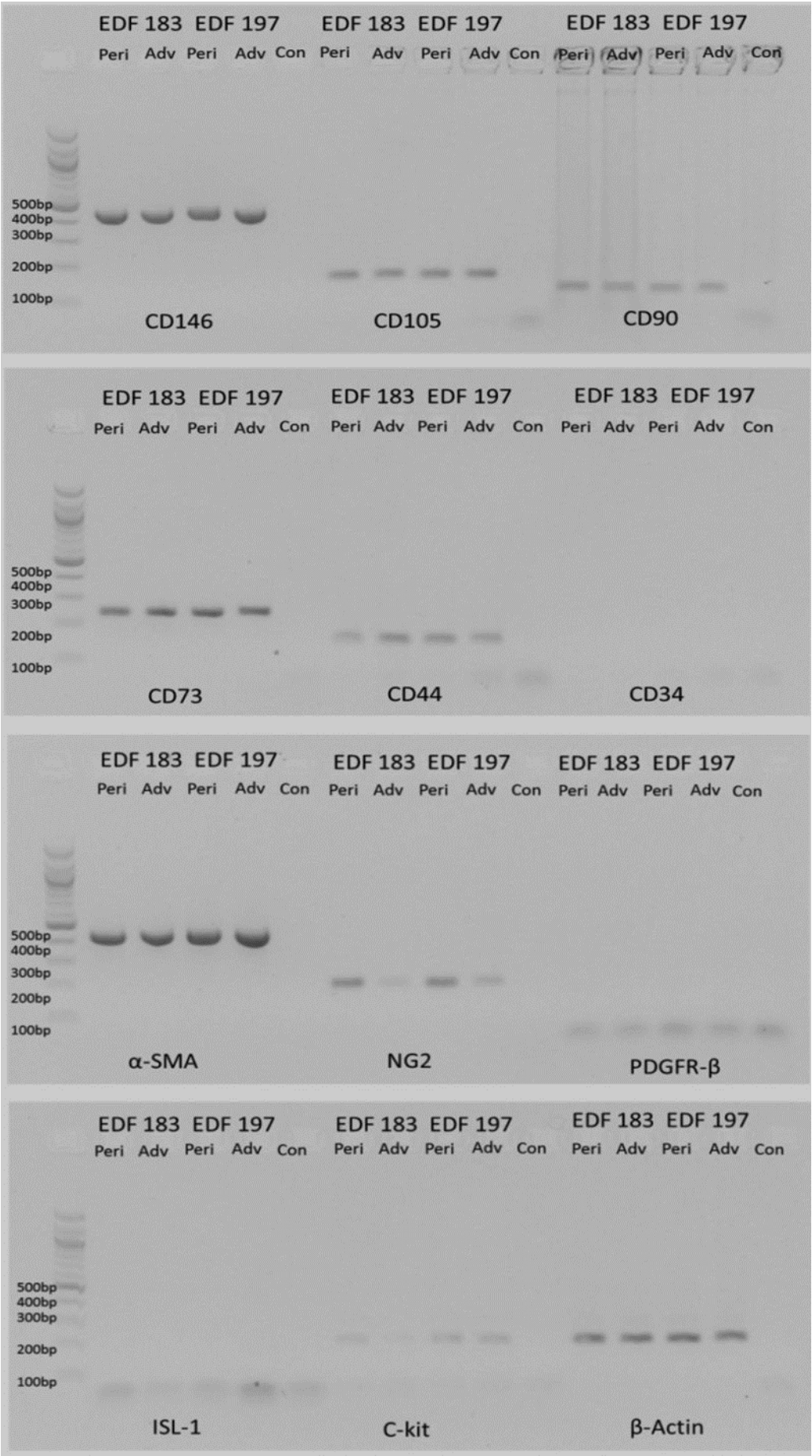


Figure 4-7 Conventional RT-PCR electrophoresis gel for pericyte and MSC marker genes in cell transplantation candidate populations of human foetal cardiac pericytes (peri) and adventitial cells (adv) from donors EDF183 and EDF197 at injection passage. There was uniform expression of pericyte markers CD146, NG2 and α -SMA by all populations. PDGFR- β and CD34 were no longer expressed by any of the populations. MSC markers CD44, CD73, CD90 and CD90 were expressed in all populations. Weak c-kit expression was present in all populations. Isl-1 was not expressed by any of the populations. Ladder bands are 100bp apart. Con = negative control.

4.5.3 Preparation and storage prior to injection results in approximately 20% loss of cells

Eighty one percent of pericytes and 100% adventitial cells were determined to be viable following trypsinisation and prior to loading of the syringe. This dropped to 63% and 78% respectively after a 4 hour delay indicating a loss of approximately 20% of live cells in each group.

4.5.4 The majority of surgical complications and peri-operative mortality result from cardiac arrhythmias

Of the 53 NOD/SCID mice used in the main study 32 (60%) survived until the 21 day endpoint and 28 had detectable infarcts on ultrasound. Seven animals died under anaesthesia and 14 were found dead or were euthanased within 7 days of surgery. No differences in survival were seen between the treatment groups (Figure 4-8). The Charles River NOD/SCID mice used in the final study were notably less stable under anaesthesia compared to C57/BL6 mice and the NOD/SCID Harlan mice used in the preliminary studies taking significantly longer to recover post operatively. Whereas C57/BL6 mice regained consciousness and mobility within 5 to 10 min of stopping isoflurane administration the NOD/SCID animals frequently required 20 to 50 min and supplemental oxygen. Intra-operative surgical complications are detailed in Table 4-6. These included: haemorrhage from cardiac puncture during ligation; fatal arrhythmias on injection of cells or vehicle; laryngospasm on intubation; pneumothorax on intubation; inadvertent extubation during surgery; and failure to induce infarction. Arrhythmias were the most common complication and more commonly seen in cell injected animals. These were evident during surgery as slowing and irregularity of the heart-beat, frequent development of ventricular fibrillation and cyanosis of the tongue and digits. Animals that died or were euthanased were briefly necropsied. Intra-operative fatal arrhythmia/fibrillation were also the most common cause of death/euthanasia during the study and were seen predominantly in cell treated groups (Table 4-7). Other deaths/euthanasia's were attributed to: thrombosis/emboli which presented as sudden onset head tilt or limb ischaemia; respiratory complications such as pneumothorax or inadvertent extubation; non-specific failure to recover from anaesthesia; and unexplained deaths

of unknown cause. Cardiac tissue from several of the animals that died of intra-operative arrhythmia/fibrillation was processed to histological sections and stained with anti-GFP antibody to reveal numerous cell emboli throughout the myocardium (Figure 4-9). Unlike in C57/Bl6 mice post-operative ventricular rupture was not seen.

Surgery was performed over a 4 week period and there was an apparent trend for increased survival this period suggesting an improvement in anaesthetic or surgical technique however statistical analysis failed to confirm this (Figure 4-10). To compare survival data between surgical weeks, Fisher's exact tests were performed using R.

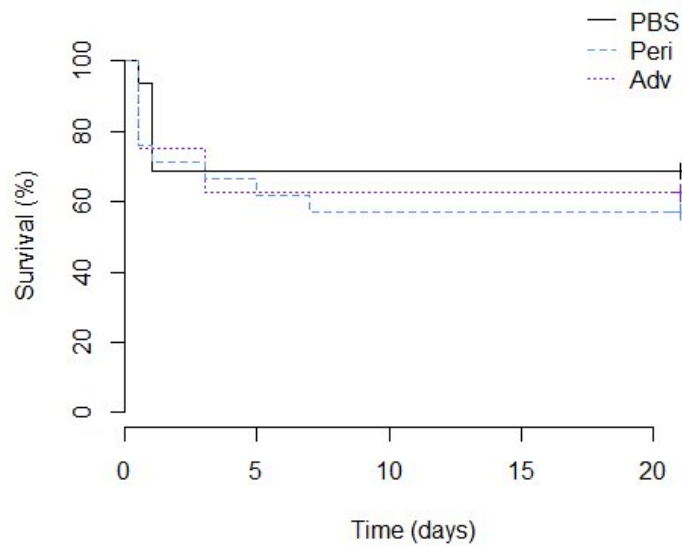


Figure 4-8 Kaplan-Meier survival curve illustrating cumulative survival of NOD/SCID mice up to 21 days following induction of myocardial infarction and transplantation of cells or PBS. Animals in the pericyte (peri) and adventitial cell (adv) treated groups exhibited slightly lower survival over 21 days compared to the PBS treated control animals. All losses in the PBS control group occurred within 2 days whereas losses in the cell treated groups continued up to 7 days post-surgery. PBS n=14, Peri n=22, Adv n=17.

Table 4-6 Intra-operative surgical complications of coronary artery ligation and cell transplantation in NOD/SCID mice according to treatment group. Values given are absolute numbers of mice and percentage of the total experimental cohort (53) in brackets. Animals in the “before injection” group developed complications before cells or vehicle were injected. Arrhythmias were the most common complication and were more prevalent in cell injected groups. PBS n=14, Peri n=22, Adv n=17.

Intra-operative complication	Vehicle control	Pericytes	Adventitial Cells	Before injection
Haemorrhage	1 (1.9%)	1 (1.9%)	-	-
Arrhythmia/fibrillation	2 (3.8%)	6 (11.3%)	4 (7.5%)	1 (1.9%)
Pneumothorax	1 (1.9%)	-	-	1 (1.9%)
Extubation	-	1 (1.9%)	1 (1.9%)	-
Laryngospasm	-	-	-	1 (1.9%)
Failure of infarction	2 (3.8%)	1 (1.9%)	1 (1.9%)	-

Table 4-7 Cause of death/euthanasia following coronary artery ligation and cell transplantation in NOD/SCID mice according to treatment group. Values given are absolute numbers of mice with percentage of total experimental cohort in brackets. Losses were highest in the pericyte treated group and cell treated groups had a higher incidence of death associated with arrhythmia/fibrillation. PBS n=14, Peri n=22, Adv n=17.

Cause of death	Vehicle control	Pericytes	Adventitial Cells	Before injection
Arrhythmia/fibrillation	-	3 (5.7%)	2 (3.8%)	-
Systemic thrombosis/emboli	1 (1.9%)	2 (3.8%)	1 (1.9%)	-
Respiratory complications	-	1 (1.9%)	1 (1.9%)	3 (5.7%)
Non-specific failure to recover	-	1 (1.9%)	-	-
Unknown	3 (5.7%)	3 (5.7%)	2 (3.8%)	-

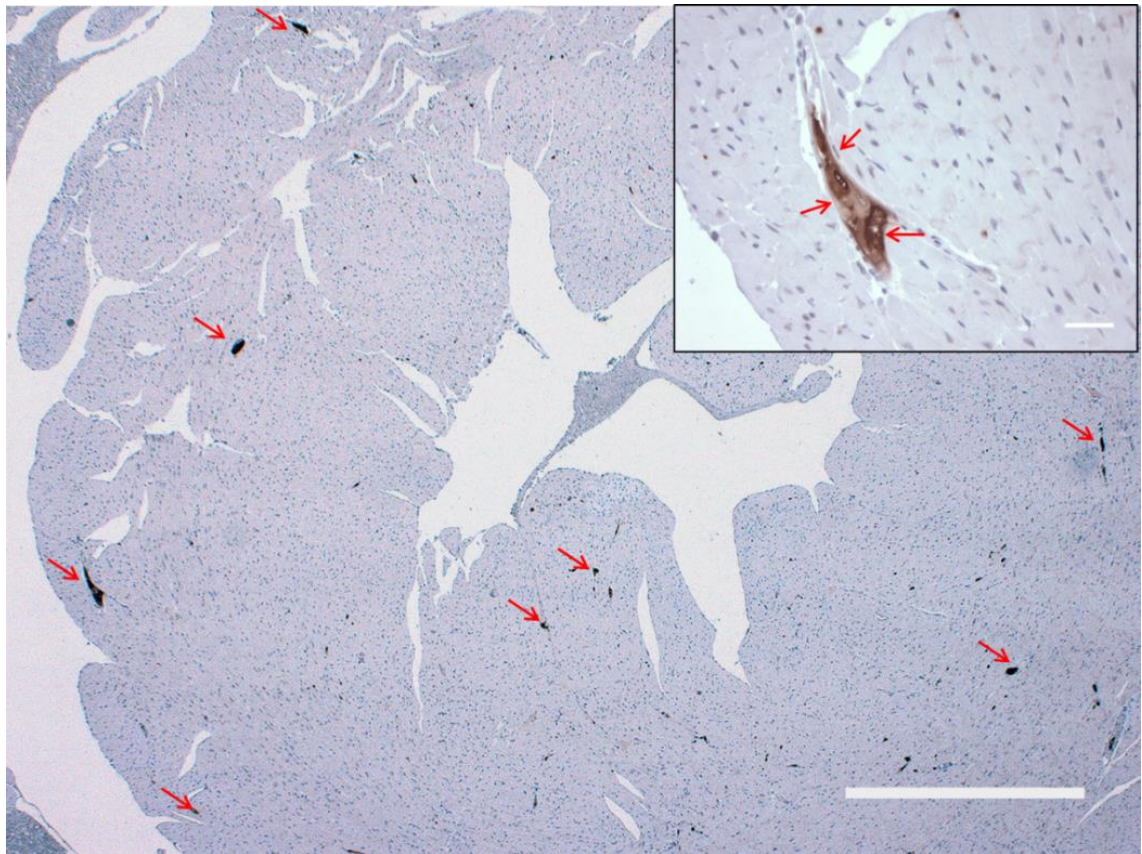


Figure 4-9 Brightfield image of the myocardium of a NOD/SCID mouse that died shortly after injection of pericytes DAB stained with anti-GFP antibody. GFP⁺ cells are stained brown and are highlighted with red arrows. Stem cell emboli brown staining GFP⁺ cells are present within the myocardial vessels. High power inset shows cluster of GFP⁺ cells within a vessel. Main image scale bar = 500μm. Inset image scale bar = 25μm.

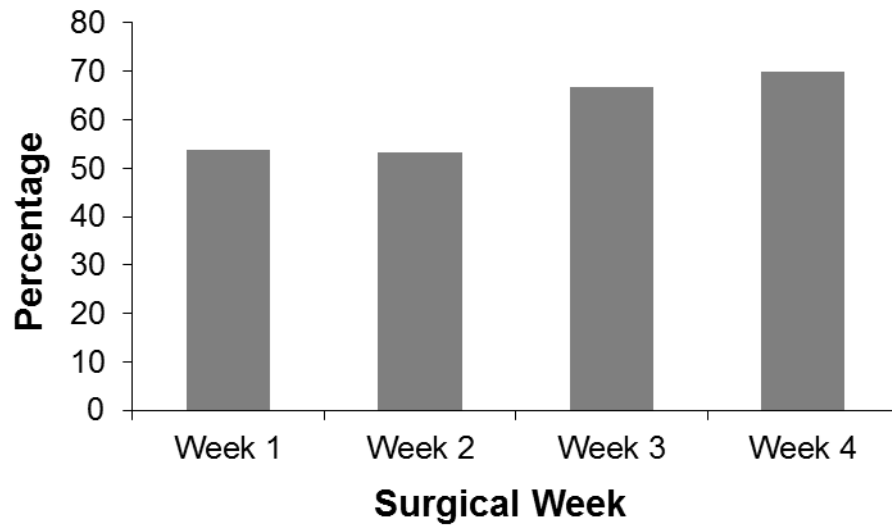


Figure 4-10 Survival of NOD/SCID mice undergoing coronary artery ligation surgery and cell transplantation according to the week of surgery. Columns represent percentage of mice undergoing surgery that week which survived to the 21 day endpoint. No statistically significant differences were found between surgical weeks. Week 1 n=13, week 2 n= 15, week 3 n=15, week 4 n =10. Fisher's exact tests.

4.5.5 Variability is present in the size of the initial myocardial injury

4.5.5.1 Mice were of similar body weight on the day of surgery

Mean body weights on the day of surgery for animals in each treatment groups were compared and no statistically significant differences ($p>0.05$) were noted (Figure 4-11).

4.5.5.2 Troponin I was not a suitable indicator of initial myocardial injury

In the first 25 animals subjected to surgery the initial infarct size was estimated and compared to 24 hr post infarction serum troponin I level with no association evident ($p>0.05$) (Figure 4-12).

4.5.5.3 Apex to ligature distance was a suitable indicator of initial myocardial injury size

Comparison of ligature distance to infarct size demonstrated a significant association between increased infarct size and increased ligature distance (large/small $p<0.001$; large/medium $p<0.01$) (Figure 4-13).

Analysis of ligature to apex distance revealed a decrease over the surgical period suggesting a reduction in the size of the initial injury (week 2/week 1 $p>0.05$; week 3/week 1 $p<0.001$; week 4/week 1 $p<0.001$; week 3/week 2 $p<0.01$; week 4/week 3 $p<0.001$) (Figure 4-14).

Surgical groups were randomised during the study and when examined as function of treatment there was however no significant difference ($p>0.05$) in mean ligature distance between groups (Figure 4-15).

4.5.6 Outliers representing extremes of initial myocardial injury were excluded using apex to ligature distance

To exclude extremes of myocardial injury and minimize variation in initial injury size between groups, animals in the cell treated groups with apex to ligature distances of greater than the upper (5.5mm) and lower (3.4mm) extremes noted in the PBS control group were excluded from the study. This resulted in the removal of

four animals from the pericyte treated group and three from the adventitial treated group (Table 4-8) leaving 7 in the PBS treated group, 7 in the pericyte treated group and 6 in the adventitial cell treated group.

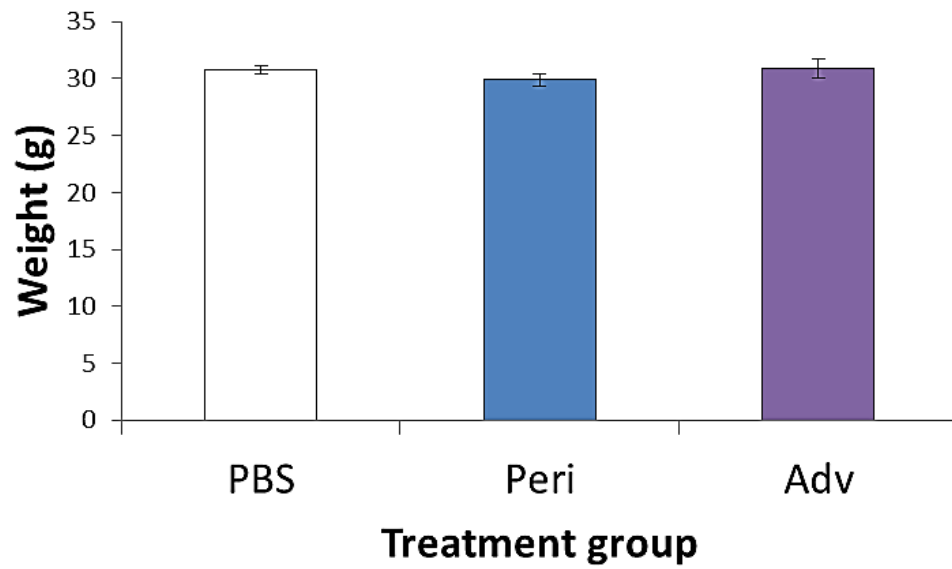


Figure 4-11 Mean body weights of NOD/SCID mice on the day of coronary artery ligation surgery in each of the treatment groups. No statistically significant differences were present between groups. Columns represent mean $\pm$ SEM. PBS n=14, Peri n=22, Adv n=17. One way ANOVA followed by Tukey's post hoc analysis.

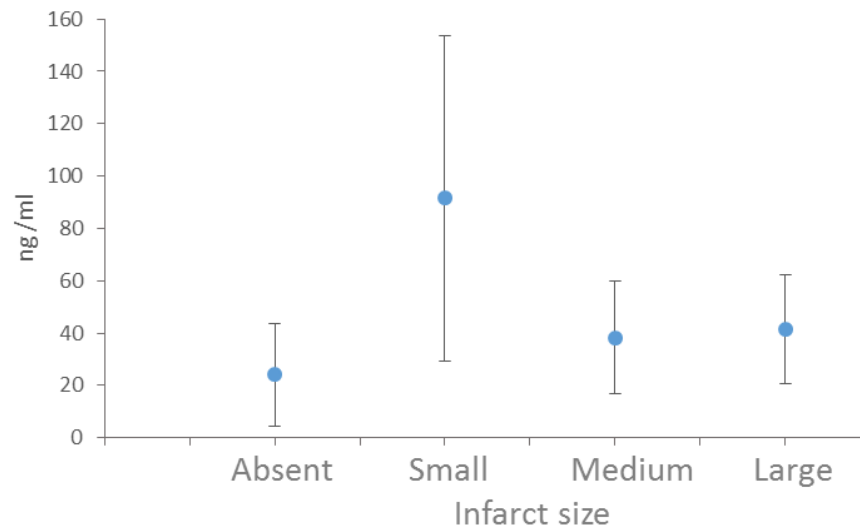


Figure 4-12 Estimated infarct size versus 24 hr serum Troponin I concentrations (ng/ml) in NOD/SCID subject to coronary artery ligation and PBS injection or cell transplantation. No association was evident between infarct size and serum troponin I concentrations. Blue dots represent mean $\pm$ SEM. Absent n=4, Small n=4, Medium n=6, Large n=11. One way ANOVA followed by Tukey's post hoc analysis.

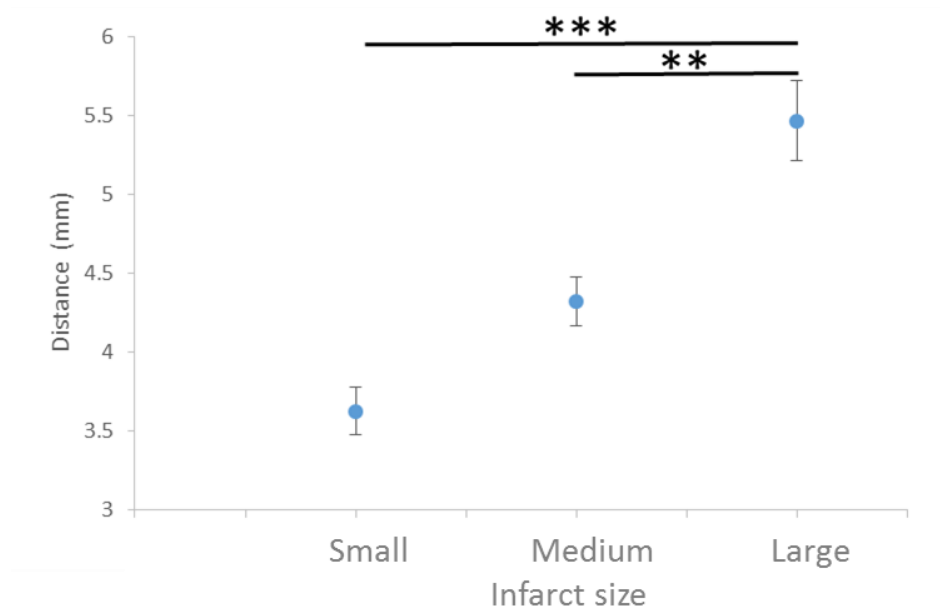


Figure 4-13 Estimated infarct size versus ligature to heart apex distance (mm) in PBS injection and cell transplantation study NOD/SCID mice 21 days after coronary artery ligation surgery. An increase in ligature distance was associated with an increase in macroscopic infarct size. Small n=8, medium n=9, large n=11. Blue dots represent mean $\pm$ SEM. * $p < 0.001$, ** $p < 0.01$. One way ANOVA followed by Tukey's post hoc analysis.**

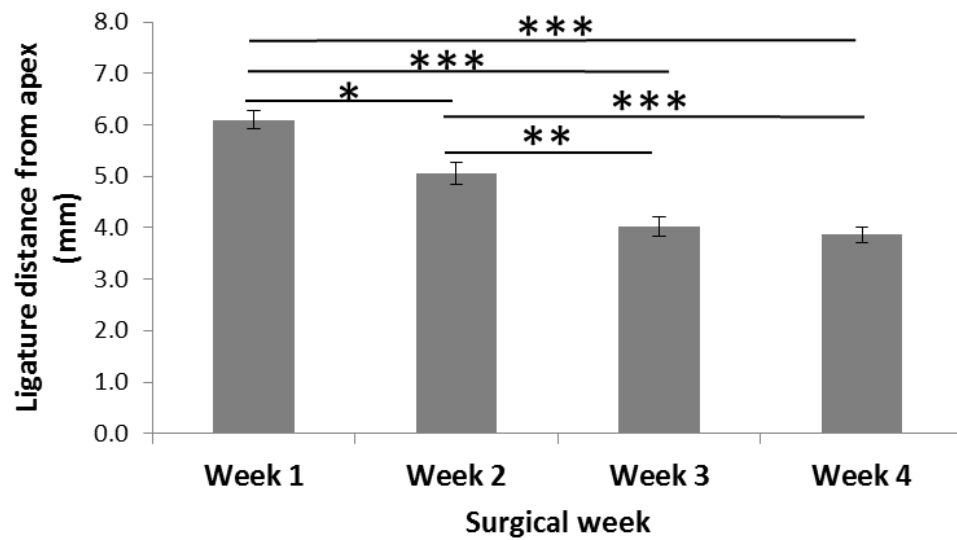


Figure 4-14 Mean ligature to apex distance (mm) according to week of coronary artery ligation surgery and PBS injection or cell transplantation in NOD/SCID mice. The distance ligatures were placed from the apex decreased with each week of surgery during the experimental period. Columns represent mean $\pm$ SEM. Week 1 n=6, week 2 n=6, week 3 n=10, week 4 n=6. * p <0.05, ** p <0.01, * p <0.001. One way ANOVA followed by Tukey's post hoc analysis.**

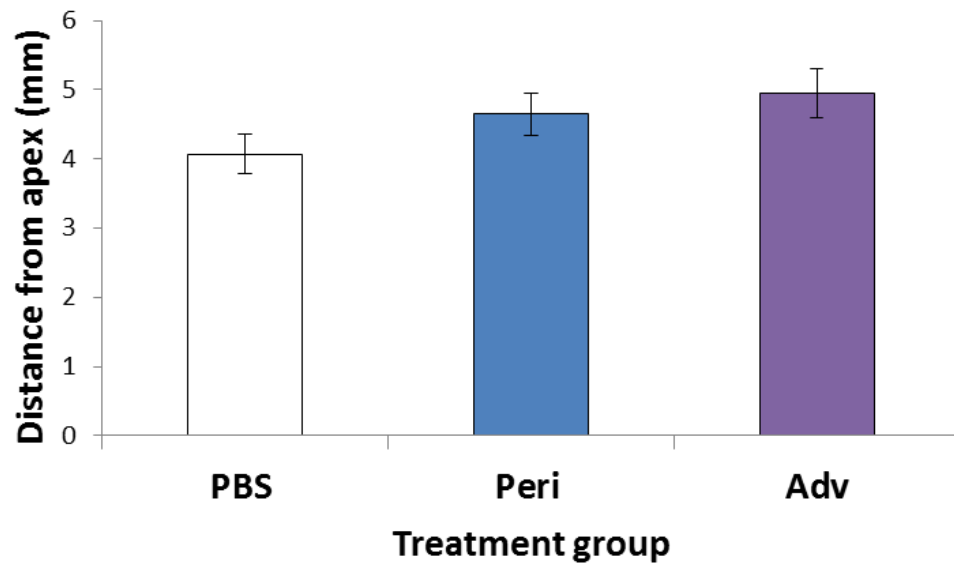


Figure 4-15 Mean ligature to apex distance (mm) according to PBS injection or cell transplantation treatment group in NOD/SCID mice subject to coronary artery ligation. No statistically significant differences detected in ligature distance between groups. Columns represent mean $\pm$ SEM. Vehicle control (PBS) n=7, Pericytes (Peri) n=11, Adventitial cells (Adv) n=9. One way ANOVA followed by Tukey's post hoc analysis.

Table 4-8 Exclusion of animals subject to extremes of initial myocardial injury based on ligature distance to apex. Individual ligature distances (mm) are presented for each animal in the three treatment groups. Animals with ligature distances in the grey shaded boxes were excluded from the study as these exceeded the upper and lower values in the PBS control group.

PBS	Pericytes	Adventitial cells
5.5	6.3	6.4
4.5	5.9	6.4
4.0	5.8	5.5
3.9	4.8	5.2
3.8	4.7	5.0
3.6	4.7	4.8
3.4	4.4	4.0
	4.1	4.0
	3.6	3.3
	3.5	
	3.3	

4.5.7 Transplantation of human cardiac perivascular cells into the infarcted heart results in reduced fractional shortening and ejection fraction

Complete 21 day high frequency ultrasound data is presented in Table 4-9. No differences were noted between groups with regards left ventricular diastolic or systolic endocardial area data (Figure 4-16, A & B).

The left ventricular endocardial area change (EAC) of the adventitial treated group was significantly reduced ($p<0.05$) compared to the pericyte treated group (Figure 4-16, C).

There was a significant reduction ($p<0.05$) in left ventricular fractional area change of the adventitial treated group compared to pericyte treated group (Figure 4-16, D).

Left ventricular fractional shortening was significantly reduced in both adventitial cell ($p<0.001$) and pericyte ($p<0.01$) treated groups compared to the PBS control (Figure 4-16, E).

There was a significant reduction in left ventricular ejection fraction in adventitial cell treated animals compared to PBS injected controls ($p<0.05$) and pericyte treated animals ($p<0.05$) (Figure 4-16, F).

Table 4-9 Complete 21 day high frequency ultrasound data for PBS control (n=7), pericyte (n=7) and adventitial cell (n=6) treated groups. EF = ejection fraction. EAC = endocardial area change. FAC = fractional area change. FS = fractional shortening. EAd = diastolic endocardial area. EAs = systolic endocardial area. CO = cardiac output.

Treatment Group	I.D.	EF (%)	EAC (mm)	FAC (mm)	FS (%)	EAd (mm ²)	EAs (mm ²)	CO (ml/min)
PBS	C3	42.90	8.02	28.82	26.04	27.84	19.82	16.26
PBS	M2	46.30	9.43	29.49	35.41	31.97	22.54	20.24
PBS	M3	54.26	10.34	34.52	31.74	29.95	19.61	22.65
PBS	M4	52.79	9.14	33.25	32.55	27.49	18.35	18.26
PBS	N2	56.51	9.65	37.67	42.96	25.61	15.97	16.40
PBS	N3	51.83	9.35	34.07	29.99	27.46	18.11	17.77
PBS	N4	43.25	7.53	25.59	27.40	29.43	21.90	17.37
Peri	H2	62.43	12.27	41.91	18.38	29.28	17.01	25.54
Peri	H3	52.52	9.50	33.45	19.27	28.42	18.91	22.14
Peri	I1	35.17	6.73	22.09	13.53	30.49	23.76	16.84
Peri	J1	49.95	7.60	33.55	26.00	22.65	15.05	13.48
Peri	J2	35.97	8.55	25.34	12.31	33.74	25.19	18.96
Peri	J3	62.27	12.02	45.19	16.93	26.59	14.57	22.69
Peri	L1	56.47	9.58	37.67	34.87	25.43	15.85	19.75
Adv	E2	37.75	8.12	27.93	13.09	29.06	20.95	14.82
Adv	F3	38.12	6.61	23.83	19.32	27.74	21.13	14.76
Adv	F4	23.23	5.27	13.75	4.31	38.33	33.06	17.96
Adv	I2	49.60	8.05	32.50	13.70	24.77	16.72	16.92
Adv	I3	36.11	7.02	21.32	8.22	32.94	25.92	20.77
Adv	K2	33.39	6.69	21.28	6.32	31.44	24.75	15.83

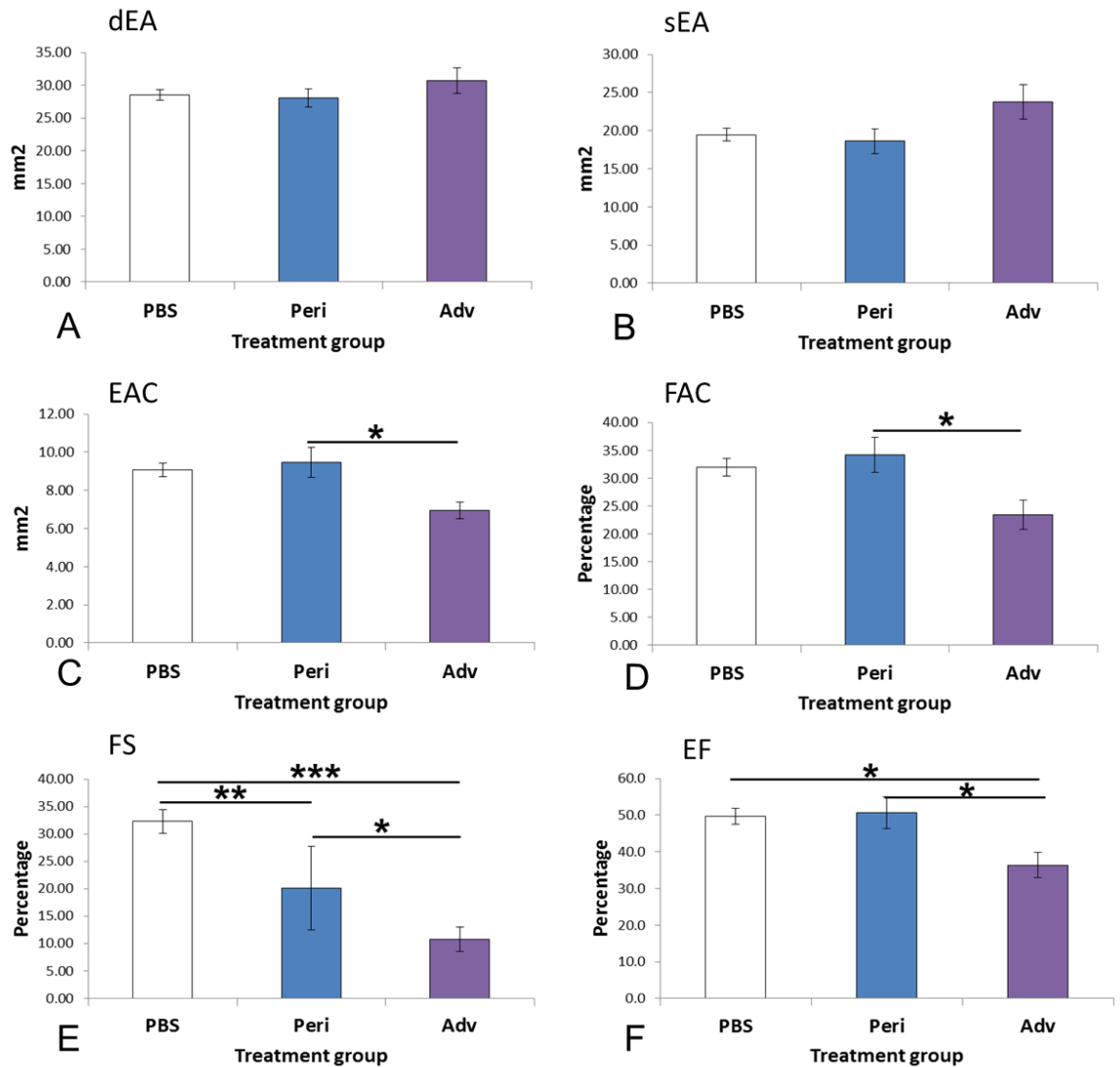


Figure 4-16 High frequency ultrasound assessment of the cardiac function of NOD/SCID mice 21 days after coronary artery ligation and PBS injection or cell transplantation. No differences were detected between groups in A - Diastolic endocardial area (dEA) or B - systolic endocardial area (sEA). C - Endocardial area change (EAC) and D - Fractional area change (FAC) were reduced in adventitial treated animals compared with pericyte treated animals. E -Fractional shortening (FS) was reduced in both cell treated groups compared to PBS treated controls and between adventitial and pericyte treated animals. F - Ejection fraction (EF) was reduced in adventitial treated animals compared to both PBS treated controls and pericyte treated animals. Columns represent mean $\pm$ SEM. PBS n =7, Peri n=7, Adv n=6. *p<0.01, **p<0.05, *p<0.05. One way ANOVA followed by Tukey's post hoc analysis.**

4.5.8 Human cardiac perivascular cell transplantation does not appear to have a beneficial effect on cardiac morphology post infarction

Quantifying the collagen area via the region of interest method revealed a significant increase ($p < 0.05$) in scar size in adventitial cell treated hearts when compared to PBS controls. (Figure 4-17, A).

There was no difference ($p > 0.05$) between groups in colour threshold analysis of scar area, colour picker analysis of scar area, left ventricular wall thickness, absolute scar length or scar length as a percentage of the circumference of the ventricular free wall (Figure 4-17, B to E).

4.5.9 Human cardiac perivascular cell transplantation does to appear to improve angiogenesis post infarction

Isolectin B4 staining revealed high microvascular density in all treatment groups, however no differences ($p > 0.05$) were detected between groups (Figure 4-18)

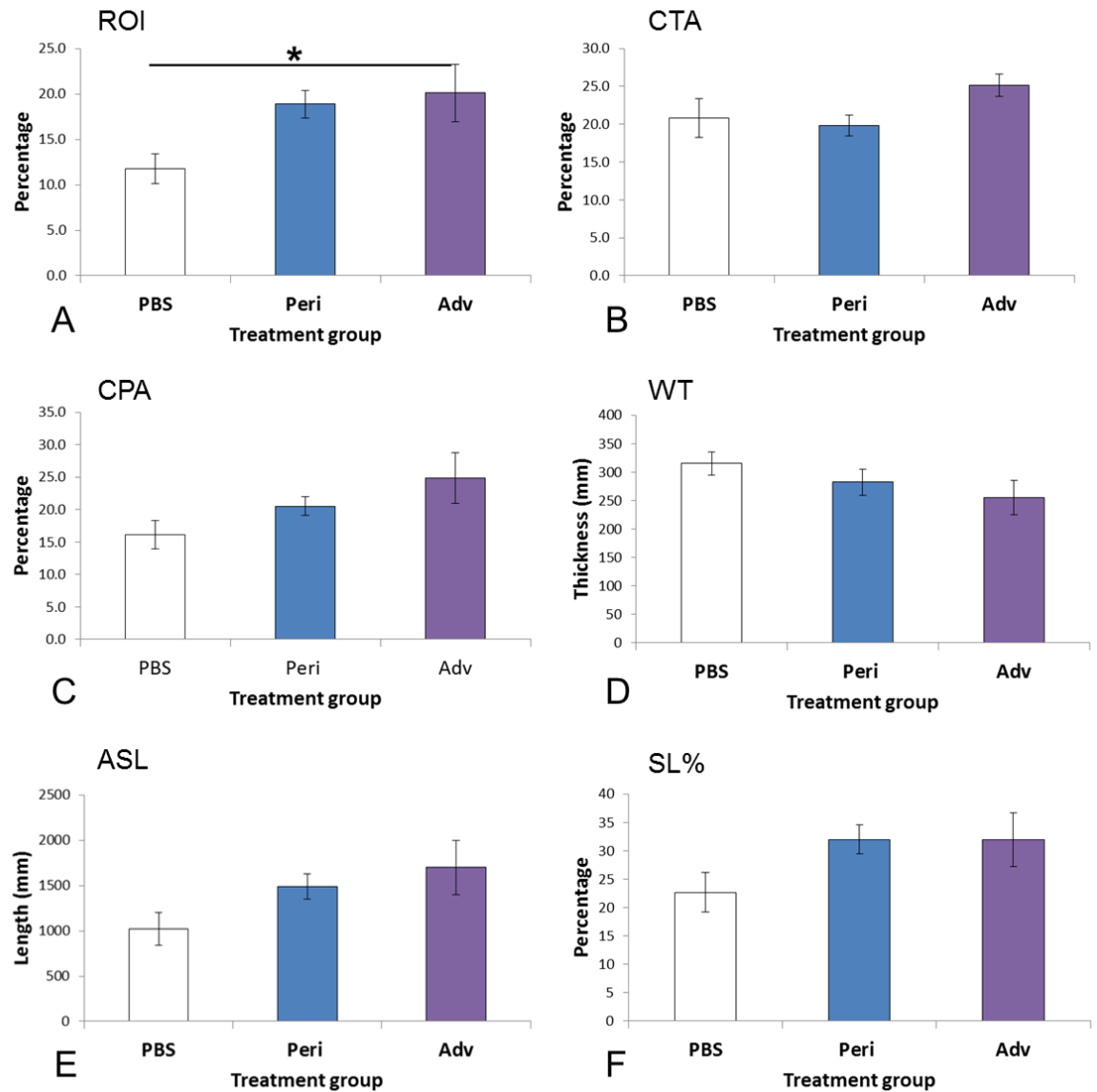


Figure 4-17 Morphological assessment of cardiac scar histology of NOD/SCID mice 21 days after coronary artery ligation and PBS injection or cell transplantation. A – Region of interest (ROI) was increased in adventitial treated animals compared to PBS treated controls. No differences were detected between treatment groups for B - Colour threshold analysis (CTA), C - colour picker analysis (CPA), D - wall thickness (WT), E - absolute scar length (ASL) or F - scar length as percentage of ventricular diameter (SL%). Columns represent mean $\pm$ SEM. PBS n =7, Peri n=7, Adv n=6. *p<0.05. One way ANOVA followed by Tukey's post hoc analysis.

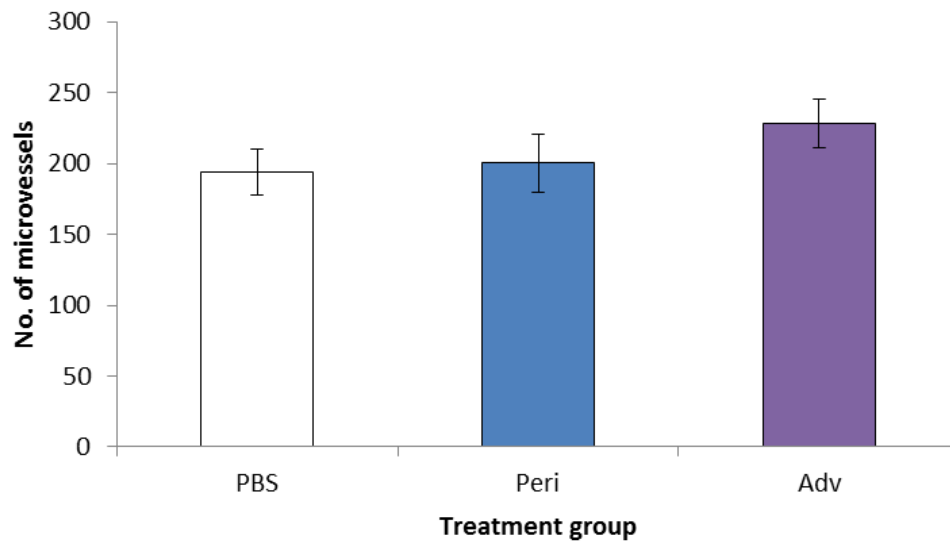


Figure 4-18 Microvessel count in the myocardial border zone of NOD/SCID mice 21 days after coronary artery ligation and PBS injection or cell transplantation. Microvessels less than 20 μ m diameter were counted in six x200 magnification fields in the border zone either side of the infarct and represented the mean per field. No differences detected between treatment groups. Columns represent mean $\pm$ SEM. PBS n =7, Peri n=7, Adv n=6. One way ANOVA followed by Tukey's post hoc analysis.

4.5.10 Transplanted human cardiac perivascular cells adopt several different distinct phenotypes in the infarcted myocardium

Overall the numbers of GFP⁺ cells retained at 21 days was both small and highly variable between hearts. Of the 9 pericyte injected hearts examined only two were found to contain cells with 32 and 36 cells detected in each. Cells were more abundant in adventitial cell injected hearts with 4 of 9 hearts containing between 61 and 244 GFP⁺ cells each. Of all GFP⁺ cells detected 88% were in the adventitial cell treated group compared to 12% in the pericyte treated group (Figure 4-19).

The GFP signal was detected in cells of three distinct morphological appearances. Cells of fibroblast morphology were present aligned in parallel and in clusters within a pauci-cellular matrix. These medium size spindle shaped cells were evident within the scar region or border zone and often in a sub-epicardial location. Anti-human nuclear antigen staining confirmed them as human origin with a single oval nucleus (Figure 4-20) and they also stained positively with vimentin, a fibroblast marker (Figure 4-21).

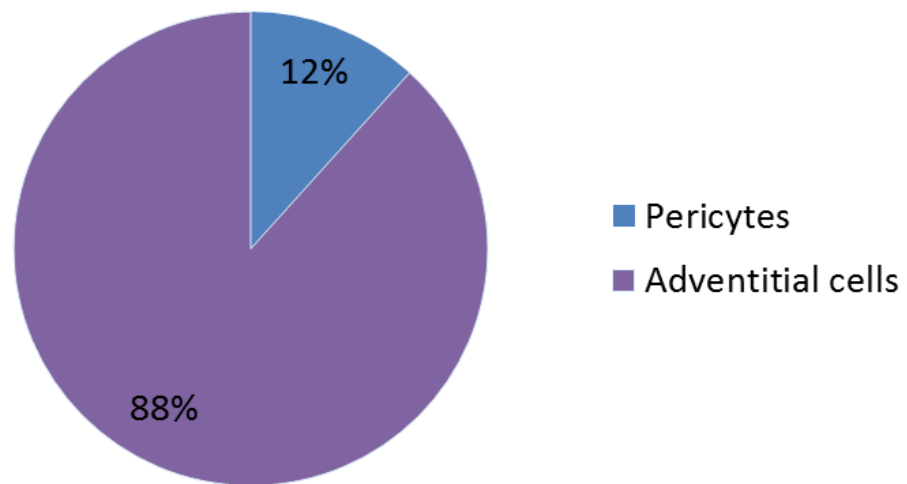


Figure 4-19 Detection of virally transduced eGFP⁺ pericytes and adventitial cells in the myocardium of NOD/SCID mice 21 days after coronary artery ligation and cell transplantation. 88% of all retained GFP⁺ cells were of adventitial cell origin compared to 12% of pericyte origin. Three transverse sections were DAB stained with anti-GFP antibody and examined per heart. Peri n=9, Adv n=9

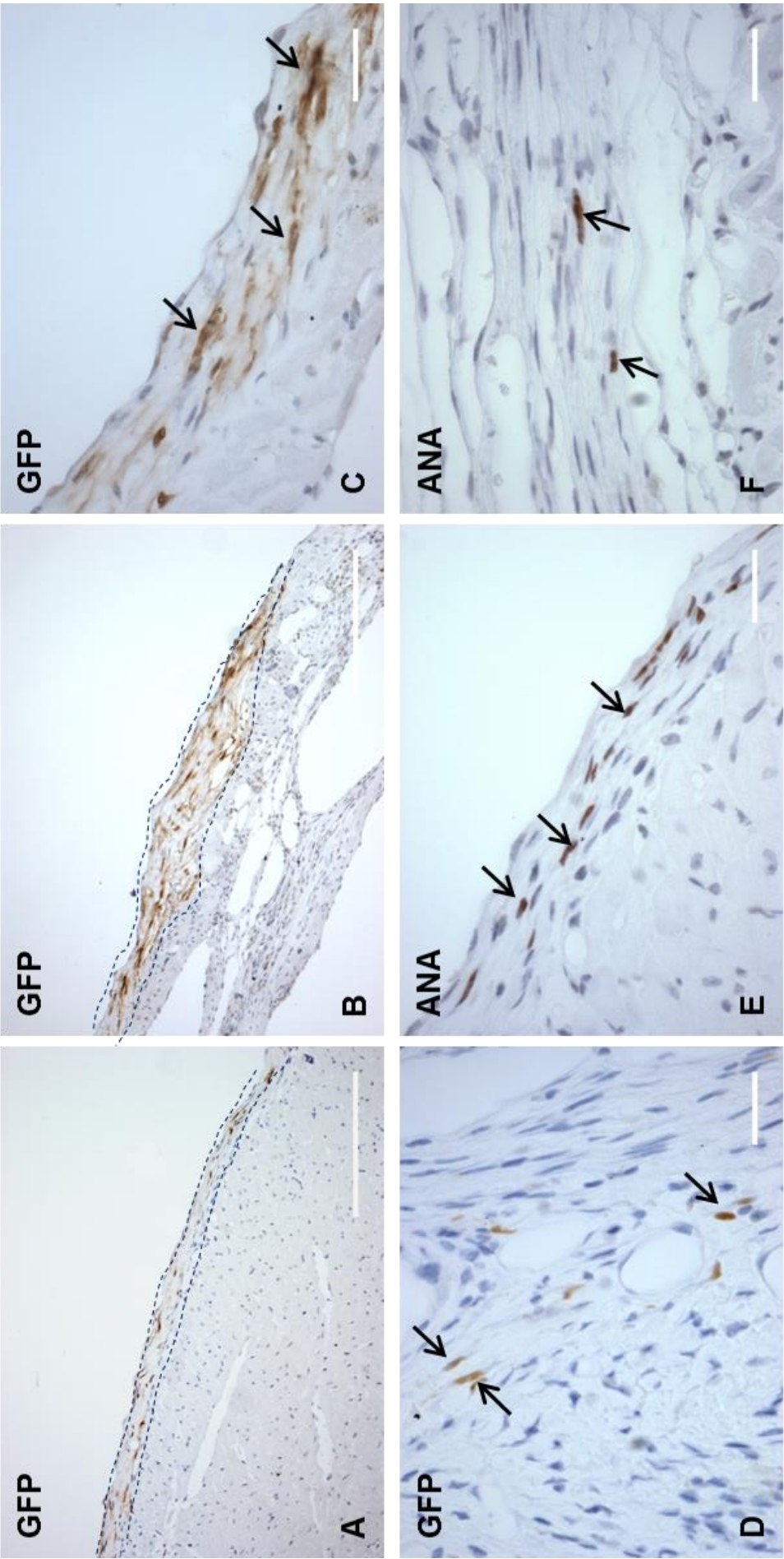


Figure 4-20 Representative brightfield images of myocardium from NOD/SCID mice 21 days after coronary artery ligation and cell transplantation DAB stained with anti-eGFP antibody and illustrating fibroblast morphology of injected cells. Green fluorescent protein (GFP) and human anti-nuclear antigen (ANA) positive cells are labelled brown. A & B - GFP⁺ cells form a sub-epicardial layer highlighted between dashed blue lines. C & D – GFP⁺ cells (black arrows) are spindle shape with parallel orientation. E & F ANA⁺ nuclei (black arrows) are oval and single conforming to a fibroblast morphology. A & B scale bars = 50µm. All other scale bars = 25µm. Peri n=9, Adv n=9.

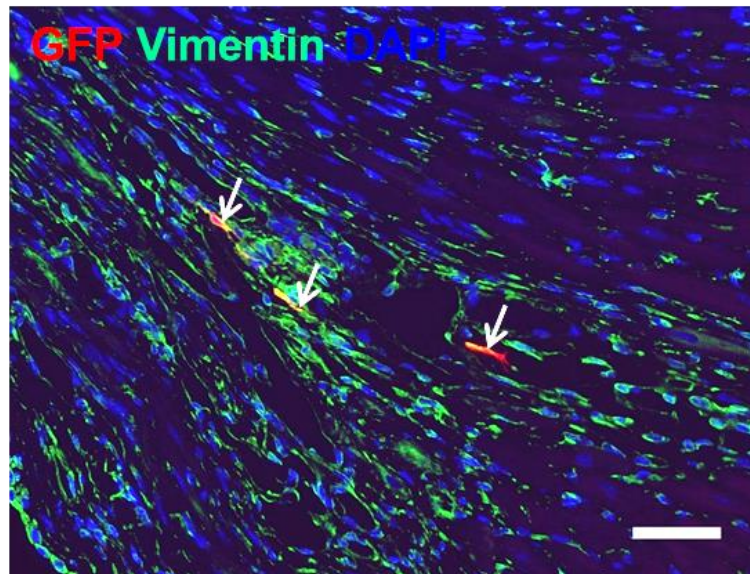


Figure 4-21. Representative immunofluorescence image of anti-GFP and anti-vimentin stained myocardium in NOD/SCID mouse 21 days after coronary artery ligation and cell transplantation. Co-expression of GFP and fibroblast marker vimentin in injected cells (white arrows) located within fibrous scar containing abundant vimentin⁺ fibroblasts. DAPI nuclear stain in blue. Scale bar = 50µm.

Cells of cardiomyocyte like morphology were present in the border regions and within the adjacent uninjured myocardium. These cells were large and present individually rather than as clusters. They were strap shaped to round depending on whether they were sectioned longitudinally/obliquely or in cross section and clear cross striations could be seen (Figure 4-22). Each cell contained a single nucleus and anti-human nuclear antigen staining was only detected in one nucleus per cell suggesting true differentiation rather than fusion with a host cardiomyocyte (Figure 4-23).

The third and final GFP positive cell morphology was macrophage-like. These medium size round cells were present in dense clusters within the scar tissue and contained moderately abundant granular GFP positive cytoplasm and an eccentric nucleus. ANA nuclear staining was negative in this population with only uptake of stain within the granular cytoplasmic contents (Figure 4-24).

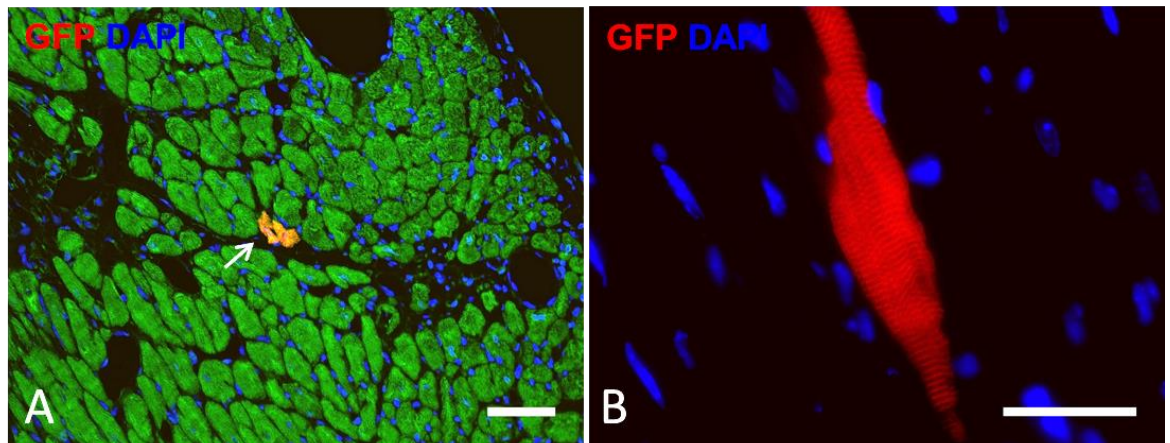


Figure 4-22 Representative immunofluorescence image of anti-GFP stained myocardium in NOD/SCID mice 21 days after coronary artery ligation illustrating cardiomyocyte morphology of injected cells. A – Transverse section of GFP⁺ cell with cardiomyocyte morphology within healthy myocardium autofluorescing in green. B – Oblique section of GFP⁺ cell with cardiomyocyte morphology with prominent cross striations. Scale bars = 50µm.

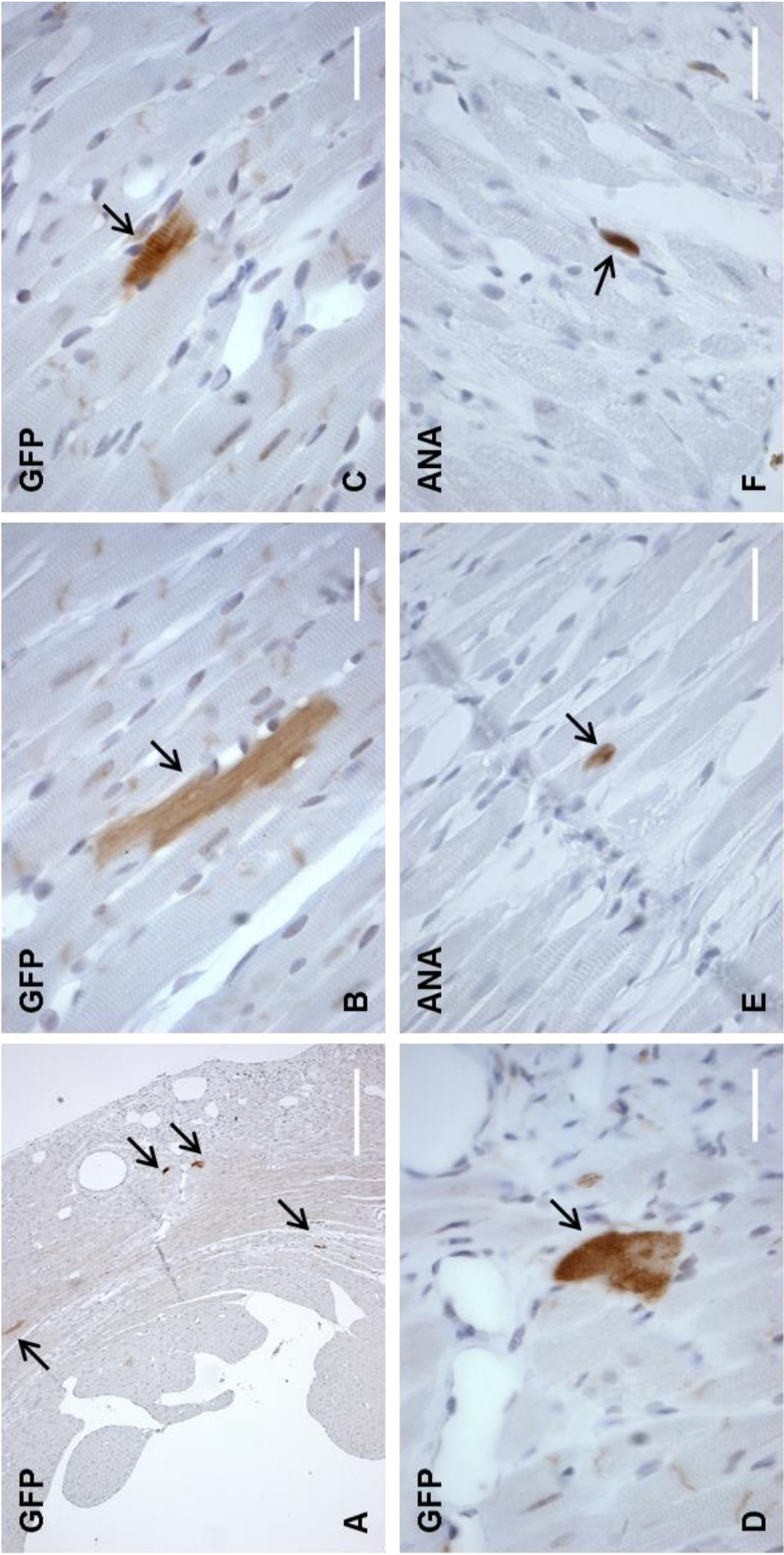


Figure 4-23 Brightfield image of myocardium from NOD/SCID mice 21 days after coronary artery ligation and cell transplantation DAB stained with anti-eGFP antibody illustrating cardiomyocyte morphology of injected cells. Green fluorescent protein (GFP) and human anti-nuclear antigen (ANA) positive cells are labelled brown. A – Low numbers of scattered GFP⁺ cells (black arrows) are present within the myocardium of the border zone. B to D – Longitudinal oblique and transverse sections of GFP⁺ cells (black arrows) with a cardiomyocyte phenotype. E & F – Human anti-nuclear antigen (ANA) staining labels single nuclei within cells of cardiomyocyte morphology. Figure A - scale bar = 500µm. All other scale bars = 50µm.

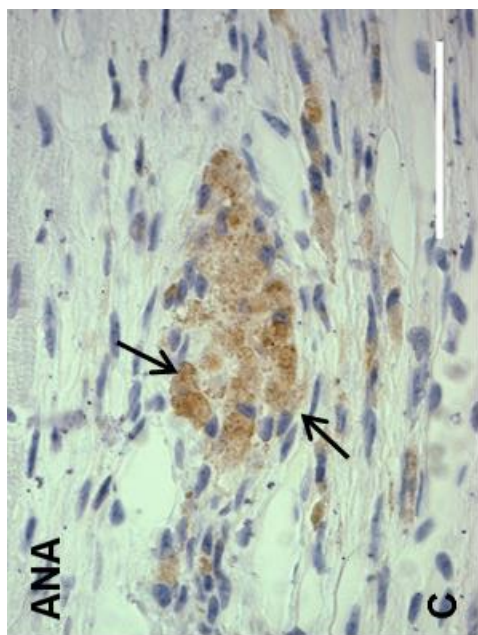
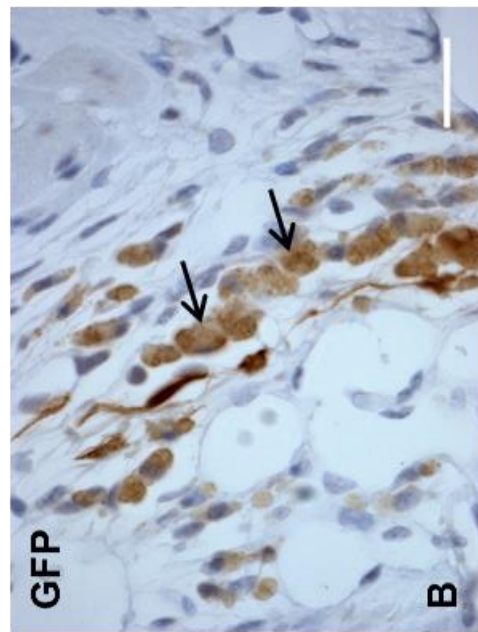
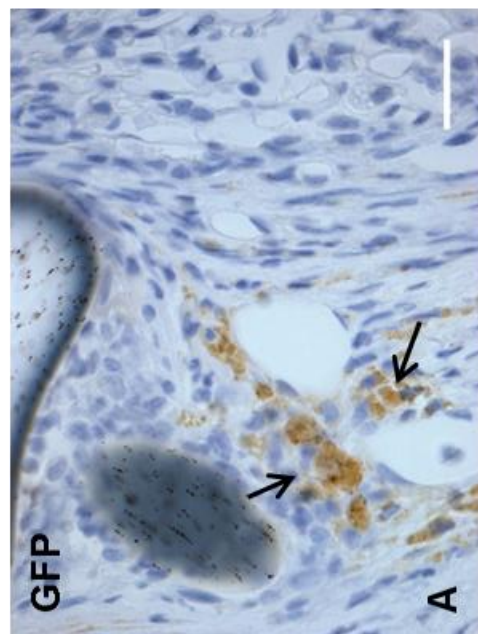


Figure 4-24 Brightfield image of myocardium from NOD/SCID mice 21 days after coronary artery ligation and cell transplantation DAB stained with anti-eGFP antibody and illustrating macrophage morphology of injected cells. Green fluorescent protein (GFP) and human anti-nuclear antigen (ANA) positive cells are labelled brown. A & B - Clusters of GFP⁺ cells (black arrows) are present within the myocardium. C - Human anti-nuclear antigen (ANA) labels the cytoplasm (black arrows) but not nuclei of clusters of cells within the myocardium. All scale bars = 50µm.

Cell counts revealed that injected cells most commonly adopted a fibroblast phenotype with 55% of all GFP⁺ cells displaying this morphology. The next most common morphology was the macrophage type (41%) whilst a cardiomyocyte morphology was seen in only 4% of GFP⁺ cells (Figure 4-25).

When each of the injected populations was examined individually it is evident that the majority of both pericytes and adventitial cells favour a fibroblast type morphology and in similar proportions (50% of all GFP⁺ pericytes versus 56% of all GFP⁺ adventitial cells). In contrast the cardiomyocyte differentiation capacity was higher in pericytes compared to adventitial cells (13% of all GFP⁺ pericytes versus 3% of all GFP⁺ adventitial cells) (Figure 4-26, Figure 4-27).

4.5.11 Human cardiac perivascular cells express pro-fibrotic markers in culture

Conventional RT-PCR revealed expression of the profibrotic genes collagen I alpha, fibroblast activated protein α , discoid domain receptor and α -SMA in both pericytes and adventitial cells from all three donors (Figure 4-28). Immunocytochemistry confirmed protein expression of these markers in both cultured pericytes and adventitial cells from all donors (Figure 4-29). Collagen I alpha expression was restricted to a subset of cells.

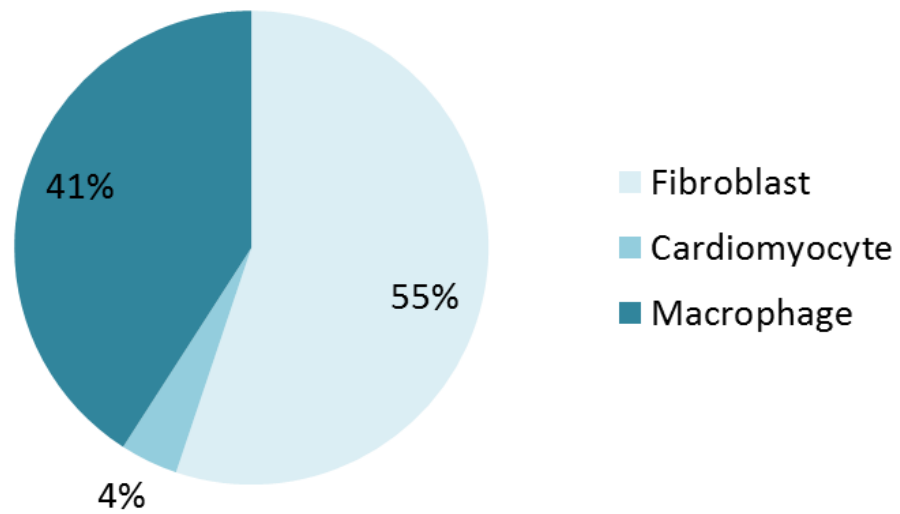


Figure 4-25 Morphology of all virally transduced eGFP⁺ cells 21 days after transplantation into the hearts of NOD/SCID mice subject to coronary artery ligation. Fibroblast morphology was most common followed by macrophage morphology. Only a minor subset adopted a cardiomyocyte morphology. Three transverse sections were DAB stained with anti-GFP antibody and examined per heart. Peri n=9, Adv n=9. Total of 582 cells were counted.

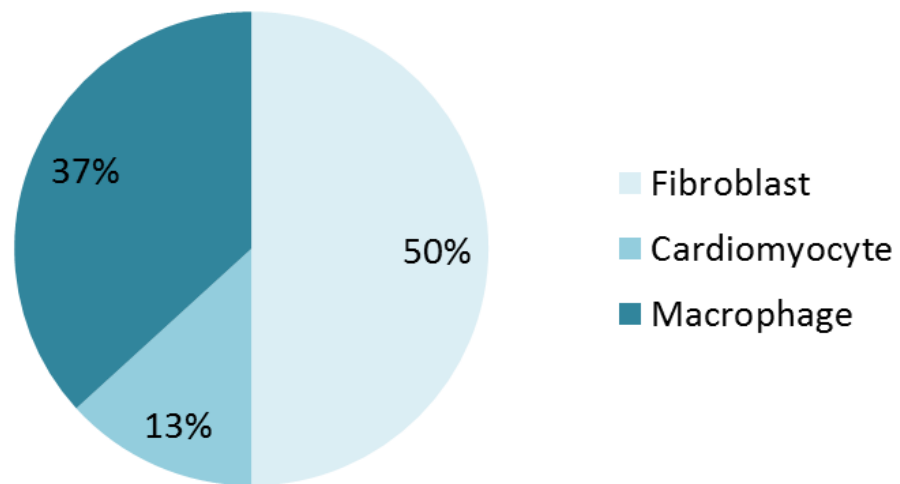


Figure 4-26 Morphology of virally transduced eGFP⁺ pericytes 21 days after cell transplantation in hearts of NOD/SCID mice subject to coronary artery ligation. The majority of injected pericytes displayed a fibroblast morphology with a macrophage morphology next most common. Three transverse sections were DAB stained with anti-GFP antibody and examined per heart. Peri n=9, Adv n=9. Total of 68 cells were counted.

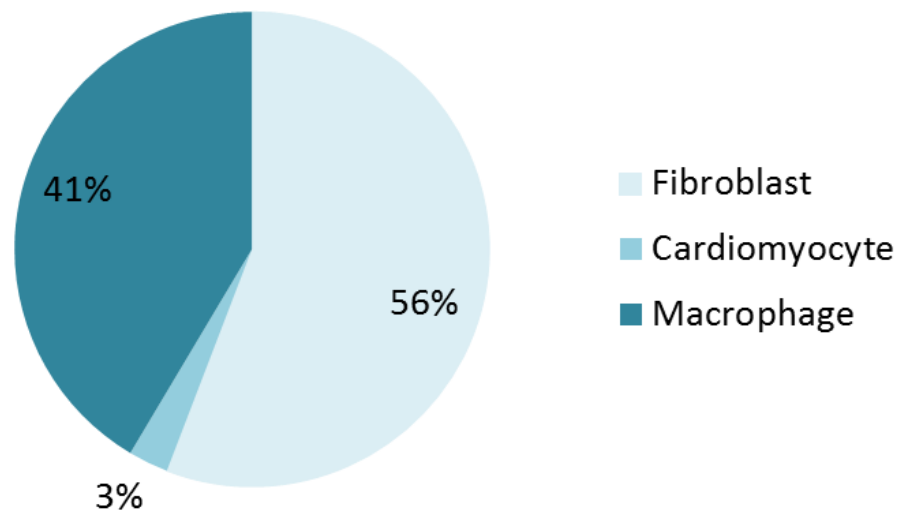


Figure 4-27 Morphology of virally transduced eGFP⁺ adventitial cells detected in the myocardium 21 days after cell transplantation in hearts of NOD/SCID mice subject to coronary artery ligation. Like pericytes the majority of injected adventitial cells displayed a fibroblast morphology with a macrophage morphology next most common. Three transverse sections were DAB stained with anti-GFP antibody and examined per heart. Peri n=9, Adv n=9. Total of 514 cells were counted.

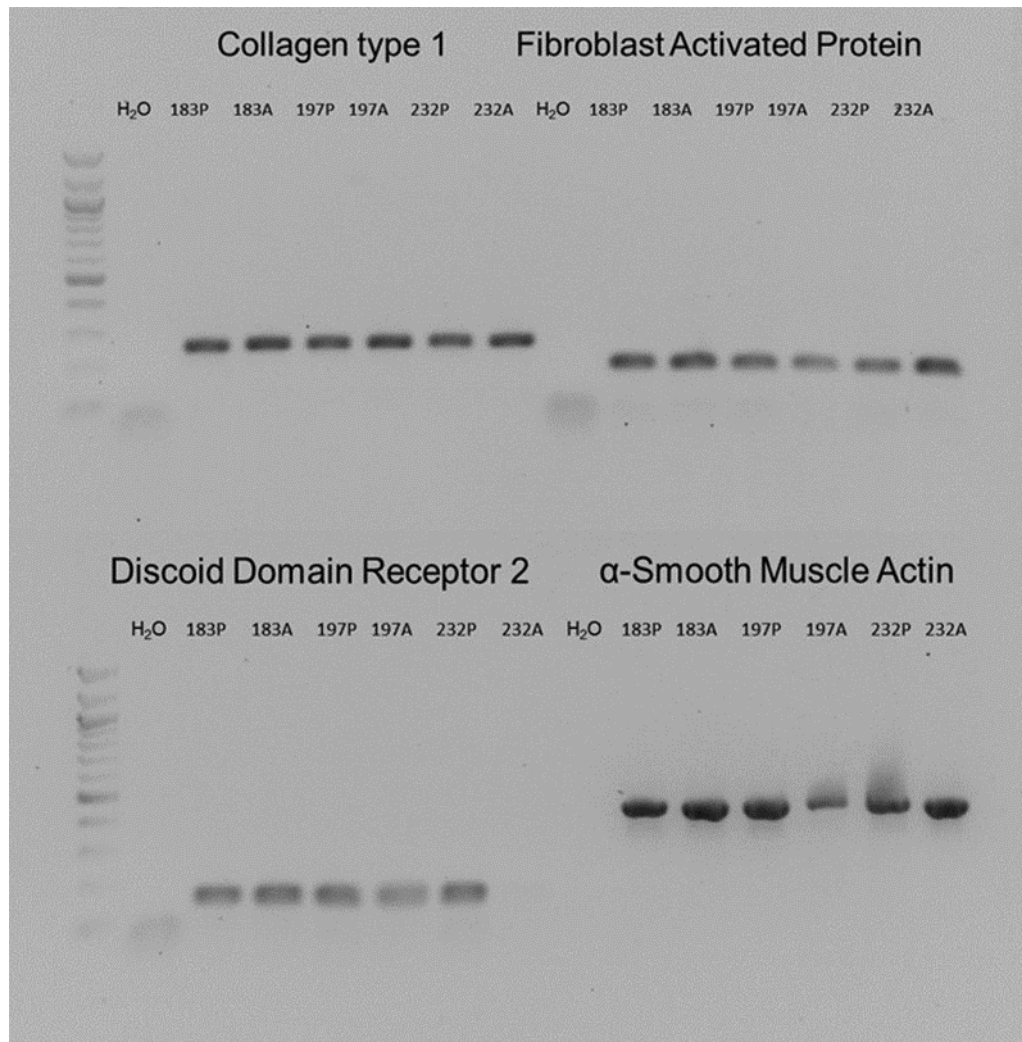


Figure 4-28 Conventional RT-PCR electrophoresis gel for pro-fibrotic marker genes in cultured pericytes and adventitial cells. Cell transplantation candidate populations of pericytes (peri) and adventitial cells (adv) at injection passage from donors EDF183 and EDF197 plus additional donor population EDF232. Pericytes and adventitial cells from all donors express mRNA of the pro-fibrotic markers collagen I alpha, fibroblast activated protein α , discoid domain receptor 2 and α -smooth muscle actin (n=3) DNA ladder bands are 100bp apart. H₂O = negative control.

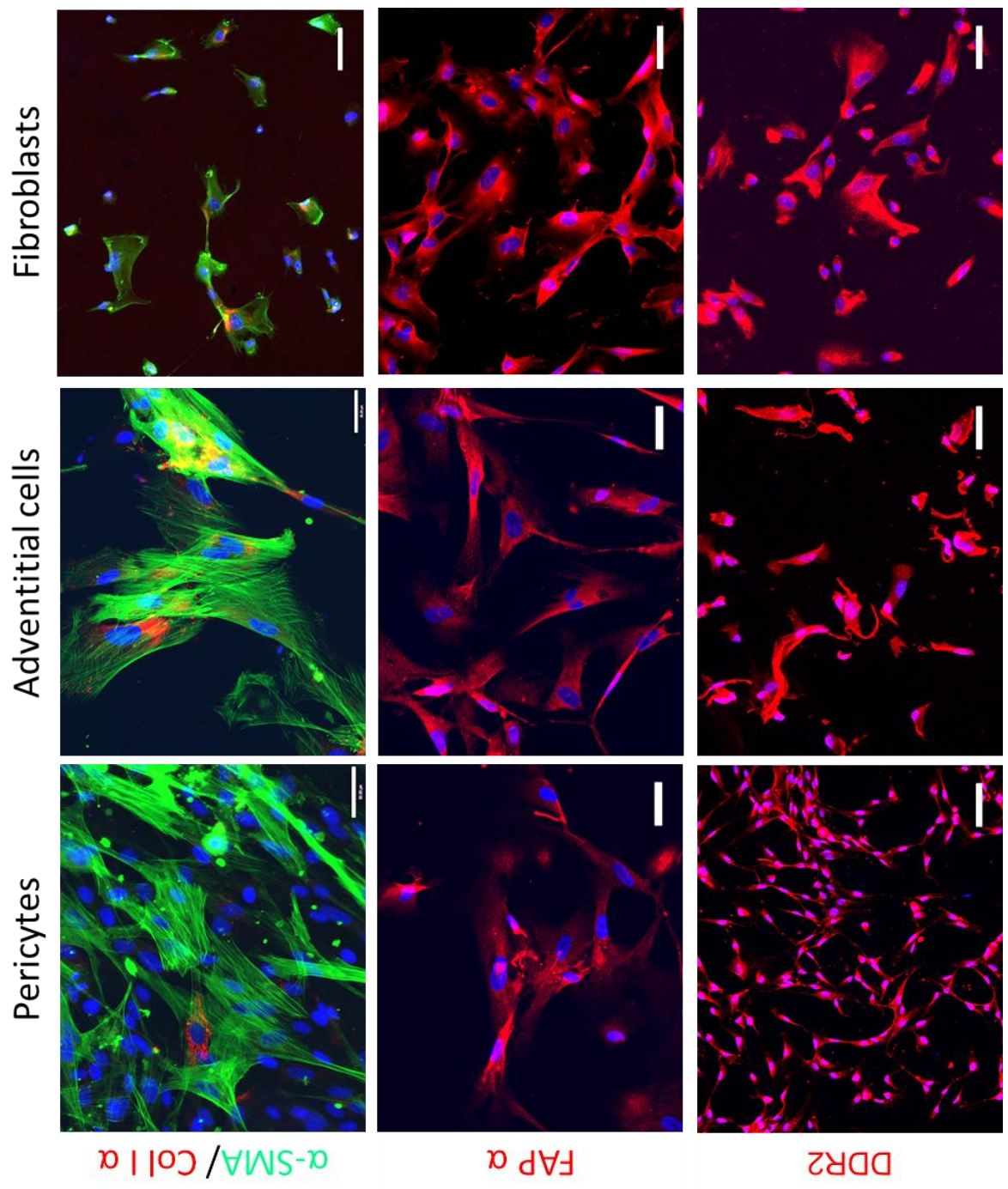


Figure 4-29 Representative immunofluorescence images of pro-fibrotic marker staining in cultured pericytes, adventitial cells (both at injection passage) and commercial human neonatal foreskin fibroblasts (Invitrogen) as a positive control. Fibroblast activated protein alpha (FAP α), discoid domain receptor 2 (DDR2) and alpha smooth muscle actin (α -SMA) were expressed by positive controls and both pericytes and adventitial cells. Collagen I alpha (Col I) was expressed by a subset of cells in each group (n=3). Scale bars all = 50 μ m.

4.5.12 Human dermal fibroblasts express pericyte and MSC markers in culture

Neonatal human dermal fibroblasts expressed mRNA of the MSC associated markers CD73, CD90, CD44 and CD105 and pericyte associated markers CD146, NG2 and PDGFR- β (Figure 4-30, Figure 4-31). Immunocytochemistry revealed expression of pericyte markers CD146, NG2 and PDGFR- β and the MSC marker CD90 (Figure 4-32).

On flow cytometry 67% of the fibroblasts labelled positively for CD146 and 74% labelled for NG2. 51% of cells co-labelled with both pericyte markers (Figure 4-33). Conventional MSC markers were highly expressed by commercial fibroblasts on flow cytometry with 100% of cells expressing CD44, 96% expressing CD73, 100% expressing CD90, 87% expressing CD105 and 0% expressing CD271 (Figure 4-34).

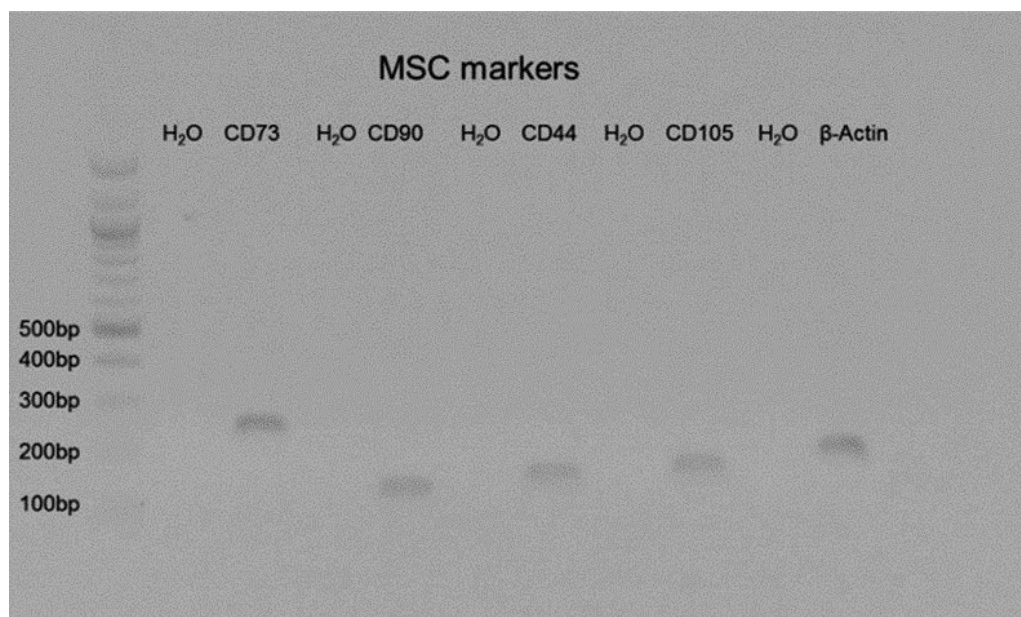


Figure 4-30 Conventional RT-PCR electrophoresis gel. Investigation of commercial human neonatal dermal fibroblasts for expression of MSC associated gene mRNA. MSC markers CD73, CD90, CD44 and CD105 were all expressed (n=1). β -actin house keeper gene (n=1). DNA ladder bands are 100bp apart. H₂O = negative control.

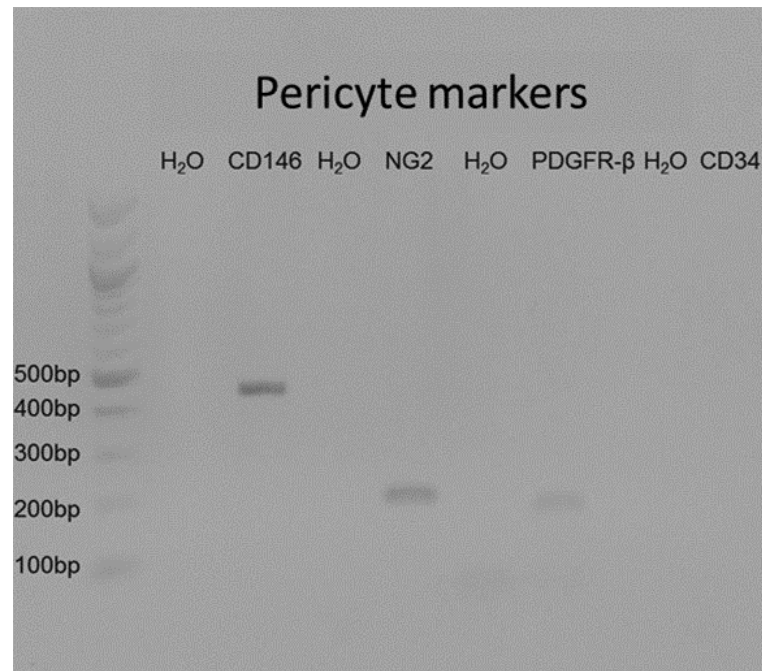


Figure 4-31 Conventional RT-PCR electrophoresis gel. Investigation of commercial human neonatal dermal fibroblasts for expression of pericyte marker mRNA. Pericyte markers CD146, NG2 and PDGFR-β were expressed (n=1). DNA ladder bands are 100bp apart. H₂O = negative control.

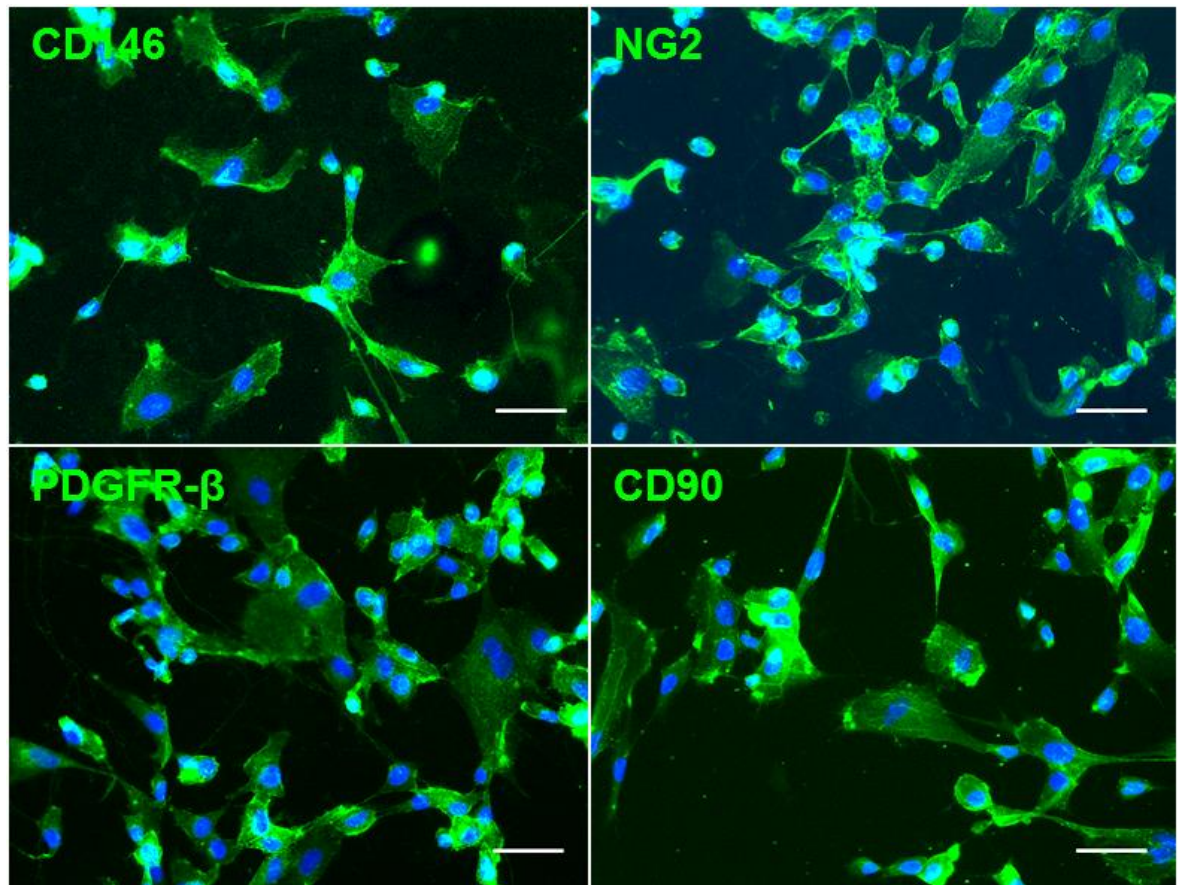


Figure 4-32 Immunofluorescence image of immunostaining of cultured human neonatal foreskin fibroblasts for anti-pericyte markers (CD146, NG2 and PDGFR- β) and CD90. Cells stain positively for all markers (n=1). DAPI nuclear staining in blue. Scale bars = 50 μ m.

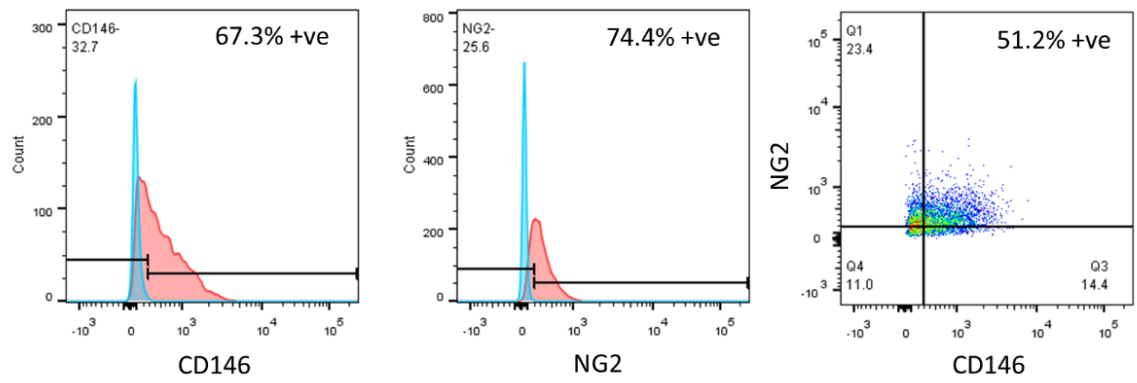


Figure 4-33 Representative flow cytometry histograms illustrating expression of pericyte markers CD146 and NG2 in a cultured commercial human neonatal foreskin fibroblast population (n=1). High levels of pericyte marker expression were evident.

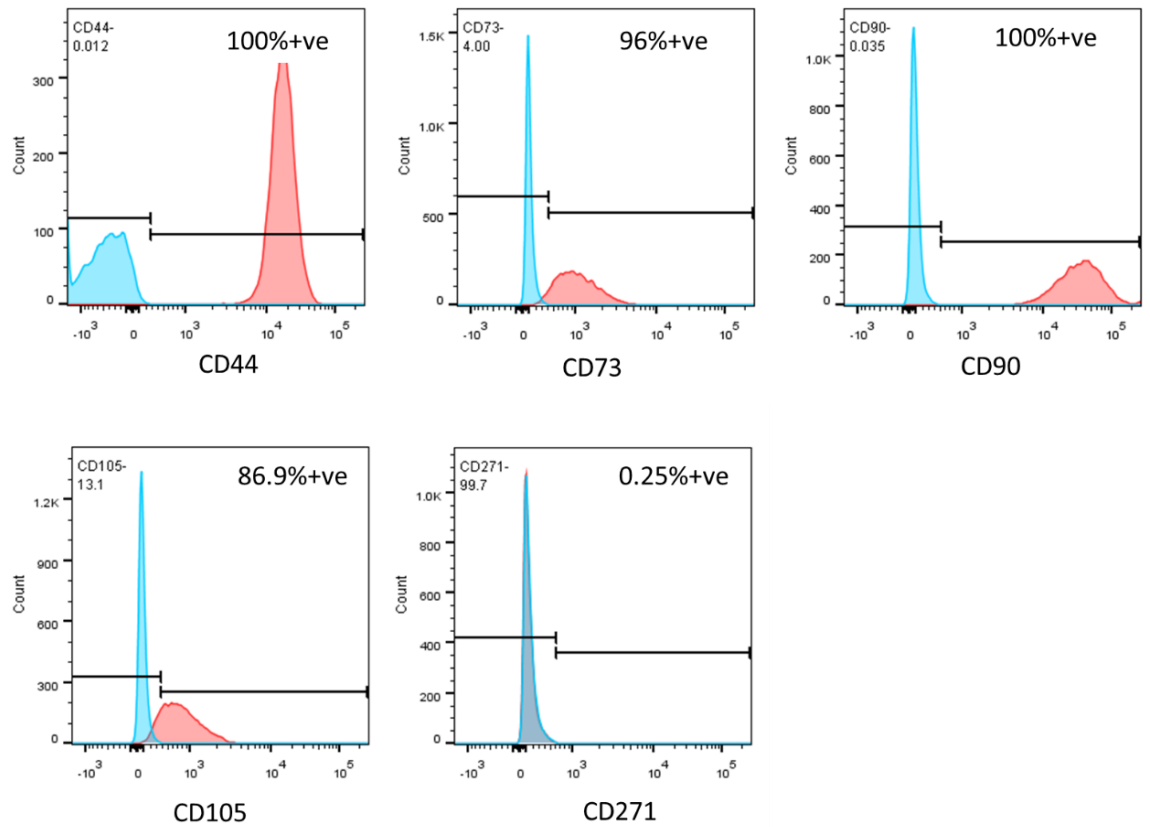


Figure 4-34 Flow cytometry histograms illustrating expression of MSC markers CD44, CD73, CD90, CD105 and CD271 in a cultured commercial human neonatal foreskin fibroblast population (n=1). High levels of MSC marker expression were present.

4.6 Summary and discussion

The specific aims set out at the start of this chapter were met by the work undertaken. The hypothesis which this work set out to investigate was that: “Foetal human cardiac perivascular cells will differentiate into cardiomyocytes when transplanted into ischaemically injured myocardium and by doing so will regenerate heart muscle, reduce scarring and improve cardiac function.”

4.6.1 Human cardiac perivascular cells maintain a stable phenotype throughout cryopreservation, thawing and expansion

Comparison of potential donor populations at cryopreservation and again prior to injection and after further expansion in culture revealed reasonably stable expression of pericyte and MSC associated markers. Cryopreservation and subsequent thawing have been reported to alter cell phenotypes and in particular the expression of cell membrane receptors (Costantini et al., 2003, Deneys et al., 1999). However with the exception of CD34 and PDGFR- β the same panel of pericyte and MSC associated marker mRNA was expressed by the candidate cell populations before cryopreservation and after thawing followed by several passages of expansion. Conventional RT-PCR however does not properly quantify gene expression and expression levels may have differed after cryopreservation. The loss of CD34 expression in culture was to be expected as this marker was also lost in adventitial cell populations from other tissues sources. The significance of the loss of PDGFR- β expression to the cell phenotype was unclear as the other pericyte markers CD146 and NG2 continued to be expressed.

4.6.2 Human cardiac perivascular cells are reasonably tolerant of prolonged storage in PBS and injection using a narrow gauge needle

Given that repeated passage of cell suspensions through a narrow gauge needle is used to lyse cells prior to RNA extraction it seemed sensible to briefly check whether ejection through similar diameter needle would result in damage of cells during transplantation. The basic assessment of pericyte and adventitial cell survival carried out suggested a loss of approximately 20% of cells. Several recent papers have

investigated the effect of cell injection techniques on the survival of human mesenchymal stem cells. These have demonstrated that passing cells through a 26 gauge needle does not affect their morphology, attachment, viability, phenotypic expression, or differentiation potential (Mamidi et al., 2012). In another study only approximately 8% of cells had died or become apoptotic 24 hr post ejection through a 30 gauge needle (Walker et al., 2010). However in the present study cells were allowed to stand in PBS (containing no serum) for 4 hr prior to injection, simulating the maximum delay from syringe loading to transplantation and this may have contributed to the greater cell death seen. An additional source of discrepancy could be the method in which dead cells were identified and counted. In this study cells were stained with Trypan blue and counted on a haemocytometer whereas the authors of the former work used Annexin V (Life Technologies) apoptosis dye and flow cytometry. The latter is likely to be more accurate. Cell loss appeared to be similar in the pericyte and adventitial cell group although the loss of viable cells from trypsinisation was greater in the pericyte population supporting the suggestion that this population is more delicate.

4.6.3 Poor post-operative survival was due to poor anaesthetic tolerance, arrhythmias and emboli

Despite the establishment of robust surgical model of myocardial infarction the post-operative survival rate of NOD/SCID mice in this study (60%) was surprisingly low. The poor response to anaesthesia in the experimental population contributed to this finding. No anaesthetic issues had been noted in the previous fifty C57/BL6 mouse infarct surgeries or indeed the tenNOD/SCID technique validation surgeries and the anaesthetic equipment and surgical protocol remained constant. All animals in these groups were of a similar age and body weight. There is little published on strain differences with relation to anaesthetic agents in rodents. A single study examined the responses of rats from eight inbred strains to three types of injectable anaesthetics (Avsaroglu et al., 2007). Significant differences were noted with two strains appearing resistant to the anaesthetic effects of medetomidine whilst ketamine proved fatal in two other strains even at the standard dose. Interestingly albino rats remained unconscious for significantly longer times than pigmented strains given the

same does of all three anaesthetics. This raises the possibility that poor tolerance of isofluorane seen in the Harlan cohort of NOD/SCID may have been specific and related to genetic differences.

The most common cause of intraoperative complications was arrhythmia. Induction of myocardial infarction alone in mice is sufficient to produce arrhythmia with significant prolongation of myocardial conduction and repolarisation times and an increase in ventricular ectopic activity (Gardiwal et al., 2009). In the current study arrhythmias were more prevalent in cell injected groups and tended to occur either on insertion of the needle or when cells or vehicle were injected into the myocardium. Insertion of the relatively large metal needle into the myocardium is likely to disrupt the normal electrophysiological activity of the heart. Injection of fluid and cells into the dense myocardium will force myofibres apart and may also disrupt electrical conduction. In addition it is possible that some cells were delivered directly into damaged vessels resulting in the formation of multiple micro emboli which would also be expected to be arrhythmogenic. Arrhythmias contributed significantly to the mortality rate as did systemic emboli/thrombosis. Emboli following systemic MSC treatment has been observed in humans with cells lodging in the pulmonary capillary beds (Ankrum and Karp, 2010). In this study animals presented with a head tilt (suggesting an ischaemic cerebral event) or pale ischaemic hind limb immediately or shortly after recovery from anaesthesia suggesting the administration of cells directly into the systemic circulation of the ventricle. Interestingly and unlike with C57/Bl6 mice post-operative cardiac rupture was not observed.

4.6.4 24 hour serum troponin I was not reliable as a measure of initial myocardial injury

24 hour serum troponin I concentrations have been shown to correlate with initial infarct size in mice (Ramani et al., 2004) and were intended to be used to exclude animals in which the myocardial injury was negligible or extreme. No correlation with the macroscopic infarct size was noted however in the NOD/SCID population and the cause of this discrepancy is unclear. This may again be related to strain differences or possibly due to additional myocardial injury resulting from needle insertion and/or cell injection. It is also possible that delays in separating and

freezing the serum samples contributed to aberrant results although all samples were stored at 4°C and processed within the ELISA test kit manufacturer's recommended 4 hour time limit.

4.6.5 Apex to ligature distance can be used to exclude extremes of myocardial injury outliers

As a result of the poor correlation between troponin I concentrations and infarct size this was not used to estimate initial injury in the main study. Ligature to apex distance instead was used to exclude injury size outliers. Heart to body weight is considered to increase in proportion to bodyweight in mice (Doevendans et al., 1998) and given that there were no significant differences in day 0 body weight between animals in each of the treatment groups it can be assumed that there were also no differences in starting heart size which may affect the use of ligature to apex distance measurements. Macroscopic infarct size and ligature distance correlated enabling the exclusion of animals with initial myocardial injuries smaller or greater than the extremes noted in the PBS control treated group. Ligature to apex distance is of course a cruder measure of injury than troponin I assay and does not account for variation in injury size relating to differences in branching points of the coronary artery between individuals (Ahn et al., 2004). Ligature to apex distance also varied over the course of the surgical period but when examined as a function of treatment group however no differences were seen between groups indicating that the results from each group can be directly compared.

4.6.6 Cardiac cell therapy using human cardiac perivascular cells reduces rather than improves left ventricular function following myocardial infarction

High frequency cardiac ultrasound analysis of mice 21 days after cell transplantation revealed no significant differences between groups in left ventricular end diastolic and systolic volumes (as indicated by endocardial area) suggesting that there was no significant difference in cardiac remodelling between groups. Left ventricular global systolic function was however affected with left ventricular endocardial area change and fractional area change significantly reduced in the adventitial treated group

compared to the pericyte treated group. Animals receiving transplants of adventitial cells exhibited a significant reduction in left ventricular fractional shortening and ejection fraction compared to the PBS controls. These last two measures are related if there is no regional impediment to normal ventricular motion. However in infarction models in which there is alteration to the normal left ventricular geometry fractional shortening is considered the most accurate measure of left ventricular global function (Gao et al., 2011). Animals receiving pericytes also exhibited a significant reduction in fractional shortening compared to controls but no change in ejection fraction. Taken together these findings suggest that transplantation of cardiac perivascular cells reduces cardiac function whilst not altering cardiac remodelling and this effect is particularly pronounced in the adventitial cell treated group. This is a surprising yet interesting finding given that all previous cell therapy studies reviewed for this thesis have universally reported a beneficial functional effect resulting from transplantation of a variety of different cell types (Smits et al., 2009, van Laake et al., 2009, Zuo et al., 2009, Lee et al., 2011, Okada et al., 2008, Kocher et al., 2001, Chen et al., 2013, Ellison et al., 2013, Simpson et al., 2012).

4.6.7 Transplantation of human cardiac perivascular cells does not result in substantial cardiac remodeling following myocardial infarction

Morphological assessment detected a significant increase in scar area in the adventitial treated group compared to PBS treated controls when measured by region of interest. This was supported by a similar but non-significant trend using the threshold analysis and colour picker analysis methods of quantifying scar area and is in conflict with the majority of other published cell transplantation studies in which a reduction in scar size is generally seen. No significant differences were noted between groups with regards scar length and wall thickness. Again contrary to the majority of cell transplantation studies (Tomita et al., 2002, Chen et al., 2013, Katare et al., 2011) no differences in microvascular density were noted between the treatment groups. These findings although again surprising are consistent with the ultrasound data which suggests a negative functional impact of cell transplantation but no significant differences in cardiac remodelling between cell treated and PBS injected control groups.

4.6.8 Retention and survival of cells following transplantation is low

Cell tracking through staining of GFP transduced cells shows that, as with other studies, cell retention following transplantation is low. This is not unexpected given that it is estimated that approximately 40% of injected cells leak out of the myocardium immediately following injection (Muller-Ehmsen et al., 2002). Care was taken to insert the needle obliquely into the myocardium so that the needle tract would collapse upon removal, however the myocardium is a dense and continuously contracting muscular structure with little capacity for expansion it is inevitable that a substantial proportion of the injected fluid will be lost. Of the retained GFP⁺ cells a substantial proportion (the second most common phenotype) appear as clustered round cells with abundant GFP⁺ material within the cytoplasm but nuclei that do not stain with human anti-nuclear antigen. These are therefore of murine origin and are likely to be host macrophages that have phagocytosed degenerate human cells. NOD/SCID mice are commonly used as recipients in xenografts transplantation studies due to their profound immune-deficient state (Dick et al., 1997). Their severe combined immunodeficiency (SCID) mutation results in impaired T and B lymphocyte development whilst the non-obese diabetic background (NOD) results in impaired natural killer cell function. Despite this sufficient functional macrophages clearly remain to mount a cellular immune response. Anti-GFP staining of regional lymph nodes, the spleen and lung would likely reveal large numbers of positive macrophages which have phagocytosed injected cells and trafficked from the heart. This dispersion of cells away from the injection site has been noted in bioluminescence tracking studies of injected cells (van der Bogt et al., 2008). The fate of injected cells is also likely to depend on the site of deposition within the injured myocardium. Post-ligation blanching varies in intensity and is sometimes not detectable at all (particularly if there has been any intraoperative blood loss) thus occasional, inadvertent, injection into the ischaemic zone is to be expected and cell deposited here are likely to rapidly die.

4.6.9 The majority of transplanted human cardiac perivascular cells adopt a fibroblast morphology in the infarcted myocardium

Adventitial cells survive better than pericytes following transplantation which is not surprising given their greater robustness in culture and apparent better tolerance of trypsinisation. It is also possible that they have a better tolerance of low oxygen conditions/handling or perhaps a less immunogenic profile, although this is yet to be investigated. Of the cells that survive transplantation the majority of both pericytes and adventitial cells develop a fibroblast morphology. These cells have single human anti-nuclear antigen positive nuclei and are vimentin positive suggesting true differentiation rather than fusion with host cells. They are frequently located within a band in the sub-epicardial layer and this may be a result of leakage of cells from the initial deeper myocardial site of deposition or may be a region with a particular tissue microenvironment that favours cell survival and fibroblast differentiation. The presence of cells deep within the scar tissue itself also suggests the presence of a microenvironment that favours a profibrotic phenotype and it is likely that cells deposited or migrating to this location will not only be exposed to lower oxygen tensions but will also have direct contact with host fibroblasts and abundant extracellular collagen. Differentiation of transplanted cells in the heart into fibroblasts has not previously been documented. Most investigators record only the differentiation of cells into cardiomyocytes (van Laake et al., 2009, Kajstura et al., 2005) or occasionally endothelial cells (Smits et al., 2009) but do not comment on differentiation to other cell types. In one study the presence of labelled cells within the scar that did not label with cardiomyocyte markers was reported but these cells were not identified further (van der Bogt et al., 2008). The relatively low numbers of GFP⁺ fibroblasts within the infarct border zone correlates with the absence of significant cardiac remodelling in the cell treated groups. It also seems unlikely, given their low numbers, that these cells will have had a direct effect on cardiac function through the production of collagenous scar tissue. Cytokines such as TNF- α , IL-6 and IL-2 have been shown to reduce cardiomyocyte contractility (Prabhu, 2004, Kelly and Smith, 1997) and it is possible that surviving transplanted fibroblasts reside within the myocardial border zone and secrete sufficient cytokines to have a

negative effect on cardiac function. Alternatively as large numbers of cells die after the initial delivery there may be a substantial release of cytokines which negatively and lastingly affect left ventricular contractility. GFP⁺ cells were not seen in a perivascular location as has been reported in a recent pericyte transplantation study (Chen et al., 2013) suggesting that cardiac perivascular cells unlike muscle pericytes do not adopt a perivascular cell phenotype following transplantation. Finally the absence of enhanced angiogenesis in cell treated groups compared to the control groups may be the result of insufficient numbers of surviving cells, secretion of inhibitory cytokines by surviving cells or conversely failure of surviving cells to secrete pro-angiogenic cytokines.

4.6.10 A minor subset of transplanted human cardiac perivascular cells adopt a cardiomyocyte morphology in the infarcted myocardium

A small proportion of injected cells develop a cardiomyocyte phenotype. Cells again have a single human anti-nuclear antigen positive nucleus suggesting true differentiation rather than fusion. Interestingly cells adopting this phenotype were only seen in the border zone of the infarct or in adjacent healthy myocardium suggesting that microenvironment of the scar itself was not conducive to cardiomyocyte differentiation. These areas, unlike the scar, also contain viable cardiomyocytes and given the results of the *in-vitro* studies it seems likely that direct cell to cell contact is required for cardiomyocyte differentiation. Pericytes appear to be better adapted than adventitial cells to the cardiomyocyte differentiation however cardiomyocyte differentiation is a rare event for both donor cell types and much less common than fibroblast differentiation. This fits with the deleterious rather than beneficial post transplantation functional findings and correlates with other transplantation studies using adult stem cells in which retention and cardiomyocyte differentiation occurs only in low numbers of cell (Simpson et al., 2012).

4.6.11 Both cardiac pericytes and adventitial cells express pro-fibrotic markers in culture

The above findings suggest that injected perivascular progenitors have both fibroblast and cardiomyocyte differentiation potential. It is clear from RNA and protein

expression analysis in chapter 3 that spontaneous cardiomyocyte differentiation in culture prior to injection does not occur. However in the initial characterisation of the cardiac perivascular cell populations the potential for fibroblast differentiation in culture was not examined. Retrospective examination of culture expanded populations was therefore carried out. As with pericyte markers no single reliable fibroblast marker exists so the following combination of pro-fibrotic markers were used: Collagen 1 alpha 1 (Col1 α 1); Fibroblast Activated Protein alpha (FAP- α); Discoid Domain Receptor 2 (DDR2); alpha smooth muscle actin (α -SMA) (Camelliti et al., 2005). Col1 α 1 is the major protein found in type I fibrillar collagen in cardiac infarct scars and is expressed by fibroblasts. FAP- α is a transmembrane serine protease expressed on activated fibroblasts in tissue undergoing remodelling of the ECM. DDR2 is a receptor tyrosine kinase expressed on cardiac fibroblasts which binds fibrillar collagen and is important in cell migration. α -SMA is a cytoplasmic cytoskeletal protein found in vascular smooth muscle cells and a subset of perivascular progenitors. It is considered a pericyte marker by some researchers (Armulik et al., 2011) but is also strongly expressed by contractile myofibroblasts. The expression of mRNA and proteins of all of these pro-fibrotic markers was seen in each of the three populations of pericytes and adventitial cells examined and suggests the adoption of a fibroblast like phenotype by human foetal perivascular cells in culture conditions. Thus it is possible that cardiac perivascular cells are fibroblast progenitors as has been suggested in several other organs (Goritz et al., 2011, Dulauroy et al., 2012). Alternatively the initial FACS population maybe contaminated with mature fibroblasts that proliferate in culture and outgrow the other cells. In either case using hard plastic substrates and medium containing 20% serum with high levels of TGF- β is likely to promote and accelerate fibroblast growth (Henderson et al., 2013).

4.6.12 Significant overlap exists between pericyte, MSC and pro-fibrotic markers

Examination of a commercial human dermal fibroblast line revealed expression of genes and proteins associated with both pericyte and MSC phenotypes. This raises the question as to how specific the current panel of pericyte, MSC and fibroblast

markers are and suggests substantial overlap in these populations in the heart. It also supports the theory that the initial cell isolation selected for a population of fibroblast progenitors. In a previous study human lung and skin fibroblasts were shown to express MSC markers but not CD146 (Halfon et al., 2011). With the addition of connective tissue growth factor human bone marrow MSCs have been shown to differentiate into fibroblasts in culture (Lee et al., 2010). In another study CD146⁺ retinal perivascular cells have been shown to share expression of pericyte and MSC markers with fibroblasts and MSCs (Covas et al., 2008). With regards differentiation capability, it has been demonstrated that human dermal fibroblasts are able to differentiate into adipocytes, chondroblasts and osteoblasts *in-vitro* whereas human embryonic lung fibroblasts are not (Alt et al., 2011, Brohem et al., 2013). These studies confirm that substantial overlap exists between dermal fibroblast and MSC populations and further support the suggestion that cardiac perivascular cells are fibroblast progenitors.

4.6.13 Conclusion

The findings of this part of the project partially confirmed the initial hypothesis in that transplanted perivascular cells did indeed differentiate into cardiomyocytes. However this was seen only in a minor subset of the population and contrary to the proposed hypothesis cell transplantation had a detrimental rather than beneficial effect on cardiac function. This worsening of the left ventricular function was especially prominent in the adventitial cell treated group. Injected cells were retained in low numbers and, in both groups, preferentially differentiated into fibroblasts. Examination of cultured cells revealed expression of pro-fibrotic markers and there was overlap between MSC, fibroblast and pericyte marker expression seen in control dermal fibroblasts. These findings suggest heterogeneity of the starting population of perivascular cells with the majority being fibroblast progenitors that favour a pro-fibrotic differentiation pathway.

Chapter 5: α_v integrin expression by cardiac PDGFR- β^+ perivascular cells regulates cardiac fibrosis *in vivo*

5.1 Introduction

The preferential differentiation, *in-vitro* and *in-vivo*, of human foetal cardiac pericytes and adventitial cells into fibroblasts type cells suggests that the cardiac perivascular cell compartment contains a population of pro-fibrotic progenitors. This pro-fibrotic role of pericytes and other perivascular cells has been proposed in the spinal cord (Goritz et al., 2011) and the kidney (Lin et al., 2008). In the human tissue studies described in chapters one and two of this thesis CD146 was used as the principle marker of the pericyte phenotype. In murine tissues however CD146 is predominantly expressed by endothelial cells with only limited expression in pericytes (Schrage et al., 2008).

Platelet derived growth factor beta (PDGFR- β) by contrast is expressed by pericytes in both human and murine tissue (Winkler et al., 2010) and is therefore a more reliable indicator of the perivascular cell population in mouse models. Platelet derived growth factors are a family of proteins synthesised by a variety of cells types including endothelial cells, monocyte/macrophages, smooth muscle cells, neurons, trophoblasts, pericytes and fibroblasts. They act as mitogens for connective tissue cells such as fibroblasts, smooth muscle cells and glial cells and additionally may induce chemotaxis, contraction, production of ECM and differentiation in target cells (Leveen et al., 1994). Three isoforms of PDGF (AA, AB and BB) are secreted as biologically active molecules whilst isoforms PDGF-CC and PDGF-DD are produced as precursor proteins requiring cleavage to become active (Cotran et al., 1993). These molecules bind two cell surface receptors PDGFR- α and PDGFR- β . The type- α receptor binds the A or B chain of the PDGF dimer whilst the type- β receptor only binds the B chain (Leveen et al., 1994).

Surface integrin receptors meanwhile have been shown to play an important role in the regulation of tissue fibrosis in a variety of organs (Horan et al., 2008, Hahm et al., 2007, Balasubramanian et al., 2012). Genetic deletion of surface integrins on

PDGFR- β expressing cells in the murine liver has recently been shown to significantly modulate hepatic fibrosis (Henderson et al., 2013). It is plausible that a similar mechanism may also be present in the heart and so the final part of this study was designed to investigate the role integrins play in the cardiac pro-fibrotic perivascular cell population using mice (provided by Dr Neil Henderson, Centre for Inflammation Research, The University of Edinburgh, UK) in which the common α_v integrin subunit (shared by five β -subunit binding partners: $\alpha_v\beta_1$; $\alpha_v\beta_3$; $\alpha_v\beta_5$; $\alpha_v\beta_6$; and $\alpha_v\beta_8$) (Henderson et al., 2013) had been genetically deleted on PDGFR- β^+ cells.

5.2 Specific hypothesis

The work in this section was based on the hypothesis that “Murine cardiac PDGFR- β^+ perivascular cells become fibroblasts following injury in a process mediated by α_v integrin expression.”

5.3 Specific aims

The specific aims for this part of the study were:

- To determine the accuracy of PDGFR- β reporting in mTmG reporter mice.
- To identify the PDGFR- β^+ cell population in the normal heart.
- To identify the PDGFR- β^+ cell population in the hypertrophied heart.
- To investigate the pro-fibrotic potential of PDGFR- β^+ cells *in-vitro*.
- To investigate the role of α_v integrin expression in the pro-fibrotic potential of PDGFR- β^+ cells *in-vitro*.
- To investigate the role of α_v integrin expression by PDGFR- β^+ cells in the regulation of cardiac fibrosis *in-vivo*.

5.4 Specific materials and methods

5.4.1 Genetically modified mice

Details of the genetically modified mice used in this part of the study are presented in 2.19.

5.4.2 Assessment of PDGFR- β reporting accuracy

Sections of unstained myocardium from the hearts of six twelve week old mTmG mice were examined using wide field immunofluorescence microscopy. A total of 3503 cells were examined and the numbers of cells co-localising the reporter signal with PDGFR- β antibody labelling, or expressing the reporter signal or antibody signal only were counted.

5.4.3 Isolation of PDGFR- β^+ cells from mTmG reporter mouse and non-reporting α_v integrin knockout and wild type mouse hearts

Cells were isolated from reporter mouse and non-reporting α_v -integrin knockout and wild type mouse hearts using FACS as described in 2.8 using the antibodies and dilutions detailed in Table 5-1. Post sort purity checks were carried out as described in 2.7.

5.4.4 Immunostaining of mTmG and Ai14 reporter mouse hearts

Mouse cardiac tissue was preserved as described in 2.3. Immunostaining was carried out as described in 2.6.1. The antibodies and dilutions used to stain mouse myocardium are detailed in Table 5-2.

Table 5-1 Primary antibodies and isotypes used for flow cytometry and FACS studies of murine heart tissue.

Antibody	Manufacturer	Raised in	Catalogue No.	Dilution
CD31 APC	eBiosciences	Rat	17-0311-82	1:100
PDGFR- α (CD140 α) APC	BD Pharmingen	Rat	562777	1:50
PDGFR- β (CD140 β) APC	eBioscience	Rat	17-1402-82	1:50
PDGFR- β (CD140 β) PE	eBioscience	Rat	17-1402-81	1:50
CD34 AF700	BD Biosciences	Rat	560518	1:100
CD146 PerCP-Cy5.5	BD Biosciences	Rat	562231	1:100
CD90.2 PerCP-Cy5.5	Molecular probes	Rat	1714897	1:100
CD45 PE- Cy7	BD Biosciences	Rat	56868	1:100
CD45 PerCP- Cy5.5	BD Biosciences	Rat	550994	1:100
CD56 APC	Abcam	Rat	Ab28335	1:100
IgG2b PerCP-Cy5.5	eBioscience	Rat	45-4031-80	1:100
IgG2b AF700	BD Biosciences	Rat	557964	1:100
IgG2b PECy7	BD Biosciences	Rat	552849	1:100
IgG2b APC	eBioscience	Rat	17-031-82	1:50
IgG2a PE	eBioscience	Rat	12-4724-41	1:50

Table 5-2 Antibodies used for immunostaining of pro-fibrotic, endothelial and pericyte markers in murine cells and tissues.

Antibody	Type	Raised in	Company	Catalogue No.	Dilution
α -SMA	Polyclonal	Rabbit	Sigma Aldrich	F3777	1:200
PDGFR- β	Polyclonal	Rabbit	Abcam	ab21234	1:50
DDR2	Monoclonal	Mouse	R&D Systems	MAB25381	1:100
FAP	Polyclonal	Rabbit	Abcam	ab28246	1:100
Isolectin GS-IB4-488	-	<i>Griffonia simplicifolia</i>	Life Technologies	I21411	1:100
CD146	Polyclonal	Rabbit	Abcam	ab75769	1:100
CD34	Monoclonal	Mouse	Abcam	ab8536	1:100
Vimentin	Polyclonal	Rabbit	Abcam	Ab92547	1:100

5.4.5 Angiotensin II model of cardiac hypertrophy and interstitial fibrosis

In order to investigate the role of α_v integrin on cardiac PDGFR- β positive cells in relation to fibrosis in the heart the 14 day angiotensin II model of cardiac interstitial fibrosis (Yamazaki et al., 2012) was used. Work by fellow PhD candidate Rachel Richardson (PhD thesis, The University of Edinburgh, UK) determined that 14 days treatment of angiotensin II in mice administered via minipump at 200ng/kg/minute is sufficient to generate cardiac hypertrophy with a quantifiable increase in interstitial fibrosis. Rachel kindly supplied formalin fixed and picro sirius red stained transverse sections of hearts from C57/Bl6 mice receiving this dose or sterile water vehicle control. Application of colour picker analysis, as described in 2.22, was used to calculate the fractional area of red collagen relative to the yellow counterstained myocardium and revealed a statistically significant increase in fibrosis in treated hearts ($p < 0.001$, one-way ANOVA with Tukey's post hoc analysis) with group sizes of 7 animals. As a result it was decided to adopt this model of angiotensin II induced interstitial fibrosis for use in the current study.

Treatment was administered over 14 days using Alzet 1002 minipumps (Alzet). The final solution used in the pumps contained 5x angiotensin II in H₂O, 2x BrdU in DMSO and H₂O diluent in a ratio of 2:5:3. Pumps were labelled and weighed then carefully loaded before being re-weighed. An increase in weight of 112mg (+/- 2) was considered evidence of accurate loading and the pumps were then placed in sterile saline in a 37°C oven overnight. Prior to pump implantation mice were conditioned to tail cuff blood pressure measurement for several days and on the day of surgery the blood pressure was again measured. The animals were then anaesthetised in an induction chamber using 3% isoflurane and maintained on gaseous anaesthesia (3% isoflurane) via a nose cone. 0.02mg/kg of buprenorphine was injected subcutaneously and the skin overlying the shoulder and caudal neck region was shaved and disinfected with povidone iodine solution on a cotton bud. A 5mm transverse incision was made in the skin in the inter-scapular region of the dorsum and a subcutaneous pocket made with the tips of a pair of blunt nosed

scissors. A minipump was inserted into this pocket before being gently manipulated to lie approximately 1cm distal to the wound. The incision was then closed with a single surgical staple and the animal was maintained on oxygen until it regained consciousness.

5.4.6 Cardiac fibroblast primary culture and flow cytometry analysis

Cardiac fibroblasts were obtained from hearts as previously published (Balasubramanian et al., 2012). Briefly the atria were removed and the ventricles of two hearts per sample were minced before being suspended in 2ml of 300 U/ml of collagenase II (Gibco). The tubes containing enzyme solution and tissue were placed on a rotator in a 37°C oven for 15 minutes then the supernatant was removed and the enzymes quenched with 10% FBS/DMEM before fresh enzyme solution was added to the tissue and the step repeated 6 times. The final solutions were filtered at 40µm centrifuged at 1000rpm for 5 minutes then re-suspended in 3ml of 10% FBS DMEM. The cells from each sample were then seeded in a single well of a 6 well plate that had been coated with 0.2% gelatin and incubated at 37°C for 3 hours. The media was aspirated and the wells were washed with 2mls of fresh 10% FBS/DMEM twice before cells were grown for 5 days. The media was changed every 48 hours. Some cells were seeded on 0.2% gelatin coated coverslips and at the end of 5 days these were fixed with 4% PFA and stained as described in 2.17 with anti-GFP antibody. Cells were dissociated by exposure to trypsin and stained for FACS as previously described in 2.8. Flow cytometry antibodies used were the anti-CD146, anti-PDGFR- α and anti-CD34 with corresponding isotypes as detailed in Table 5-1 and at a dilution of 1:100 with a 30 minute room temperature incubation.

5.4.7 Investigation of the pro-fibrotic response of PDGFR- β^+ cells *in-vitro*

eGFP⁺ (PDGFR- β^+) cells were isolated by FACS from pooled hearts (2 per pool) of mTmG mice and collected in EGM2 media. Cells were seeded in fresh EGM2 at a density of approximately 50,000 cells/cm² and cultured for 0, 5, 10 and 15 days. EGM2 was exchanged every 48 hours. At the end of each time period cells were washed twice with PBS then lysed with RLT buffer. RNA extraction and reverse-

transcription were carried out as described in 2.13 and 2.14. qRT-PCR for pro-fibrotic markers was carried out as described in 2.16 using primers for pro-fibrotic markers and their corresponding FAM hydrolysis probes (Roche UPL probe library) as detailed in Table 5-3. The housekeeping genes used were: 18s; β -Actin; Hprt1; Tbp1; and Rpl4. Means of the first four of these housekeepers were used. 0 day samples were obtained immediately following sorting. Five attempts and several modifications were made to try and improve RNA yield from 0 day samples, including collecting cells directly into RLT buffer, using β -mercaptoethanol (0.14M, Sigma Aldrich) RNase inhibitor in the cell lysate and carrier RNA in the extraction process.

5.4.8 Investigation of the effect of genetic deletion of α_v integrin on the pro-fibrotic response of PDGFR- β^+ cells *in-vitro*

PDGFR- β^+ cells were isolated via antibody labelling from pooled hearts (2 per pool) of α_v integrin cre⁺ knockout and cre⁻ wild type mice using FACS. Cells were collected in EGM2 then cultured in fresh EGM2 at a density of approximately 50,000 cells/cm² for 48 hours. Media was then exchanged for 10% FBS DMEM 1% P/S for a further 96 hours before RNA was extracted and reverse-transcription carried out as described in 2.13 and 2.14. qRT-PCR for pro fibrotic markers was carried out as described in 2.16 using primers detailed in Table 5-3.

5.4.9 Investigation of the effect of chemical inhibition of α_v integrin on the pro-fibrotic response of PDGFR- β^+ cells *in-vitro*

PDGFR- β^+ cells were isolated from the hearts of six mTmG mice, pooled into three samples, then split into two and seeded at 45,000 cells per cm². Cells were collected in EGM 2 and cultured in fresh EGM2 for 24 hours before being switched to 10% FBS DMEM 1% P/S containing 0.01mM of the small molecule α_v integrin inhibitor (CWHM 12) or 0.01mM of the control enantiomer molecule (CWHM 96) for 3 days. The inhibitor and enantiomer were dissolved in 50% DMSO in sterile water prior to being added to the culture media.

Cultures were then washed with PBS twice and lysed in RLT buffer. RNA extraction and reverse-transcription were carried out as described in 2.13 and 2.14. qRT-PCR was used to measure expression of pro-fibrotic genes in cultured mouse PDGFR- β^+ cells and carried out as described in 2.16. The primer details of the genes examined are given in Table 5-3.

Table 5-3 qRT-PCR primer sequences and probe details for house-keeping genes and pro-fibrotic markers in mouse cells/tissues.

Gene	Sequence	UPL Probe
18S	f CTCAACACGGGAAACCTCAC r CGCTCCACCAACTAAGAACG	77
β -actin	f CTAAGGCCAACCGTGAAAAG r ACCAGAGGCATACAGGGACA	64
Tbp1	f GGCGGTTTGGCTAGGTTT r GGGTTATCTTCACACACCATGA	107
Hprt1	f CCTCCTCAGACCDCTTTTT r AACCTGGTTCATCATCGCTAA	95
Rpl4	f GATGAGCTGTATGGCACTTGG r CTTGTGCATGGGCAGGTT	38
α -SMA	f CTCTCTTCCAGCCATCTTTCAT r TATAGGTGGTTTCGTGGATGC	58
Col1 α 2	f GCAGGTTACCTACTCTGTCCT r CTTGCCCCATTTCATTTGTCT	46
Col3 α 1	f TCCCCTGGAATCTGTGAATC r TGAGTCGAATTGGGGAGAAT	49
Fibronectin	f CGGAGAGAGTGCCCCTACTA r CGATATTGGTGAATCGCAGA	52
Itgav	f GGTGTGGATCGAGCTGTCTT r CAAGGCCAGCATTTACAGTG	21
PDGFR- β	f TCAAGCTGCAGGTCAATGTC r CCATTGGCAGGGTGACTC	67
TGF- β	f AGGAGCCTGCTCCAGAGTG r GAGGTGACAGAGTGGCAGGT	66
DDR2	f CGAAAGCTTCCAGAGTTTGC r GCTTCACAACACCACTGCAC	55

5.4.10 Investigation of the effect of genetic deletion of α_v integrin in PDGFR- β expressing cells on cardiac fibrosis *in-vivo*

Male α_v integrin cre⁺ knockout mice and α_v integrin cre⁻ wild type mice of between 10 and 12 weeks of age were randomly allocated to receive either 200ng/kg/minute of angiotensin II (Sigma Aldrich) in combination with 15mg/g/min of bromodeoxyuridine (to identify proliferative cells) or a vehicle control in which the angiotensin II was replaced by sterile water. Experimental numbers in each of the groups were as follows: Cre⁺ AngII 10 animals; Cre⁺ Veh 8 animals; Cre⁻ AngII 8 animals; Cre⁻ Veh 9 animals.

5.4.11 Blood pressure and body weight measurements

Prior to surgery mice were conditioned on three occasions to tube restraint and tail cuff application. Five minutes prior to assessment they were warmed in a small animal recovery chamber (Harvard Apparatus) and tail cuff blood pressures were then measured using a photoplethysmograph (Harvard Apparatus). Four diastolic and four systolic tail vein pressures were obtained and diastolic values were discarded whilst the mean of the systolic values was recorded. All measurements were taken in the morning on the day of surgery then again at days 3, 7, 10 and 14 post-surgery. Body weights were recorded at the same time.

5.4.12 Ultrasound assessment

Mice were subjected to left ventricular B-mode ultrasound at 14 days as previously described in 2.21.

5.4.13 Interstitial fibrosis assessment

At 14 days and following measurement of blood pressure and cardiac ultrasound the mice were subject to schedule one euthanasia with rising levels of CO₂. The hearts were harvested as described in 2.2 and preserved and processed as described in 2.3 and 2.5. Six μ m thick paraffin sections were cut from the upper and lower surfaces of the middle transverse slice of the heart and stained with picrosirius red. Interstitial collagen was measured as described in 2.22 using the colour picker technique 2 and

the area occupied by collagen was calculated as a percentage of the total area of the left ventricular free wall, inter-ventricular septum and right ventricular free wall.

5.4.14 Assessment of myocardial gene expression

Frozen tissue from the apex of each heart was thawed and homogenized. RNA was extracted from the homogenate and reverse-transcribed as described in 2.13 and 2.14. The mRNA expression of pro-fibrotic genes was measured by qRT-PCR as described in 2.16.

5.5 Results

5.5.1 Antibody labelling reveals appropriate PDGFR- β reporting in mTmG reporter mice

In the left and right ventricles of mTmG reporter mouse hearts over 90% of the eGFP⁺ cells also labelled with the anti-PDGFR- β antibody. Over 90% of the cells identified as PDGFR- β ⁺ by antibody binding were also found to express eGFP (Figure 5-2).

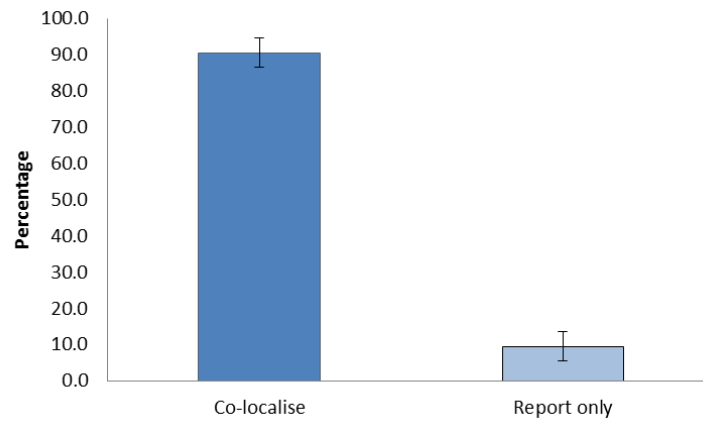


Figure 5-1 Histogram illustrating the percentage of eGFP reporting cells in the left and right ventricles of mTmG mouse heart sections that also labelled with anti-PDGFR- β antibody. 91% ($\pm 4.1\%$) of eGFP reporting cells were anti-PDGFR- β antibody positive whilst only 9% ($\pm 4.1\%$) expressed the reporter signal only (n=6). Columns represent mean $\pm$ SEM.

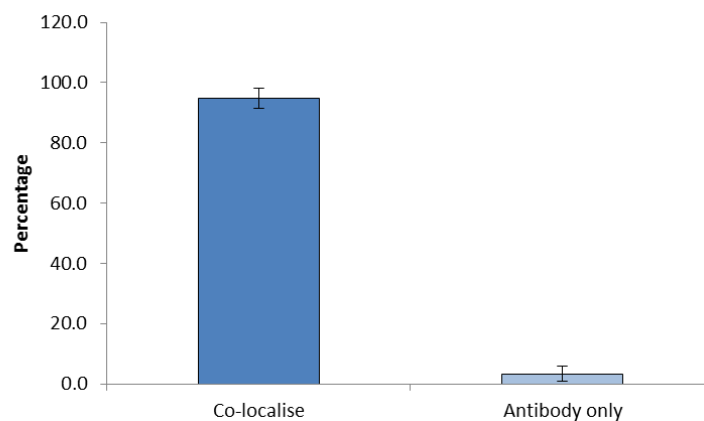


Figure 5-2 Histogram illustrating the percentage of anti-PDGFR- β antibody positive cells in the left and right ventricles of mTmG reporter mouse heart sections that also express the eGFP reporter signal. 94.8% ($\pm 3.4\%$) of anti-PDGFR- β antibody positive cells also report eGFP whilst only 3.3% ($\pm 2.5\%$) expressing the antibody signal only (n=6). Columns represent mean $\pm$ SEM.

5.5.2 Modest numbers of PDGFR- β ⁺ cells were obtained on FACS of digested mouse ventricles

PDGFR- β ⁺ cells were collected from unstained mTmG reporter mouse hearts. As expected, the majority of digested cells were td tomato⁺ and did not express eGFP. The eGFP⁻/td tomato⁻ population (Figure 5-3) were considered likely to be erythrocytes that have survived the red cell lysis step. Within the eGFP⁺ population a subset of cells co-expressing td tomato was also seen, however on examination of tissue sections co-expression of red and green fluorescence was not detected. These events were therefore considered likely to be eGFP⁺ cells bound to fragments of red fluorescent membrane as a result of incomplete digestion of adjacent cells. In order to avoid contamination only the GFP⁺/td tomato⁻ population was collected and approximately 150,000 cells were obtained per heart. Post-sort purity checks revealed consistent expression of GFP and the absence of td tomato expression in the collected population.

PDGFR- β ⁺ cells were also isolated from non-reporting α_v -integrin cre⁺ and cre⁻ mouse hearts using anti PDGFR- β (CD140 β) antibodies. Approximately 120,000 cells were obtained per heart. Post-sort purity check revealed consistent expression of PDGFR- β in collected cells.

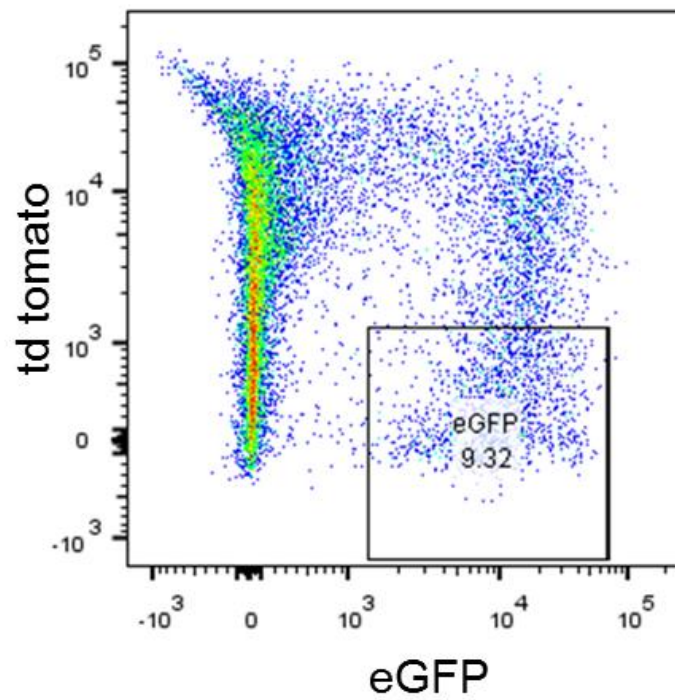


Figure 5-3 Representative dot plot of FACS gating strategy applied to unstained cells from the digested ventricles of mTmG reporter mouse hearts. The majority of fluorescent cells were negative for eGFP. Within the eGFP⁺ population a double positive eGFP⁺/td tomato⁺ subpopulation was present however only eGFP⁺/td tomato⁻ cells were collected (black square) (n>60).

5.5.3 PDGFR- β ⁺ cells in the normal myocardium reside in the perivascular compartment and are a heterogeneous population

In order to determine which cell types express PDGFR- β in normal myocardium the hearts of uninjured mTmG and Ai14 reporter mice were examined for PDGFR- β expression and co-expression of other cell surface markers by both flow cytometry and immunostaining.

5.5.3.1 PDGFR- β expression

DAB staining revealed PDGFR- β expression in cells within the pauci-cellular interstitium (endomysium) surrounding cardiomyocytes (Figure 5-4). Fluorescence microscopy confirmed the location of GFP reporting cells in the endomysium closely resembling the pattern of endothelial staining of capillaries. Rare cardiomyocytes also expressed PDGFR- β (Figure 5-5). At the endocardial surface PDGFR- β staining was evident within the narrow subendocardial layer and also appeared to be present within occasional endothelial cells (Figure 5-6). Within muscular coronary arteries PDGFR- β immunostaining labelled cells within the loose connective tissue of the adventitia and occasional cells within the media (Figure 5-7) whilst in mTmG heart sections there was widespread reporting of PDGFR- β within the smooth muscle cells of the media (Figure 5-8). This suggests a discrepancy between PDGFR- β reporter expression and the sensitivity of the antibody labelling. Reporting of cells in the media of coronary arteries was also incomplete however with a subset of cells not expressing PDGFR- β (Figure 5-8).

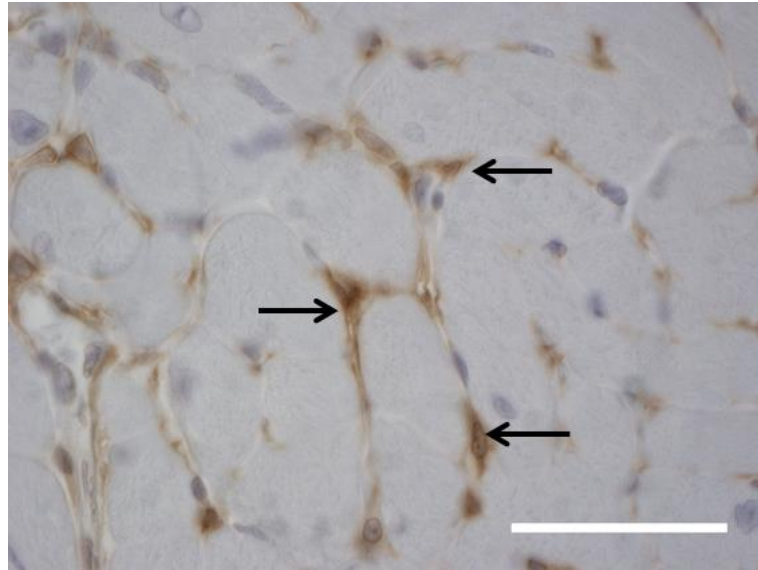


Figure 5-4 Representative brightfield photo-micrograph illustrating DAB staining of the normal myocardium using an anti-PDGFR- β antibody. Black arrows indicate PDGFR- β^+ cells within the endomysium between myofibres (n=3). Scale bar = 50um.

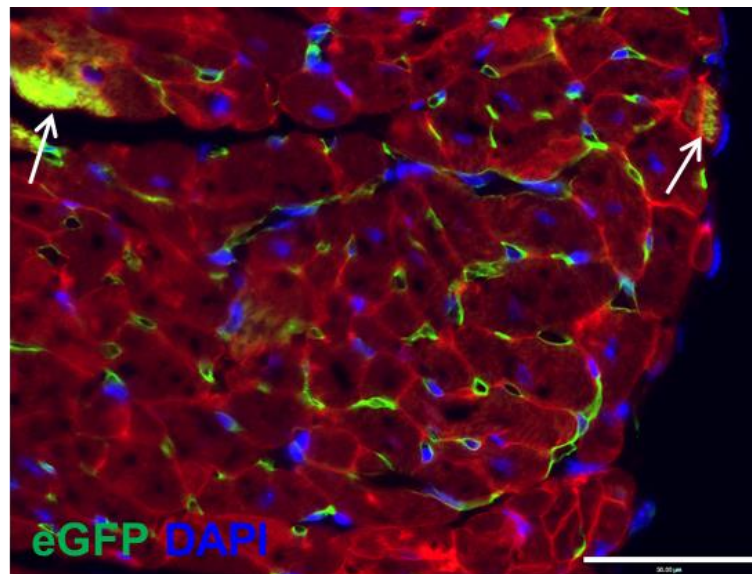


Figure 5-5 Representative wide field immunofluorescence image of mTmG reporter mouse normal myocardium. PDGFR- β^+ cells are eGFP $^+$ and fluoresce green whilst all remaining cells fluoresce red. Most eGFP $^+$ cells are within the endomysium between fibres with only rare muscle fibres expressing eGFP (white arrows) (n=3). DAPI nuclear staining in blue. Scale bar = 50 μ m.

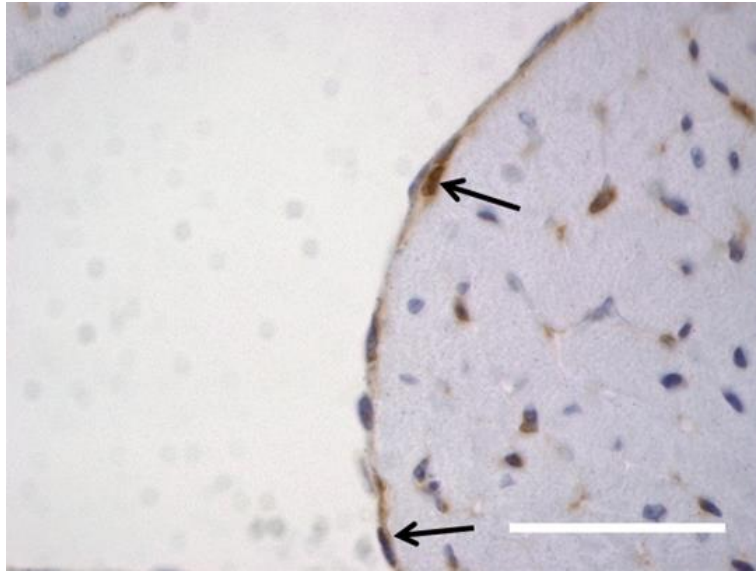


Figure 5-6 Representative brightfield photo-micrograph illustrating DAB staining of the normal myocardium using an anti-PDGFR- β antibody. Black arrows highlight PDGFR- β ⁺ cells that appeared to be both in the endocardial and subendocardial layers (n=3). Scale bar = 50 μ m.

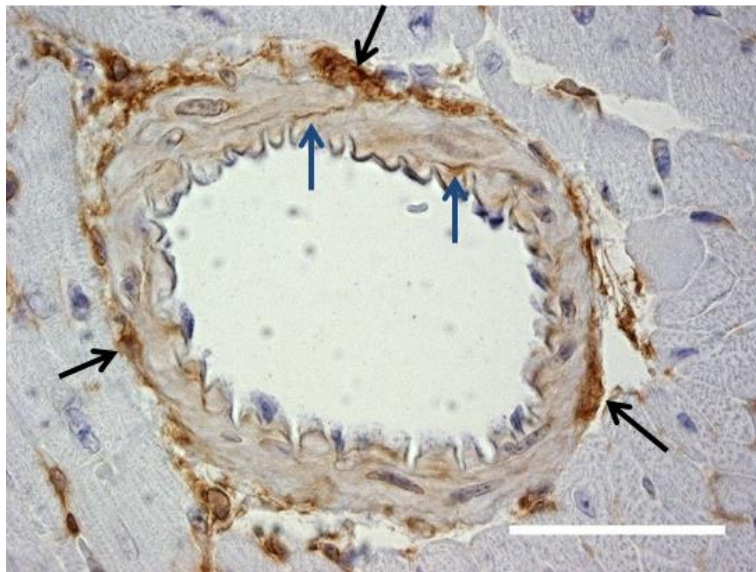


Figure 5-7 Representative brightfield photo-micrograph illustrating DAB staining of a normal coronary artery using an anti-PDGFR- β antibody. Black arrows indicate PDGFR- β ⁺ cells within the adventitia. Blue arrows indicate occasional weaker staining cells within the media (n=3). Scale bar = 50 μ m.

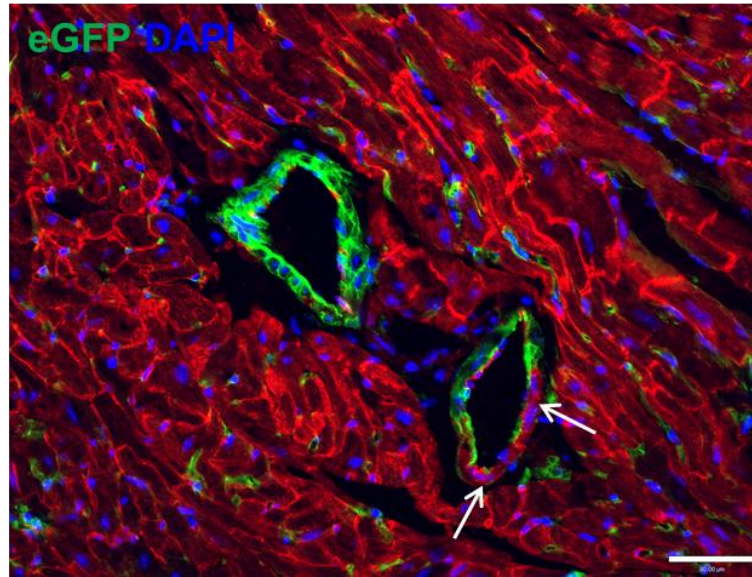


Figure 5-8 Representative wide field immunofluorescence image of mTmG reporter mouse normal myocardium. PDGFR-β⁺ cells express eGFP and fluoresce green whilst all remaining cells fluoresce red. The majority of smooth muscle cells present within the media of coronary arteries are PDGFR-β⁺ however a subset are PDGFR-β⁻ (white arrows) (n=3). DAPI nuclear staining in blue. Scale bar = 50μm.

5.5.3.2 CD31/Isolectin-B4 co-labelling

Flow cytometry analysis of PDGFR- β^+ cells from digested mTmG reporter hearts revealed 15.3% (+/-0.96) of eGFP $^+$ cells to be positive for the mature endothelial marker CD31 (Figure 5-9). Low magnification wide field microscopic examination of myocardium immunostained with the endothelial marker Isolectin B4 revealed what appeared to be overlap of staining and reporting in a proportion of cells (Figure 5-10). More detailed examination with confocal microscopy however showed that there is close association between the endothelial and PDGFR- β^+ cells but no overlap of fluorescence signals (Figure 5-11).

5.5.3.3 CD146 co-labelling

CD146 expression was seen in 11% (+/- 2.5) of PDGFR- β^+ cells from digested mTmG reporter hearts on flow cytometry (Figure 5-12). Immunostaining demonstrated apparent co-localisation of CD146 labelling and the PDGFR- β reporting in a minority of cells, likely to be perivascular cells/pericytes, supporting the flow cytometry findings (Figure 5-13).

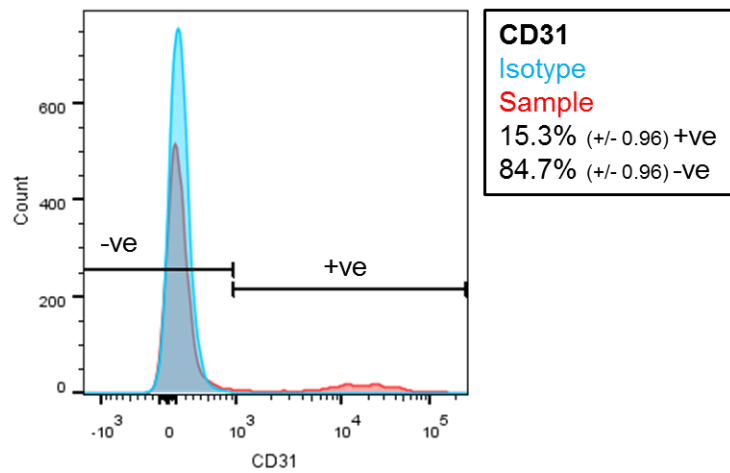


Figure 5-9 Representative flow cytometry histogram illustrating the presence of a minor CD31⁺ subpopulation of approximately 15% within the PDGFR- β ⁺ cell population isolated from digested normal mTmG reporter mouse hearts. The majority of PDGFR- β ⁺ cells are negative for CD31 (n=3).

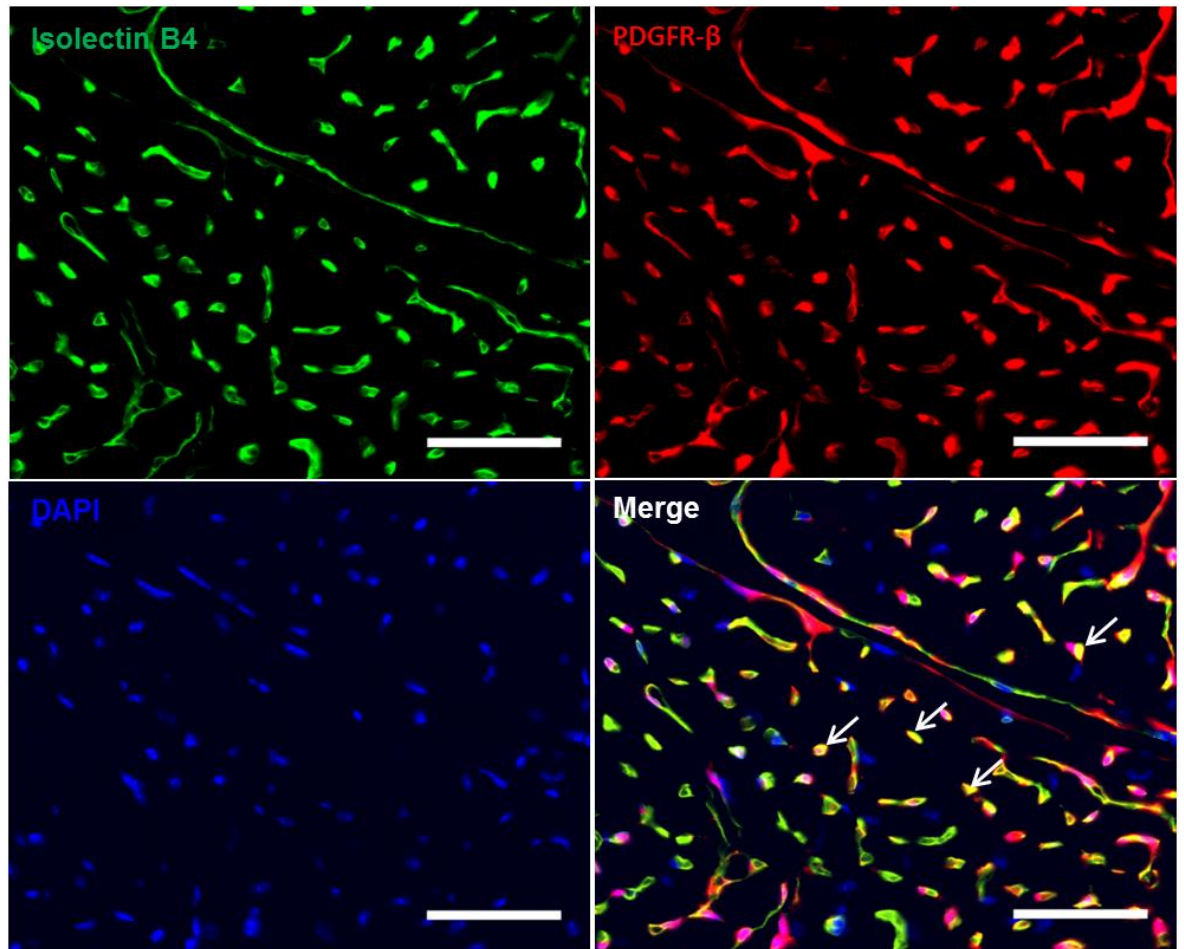


Figure 5-10 Representative wide field immunofluorescence image of anti-Isolectin B4 staining in normal Ai14 reporter mouse myocardium. On merged images there is apparent overlap of green fluorescence Isolectin B4 staining and red fluorescence PDGFR- β reporting in a minority of cells (white arrows) (n=3). DAPI nuclear staining in blue. Scale bars = 100 μ m.

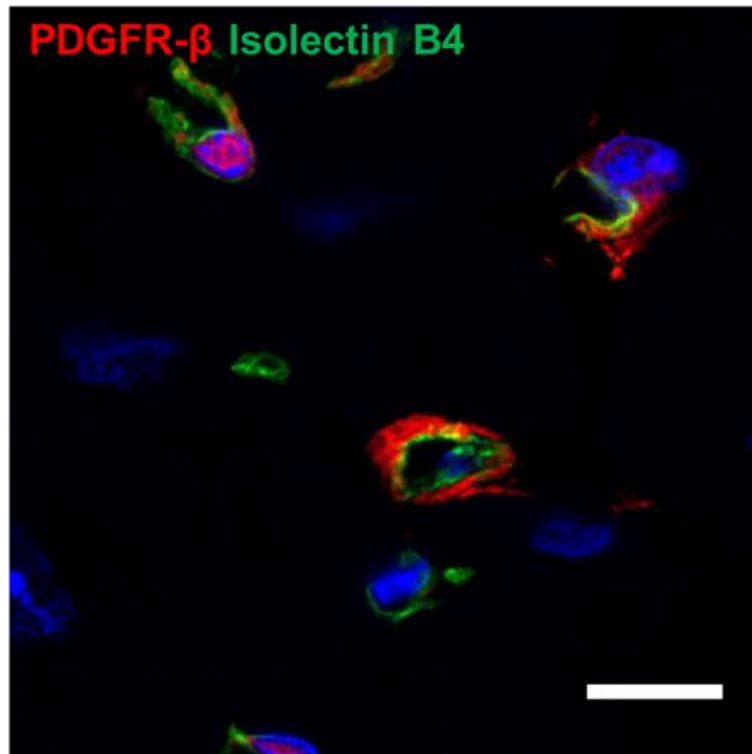


Figure 5-11 Representative confocal immunofluorescence image of anti-Isolectin B4 staining in normal Ai14 reporter mouse myocardium. Red fluorescent PDGFR- β^+ reporting cells wrap around green fluorescent Isolectin B4⁺ labelled endothelial cells of capillaries. Close association but no overlap of staining is evident (n=3). DAPI nuclear staining in blue. Scale bar = 10 μ m.

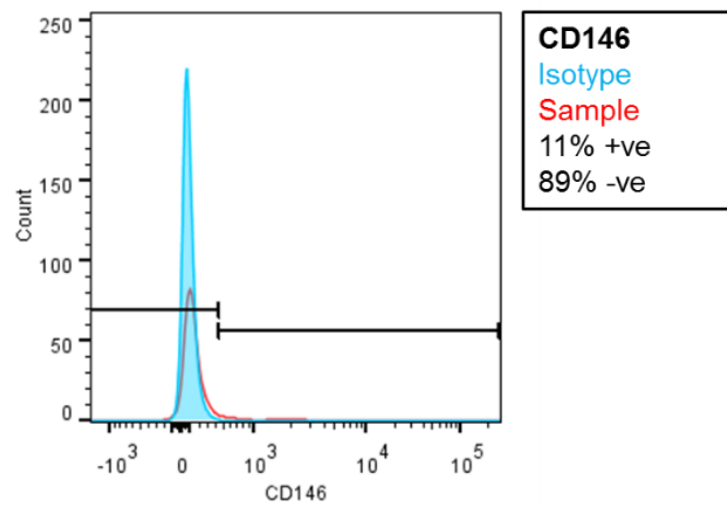


Figure 5-12 Representative flow cytometry histogram illustrating the presence of a minor CD146⁺ subset of approximately 11% within the PDGFR- β ⁺ cell population isolated from digested normal mTmG reporter mouse hearts. The majority of PDGFR- β ⁺ cells are negative for CD146 (n=3).

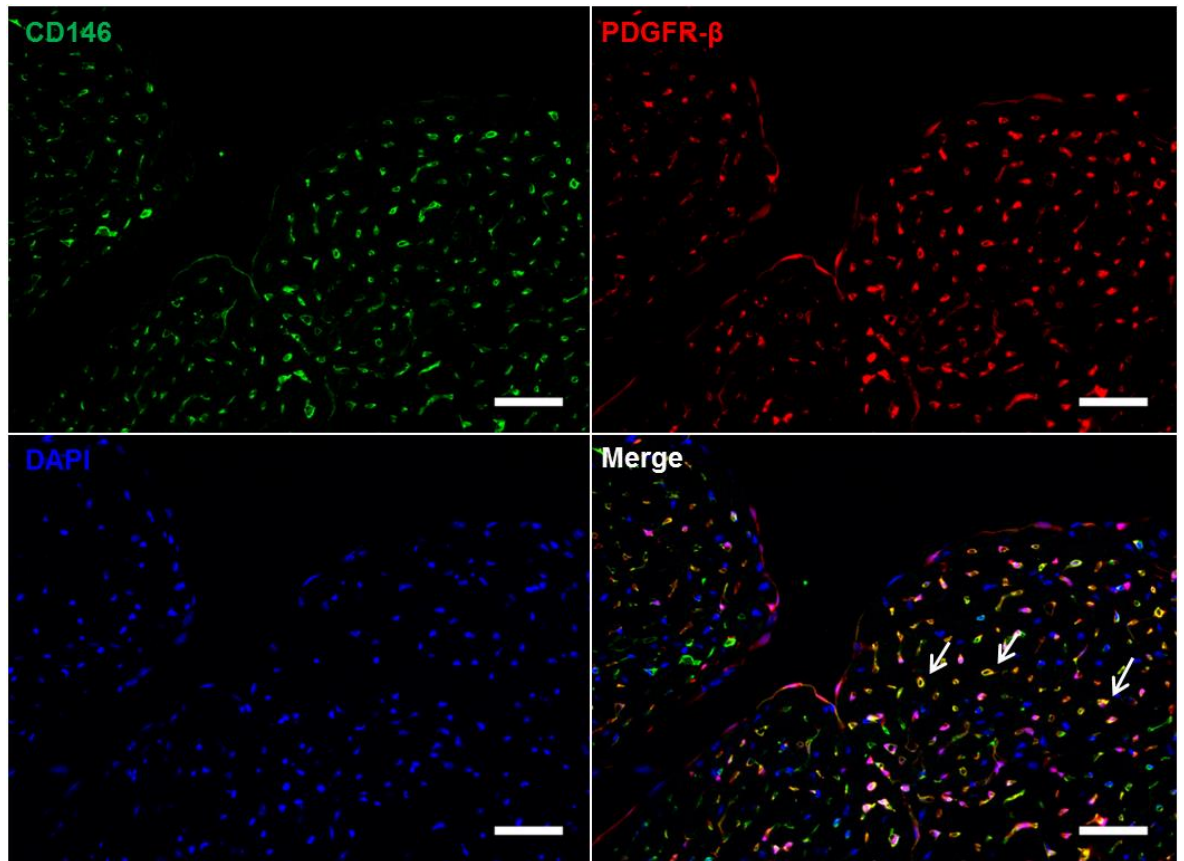


Figure 5-13 Representative wide field immunofluorescence image of anti-CD146 staining in Ai14 normal reporter mouse myocardium. On the merged image a subpopulation of green fluorescent CD146⁺ labelled cells co-localises with red fluorescent PDGFR- β reporting cells (white arrows) (n=3). DAPI nuclear staining in blue. Scale bars = 100 μ m.

5.5.3.4 CD45 co-labelling

The common leukocyte antigen CD45 was detected in 14.7% (± 0.4) of eGFP⁺ cells by flow cytometry (Figure 5-14, A).

5.5.3.5 CD34 co-labelling

46.5% (± 1.7) of PDGFR- β ⁺ cells expressed CD34 on flow cytometry (Figure 5-14, B). Co-localisation of anti-CD34 labelling and the PDGFR- β ⁺ reporter signal was confirmed on tissue staining (Figure 5-15).

5.5.3.6 CD90.2 co-labelling

CD90.2 (Thy-1.2) was expressed in 16% (± 0.67) of PDGFR- β cells when analysed by flow cytometry (Figure 5-14, C).

5.5.3.7 CD56 co-labelling

CD56 was not detected on PDGFR- β ⁺ cells by flow cytometry (Figure 5-14, D).

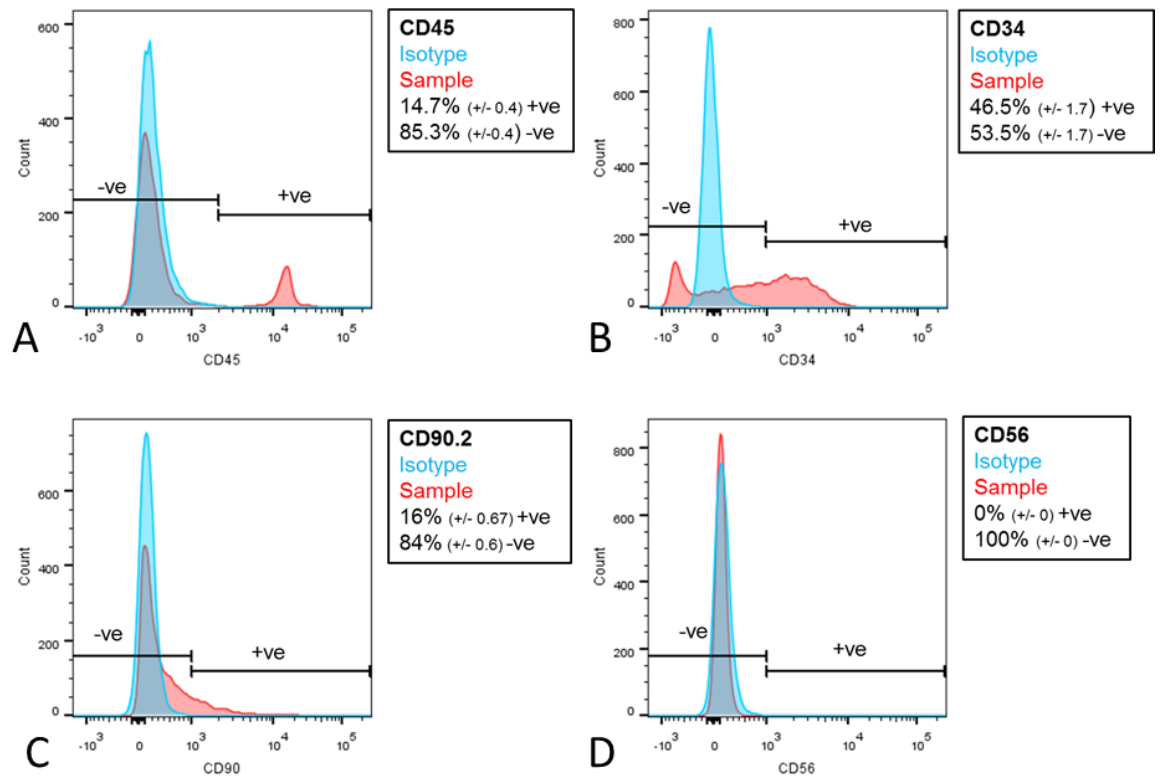


Figure 5-14 Representative flow cytometry histograms illustrating the presence of subpopulations of CD45 (A), CD34 (B) and CD90.2 (C) expressing cells within the PDGFR- β^+ population isolated from digested normal mTmG reporter mouse hearts. The CD34 $^+$ population forms a significant subset of approximately 47% whilst CD56 (D) expression was not detected (n=3).

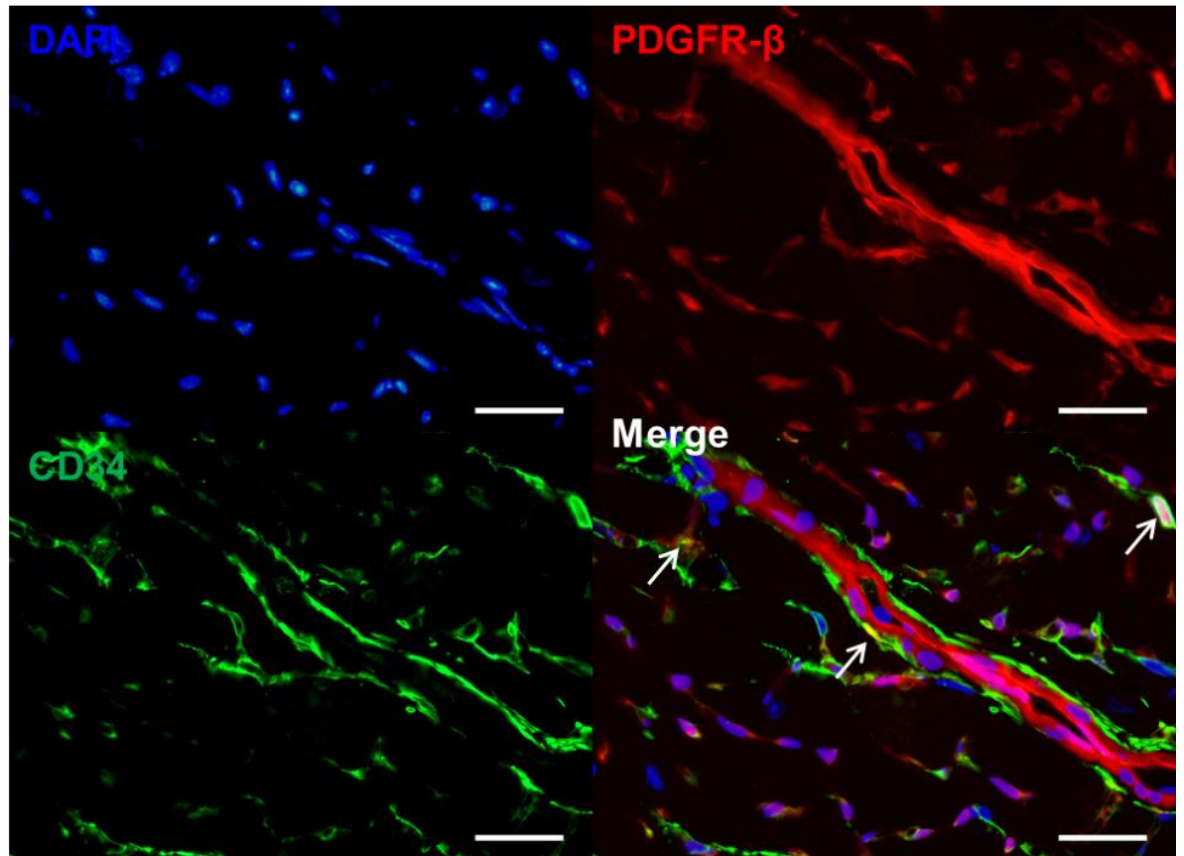


Figure 5-15 Representative wide field immunofluorescence image of green fluorescent anti-CD34 staining in normal Ai14 reporter mouse myocardium. On merged image yellow arrows indicate red fluorescent PDGFR- β^+ cells that also label for CD34 (n=3). Scale bars = 100 μ m.

5.5.3.8 PDGFR- α co-labelling

58.1% (± 1.08) of PDGFR- β^+ cells were positive for PDGFR- α on flow cytometry (Figure 5-16).

Combination staining and flow cytometry of PDGFR- β^+ cells found that PDGFR- α and CD34 co-expression was present in 33.6% (± 3.3) of the population with 8.4% (± 0.4) of cells expressing CD34 alone and 26.3% (± 1.8) expressing PDGFR- α alone (Figure 5-17).

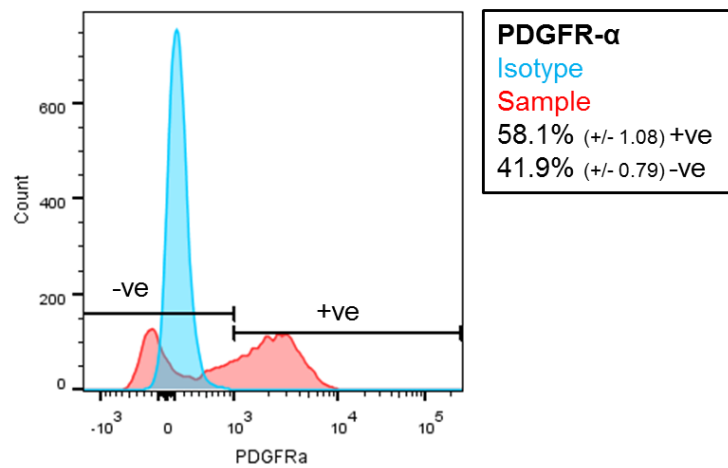


Figure 5-16 Representative flow cytometry histogram illustrating the presence of a substantial subset of PDGFR- α ⁺ cells within the PDGFR- β ⁺ population isolated from digested normal mTmG reporter mouse hearts (n=3).

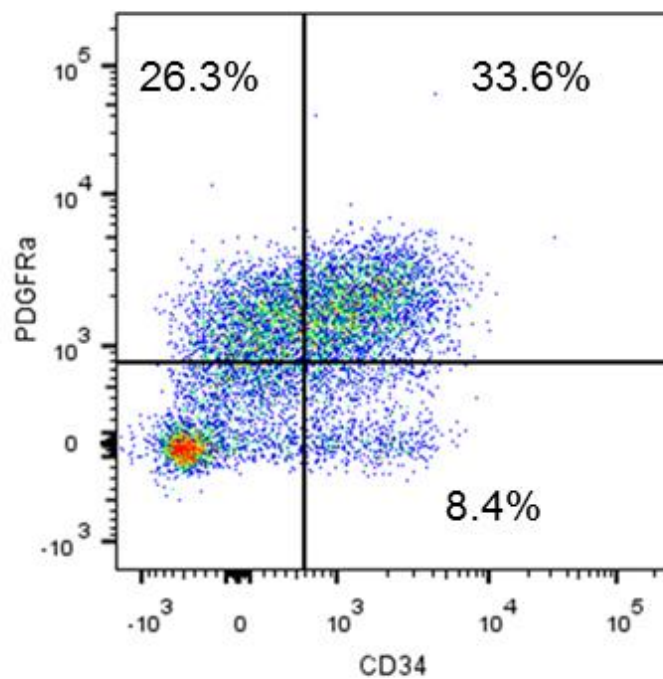


Figure 5-17 Representative flow cytometry density plot illustrating the presence of CD34⁺ and PDGFR α ⁺ single positive and double positive subpopulations within the PDGFR- β ⁺ population isolated from digested normal mTmG reporter mouse hearts (n=3).

5.5.3.9 Vimentin co-labelling

Vimentin staining of uninjured heart showed a similar pattern to that of PDGFR- β expression with tight restriction to the perivascular location and co-labelling in a subpopulation of cells (Figure 5-18, Figure 5-19, Figure 5-20). Flow cytometry analysis of vimentin expression was not carried out.

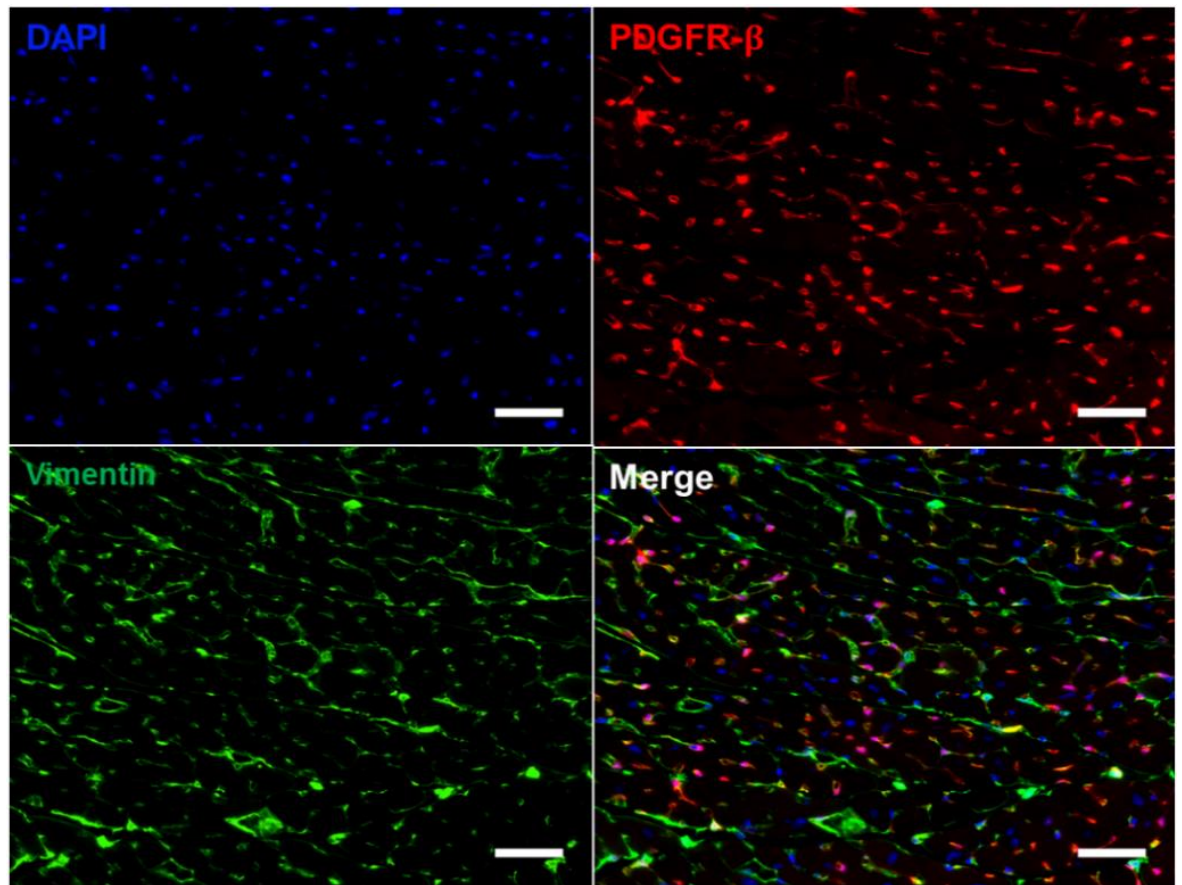


Figure 5-18 Representative wide field immunofluorescence image of green fluorescent anti-vimentin staining in normal Ai14 reporter mouse myocardium illustrating very similar patterns of vimentin labelling and red fluorescent PDGFR- β expression (n=3). DAPI nuclear staining in blue. Scale bars = 100 μ m.

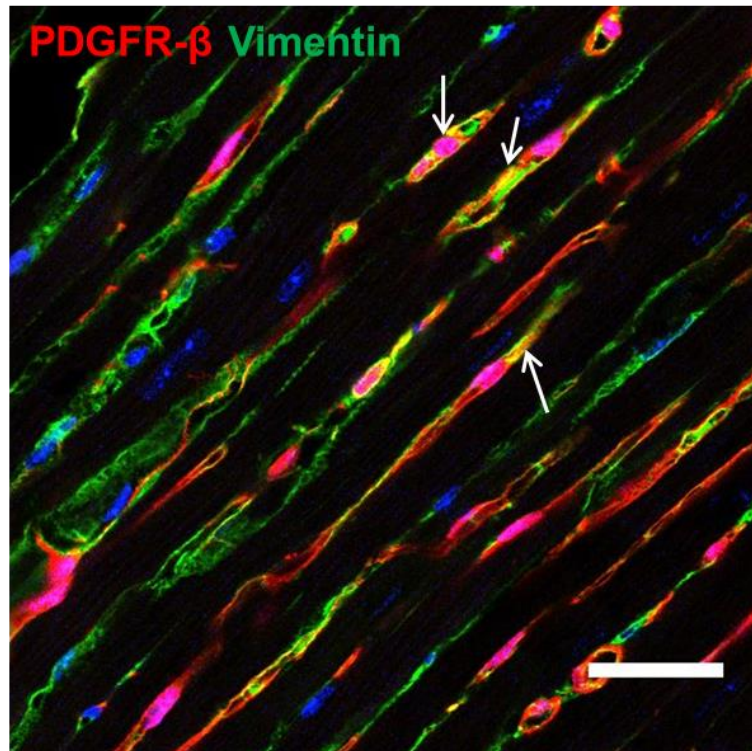


Figure 5-19 Confocal immunofluorescence image of green fluorescent anti-vimentin staining in normal Ai14 reporter mouse myocardium. Co-localisation of staining with red fluorescent PDGFR- β reporting in a subpopulation of cells is highlighted by the white arrows (n=3). DAPI nuclear staining in blue. Scale bar = 10 μ m.

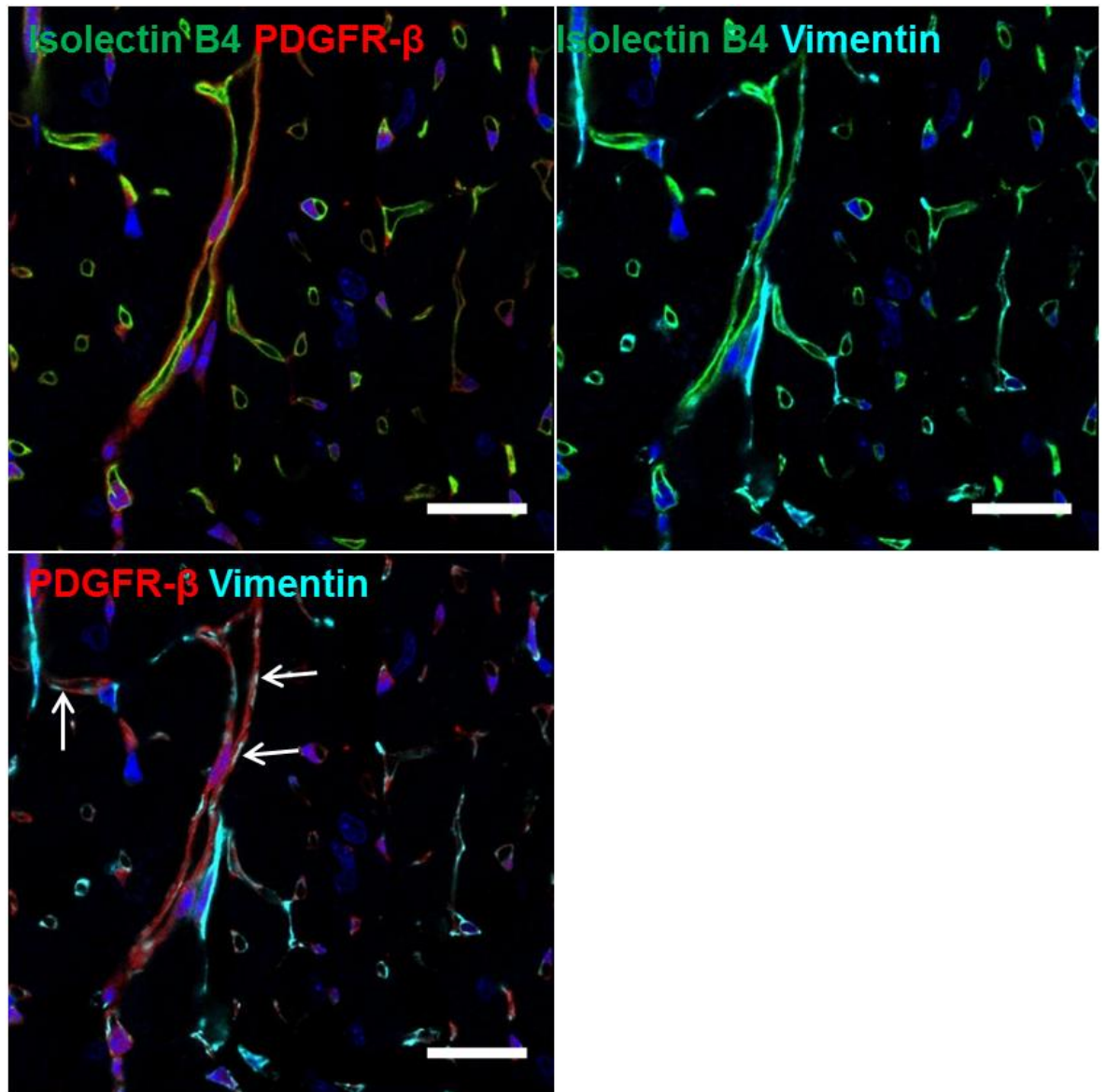


Figure 5-20 Representative confocal immunofluorescence image of cyan fluorescent anti-vimentin and green fluorescent anti-isolectin B4 staining in normal Ai14 reporter mouse myocardium. There is overlap of expression of red fluorescent PDGFR- β reporting with vimentin labelling in some (highlighted by white arrows) but not all perivascular cells (n=3). DAPI nuclear staining in blue. Scale bar = 10 μ m.

5.5.4 PDGFR- β is expressed by a subpopulation of fibroblasts in hearts of mice treated with angiotensin II

In order to determine whether activated fibroblasts in injured myocardium express PDGFR- β the hearts of mTmG and Ai14 reporter mice were subjected to 14 days treatment with 200ng/kg/minute angiotensin II (ang II) and examined for labelling of the fibroblast marker vimentin and myofibroblast marker α -SMA.

Picrosirius red staining of the myocardium demonstrated the presence of multifocal to coalescing interstitial collagen accumulates (Figure 5-21) which stained positively for collagen 1 by immunostaining (Figure 5-22). Large numbers of vimentin positive spindle shaped cells were present within these areas and were consistent with fibroblasts (Figure 5-23). Anti-GFP staining in ang II treated mTmG reporter mice confirmed PDGFR- β expression within the expanded interstitial fibroblast population (Figure 5-24). Anti-vimentin staining in mTmG mice revealed extensive labelling of cells within fibrotic regions with overlap with PDGFR- β expression in approximately 50% of cells (Figure 5-25, Figure 5-26).

Staining of ang II treated hearts revealed α -SMA expression in the media of variable size muscular arteries but not in cells in interstitial regions of fibrosis (Figure 5-27). Within the walls of muscular arteries α -SMA labelling was uniform throughout smooth muscle cells of the media. In contrast PDGFR- β expression with α -SMA co-labelling in the media was much more variable with approximately 1/4 to 1/3 of cells failing to express the PDGFR- β reporter signal (Figure 5-28).

No increase in numbers of PDGFR- β expressing cardiomyocytes was noted in ang II treated hearts.

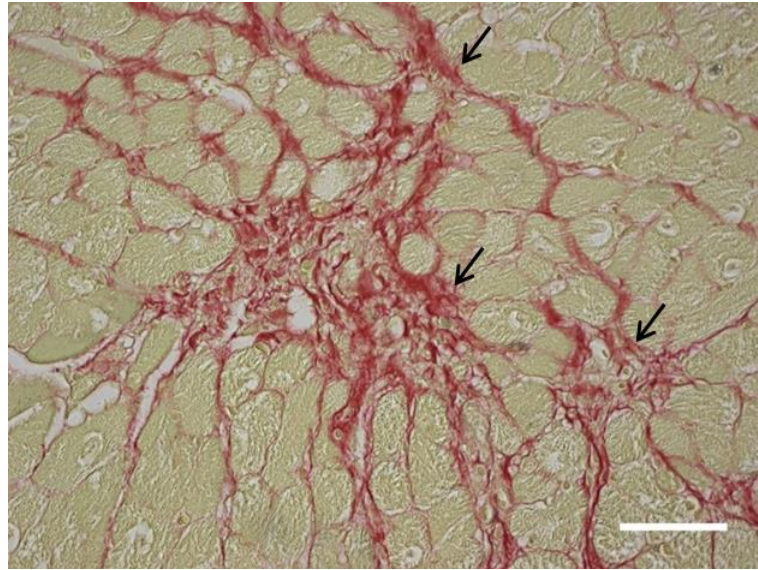


Figure 5-21 Representative brightfield photomicrograph of picosirius red stained myocardium from a mTmG reporter mouse treated for 14 days with 200ng/kg/min angiotensin II illustrating interstitial collagen (black arrows) within myocardium (n=3). Scale bar = 50 μ m.

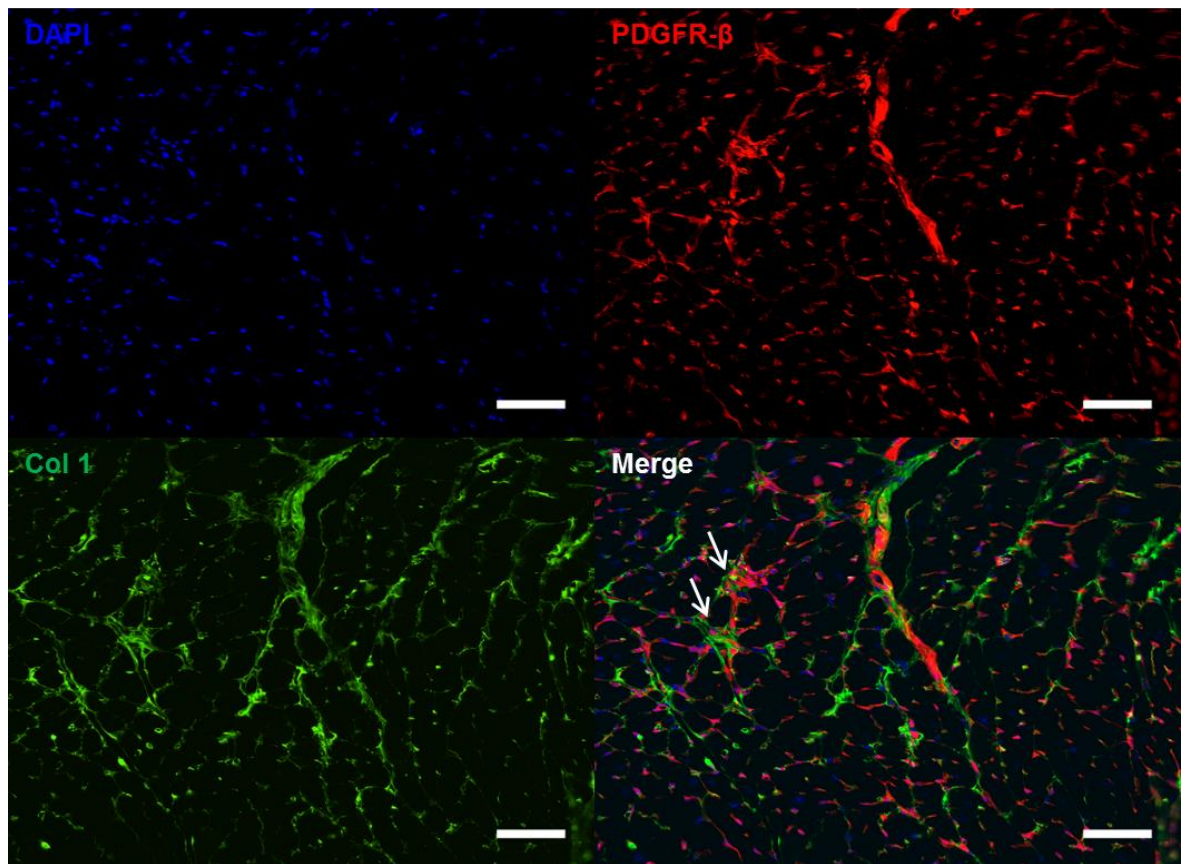


Figure 5-22 Representative wide field immunofluorescence image of green fluorescent anti-collagen 1 staining in the myocardium of an Ai14 reporter mouse treated for 14 days with 200ng/kg/minute angiotensin II. Merged image shows collagen 1 positive material in regions of expanded interstitial red fluorescent PDGFR- β staining (white arrows) (n=3). DAPI nuclear staining in blue. Scale bars = 100 μ m.

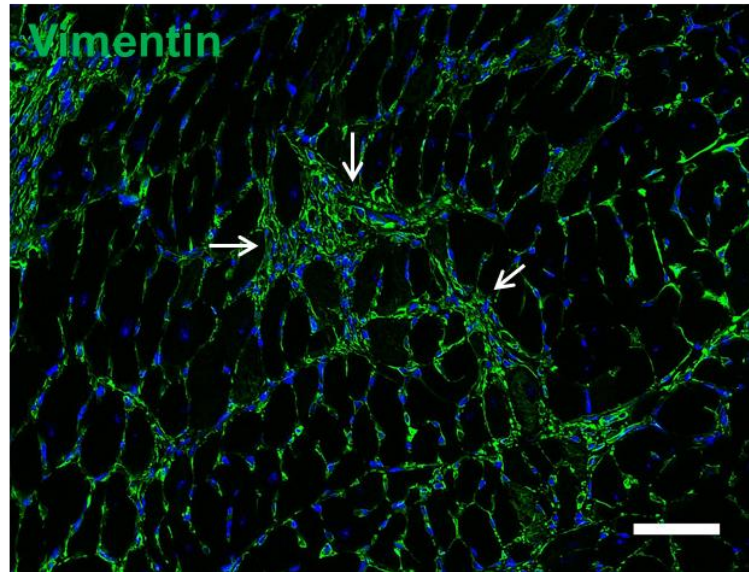


Figure 5-23 Representative immunofluorescence image of green fluorescent anti-vimentin staining in the myocardium of an Ai14 reporter mouse treated for 14 day with 200ng/kg/minute angiotensin II treated (green channel only) illustrating expansion of vimentin positive interstitial cell population (white arrows) (n=3). DAPI nuclear staining in blue. Scale bar = 100 μ m.

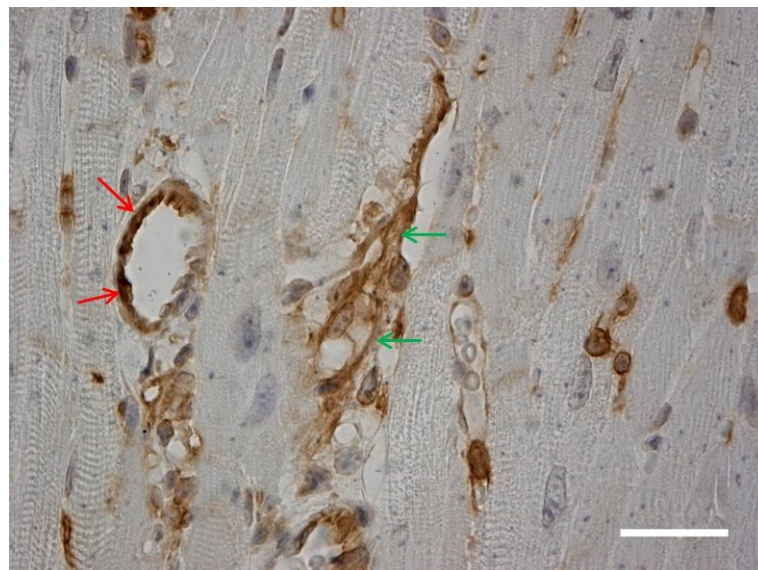


Figure 5-24 Representative brightfield photomicrograph of DAB staining using an anti-GFP antibody in the myocardium of a mTmG reporter mouse treated for 14 days with 200ng/kg/minute angiotensin II illustrating brown stained PDGFR- β^+ perivascular cells (red arrows) and interstitial fibroblasts (green arrows) (n=3). Scale bar = 25 μ m.

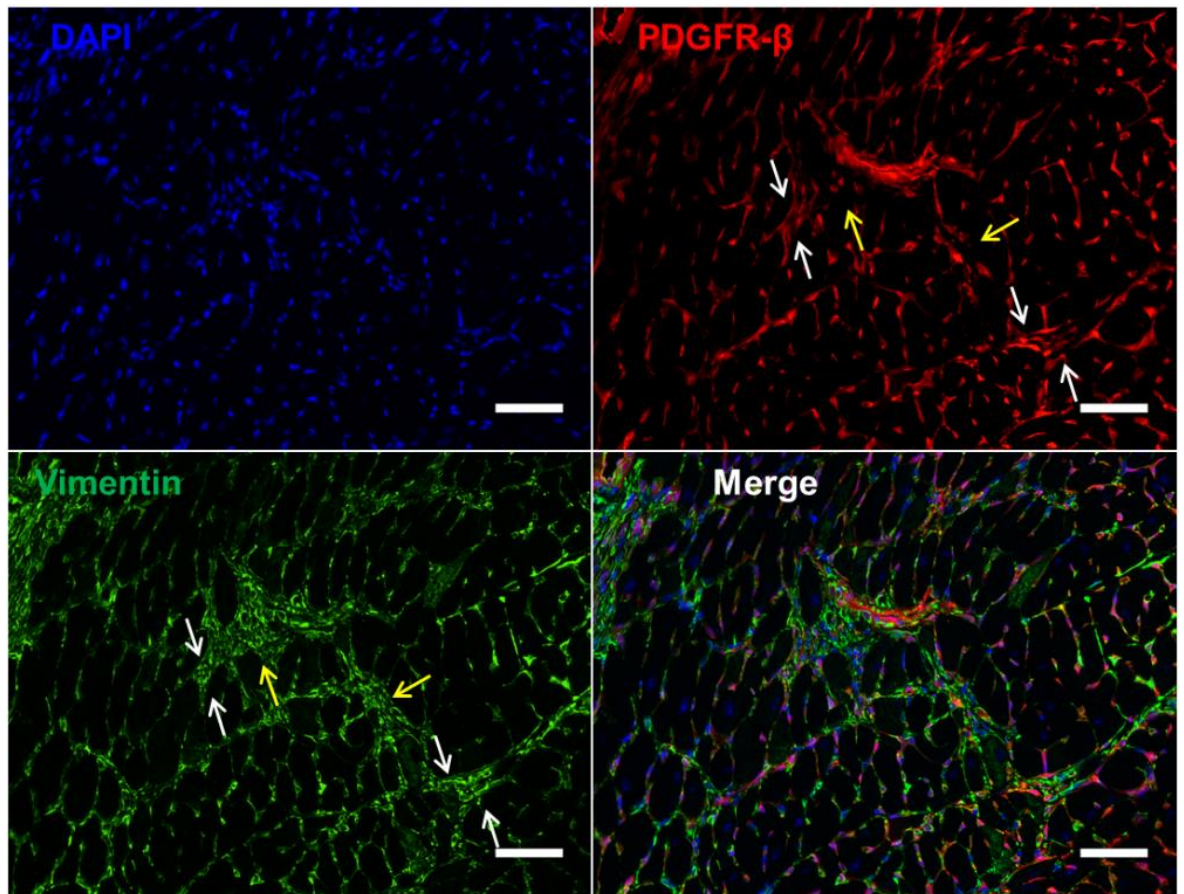


Figure 5-25 Representative immunofluorescence image of green fluorescent anti-vimentin staining of the myocardium in an Ai14 reporter mouse treated for 14 days with 200ng/kg/min angiotensin II. White arrows indicate vimentin expression in red fluorescent PDGFR- β^+ interstitial fibroblasts. Yellow arrows indicate non-PDGFR- β expressing vimentin $^+$ fibroblasts (n=3). DAPI nuclear staining in blue. Scale bars = 100 μ m.

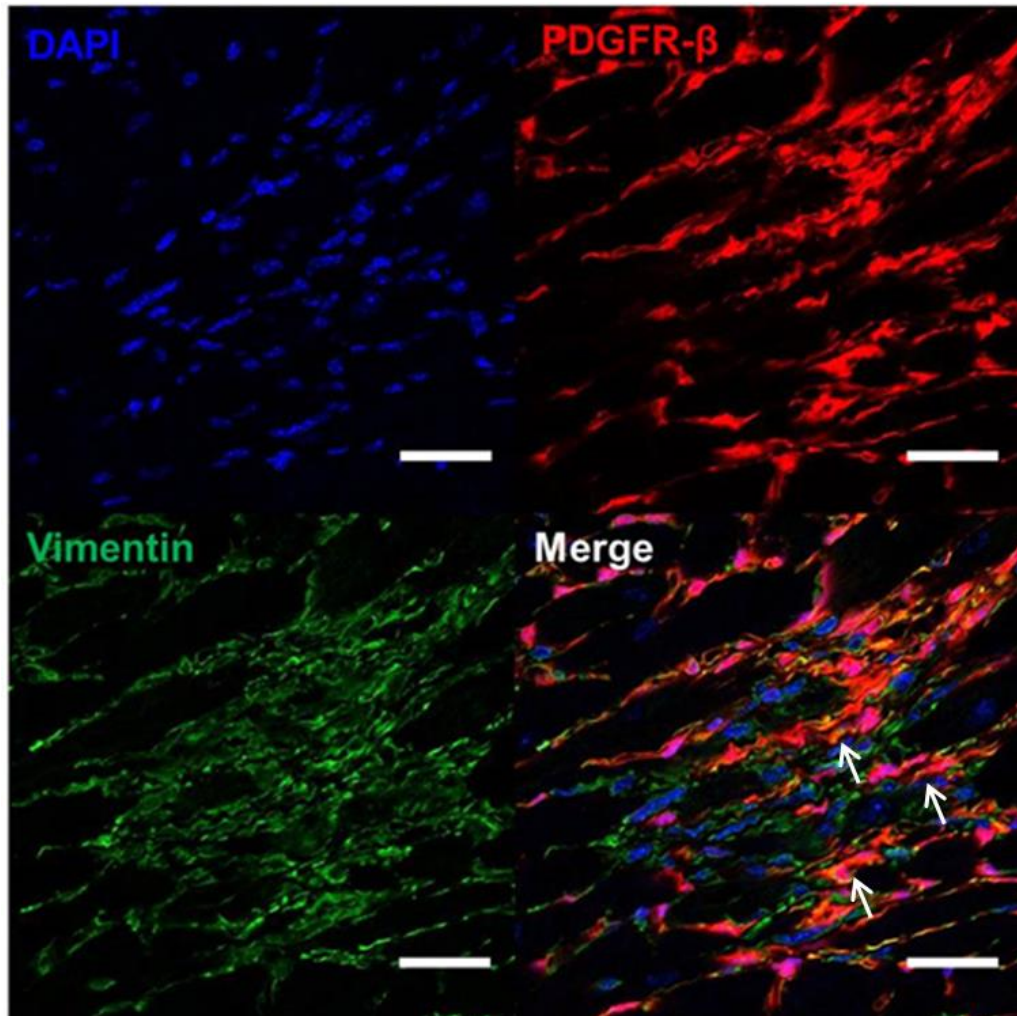


Figure 5-26 Representative confocal image of green fluorescent anti-vimentin staining of the myocardium in an Ai14 reporter mouse treated for 14 days with 200ng/kg/minute angiotensin II. In the merged image the white arrows highlight red fluorescent PDGFR- β expression by a subpopulation of vimentin⁺ fibroblasts (n=3). DAPI nuclear staining in blue. Scale bars = 10 μ m.

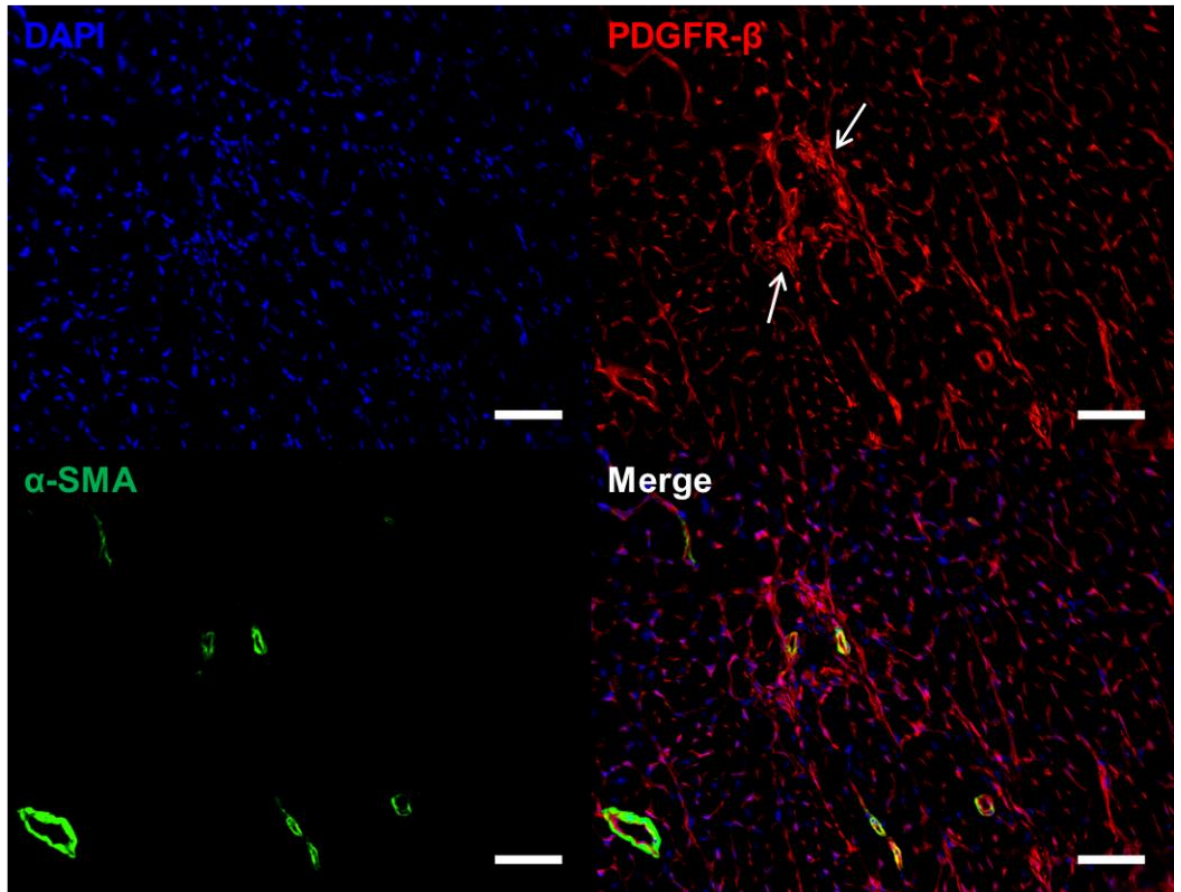


Figure 5-27 Representative immunofluorescence image of green fluorescent anti- α -SMA staining in an Ai14 reporter mouse treated for 14 days with 200ng/kg/minute angiotensin II. White arrows illustrate red fluorescent PDGFR- β ⁺ interstitial cells. α -SMA co-staining of this interstitial population is not seen (n=3). DAPI nuclear staining in blue. Scale bars = 100 μ m.

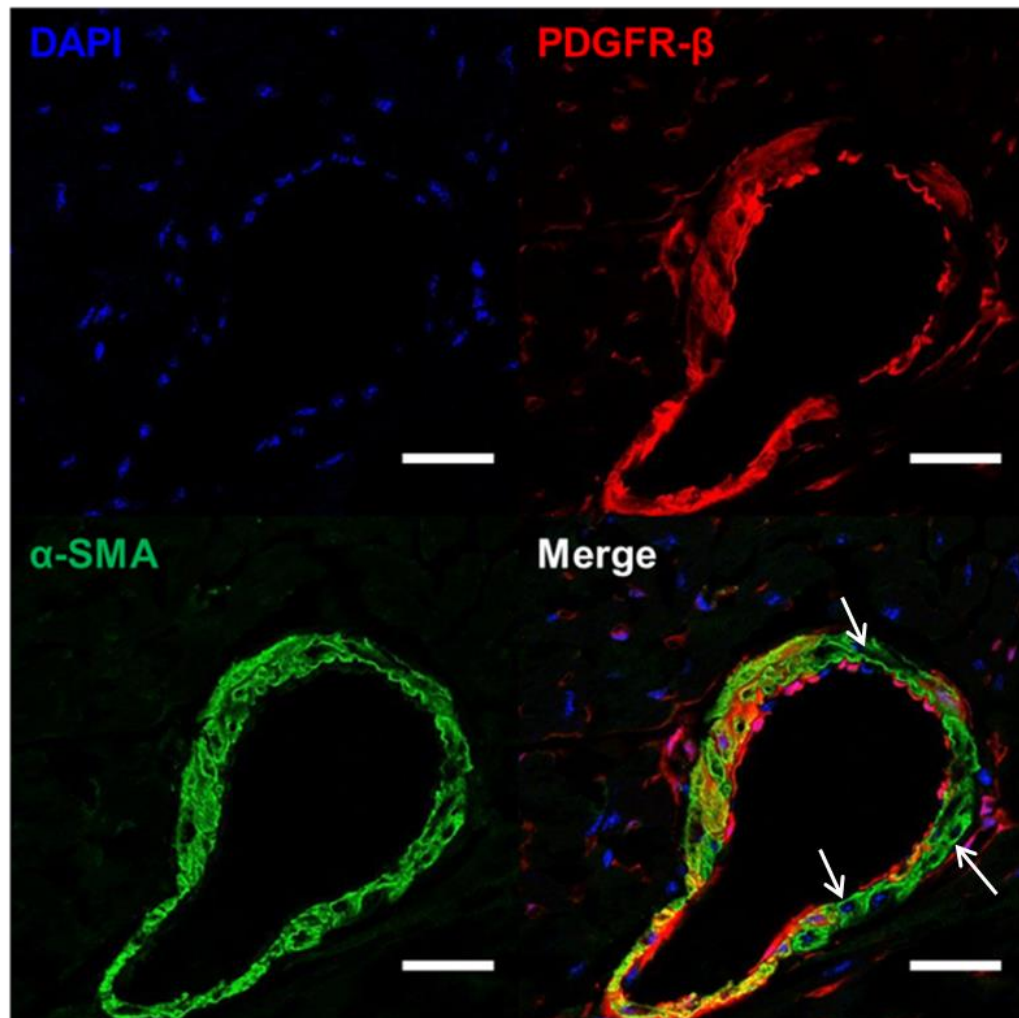


Figure 5-28 Representative immunofluorescence image of green fluorescent anti- α -SMA staining in an Ai14 reporter mouse treated for 14 days with 200ng/kg/minute angiotensin II. In merged image white arrows indicate α -SMA⁺ smooth muscle cells that do not express red fluorescent PDGFR- β reporter signal (n=3). DAPI nuclear staining in blue. Scale bars = 10 μ m.

5.5.5 PDGFR- β is expressed by a subset of cardiac fibroblasts isolated from the normal myocardium

Brightfield microscopic examination of primary cultured ventricular fibroblasts from the hearts of normal mTmG reporter mice revealed a relatively homogenous population with cells displaying a stellate morphology. When stained with an anti GFP antibody and examined under fluorescence approximately 20% of these cells labelled positively indicating PDGFR- β^+ expression (Figure 5-29).

Flow cytometry analysis of these cells confirmed this finding with 24.2% (+/-2.9) of cells expressing eGFP and were therefore PDGFR- β positive. Correspondingly 76.2% (+/-3.2) expressed td Tomato and were PDGFR- β negative (Figure 5-30). Only approximately 2.1% (+/-1.0) of the primary cultured fibroblast population expressed CD146 whilst 98.5% (+/- 0.1) of cells expressed PDGFR- α and 60% (+/- 0.5) expressed CD34 (Figure 5-31).

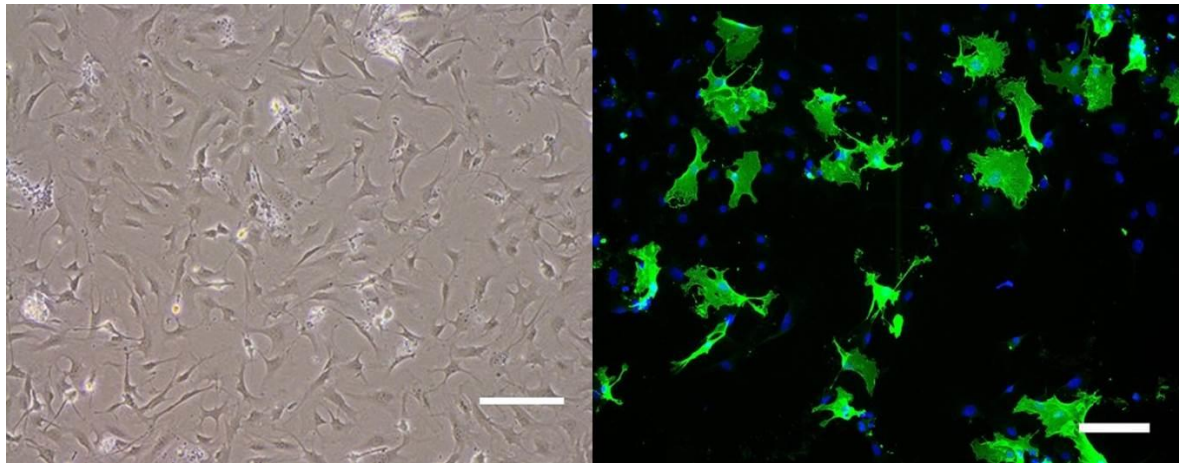


Figure 5-29 Representative brightfield and immunofluorescence images of primary cultured cardiac fibroblasts from normal mTmG reporter mouse hearts stained with an anti-GFP antibody. A minor subset of cells express eGFP and are thus PDGFR- β^+ (n=3). DAPI nuclear staining in blue. Brightfield scale bar = 20 μ m. Immunofluorescence scale bar = 10 μ m.

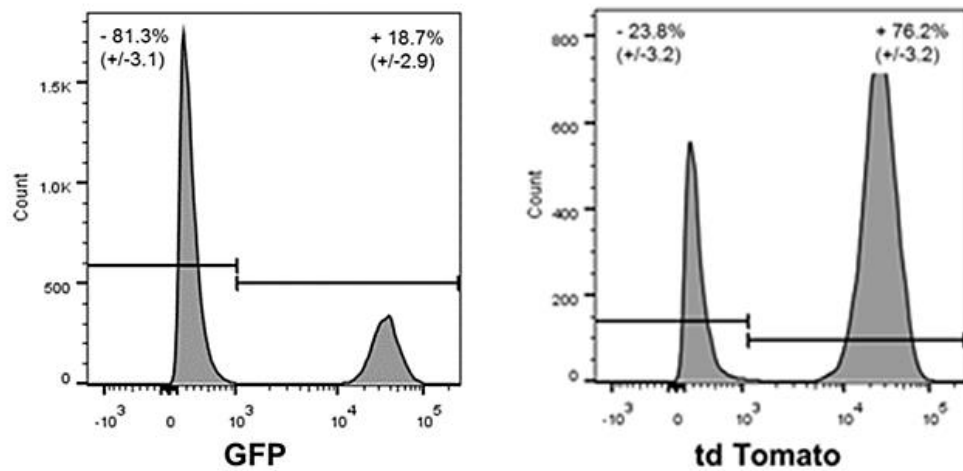


Figure 5-30 Representative flow cytometry histograms illustrating eGFP and td tomato expression by cardiac fibroblasts isolated from the hearts of normal mTmG reporter mice. The majority of cells are td tomato $^+$ with a subset of approximately 20% of eGFP $^+$ cells present (n=3).

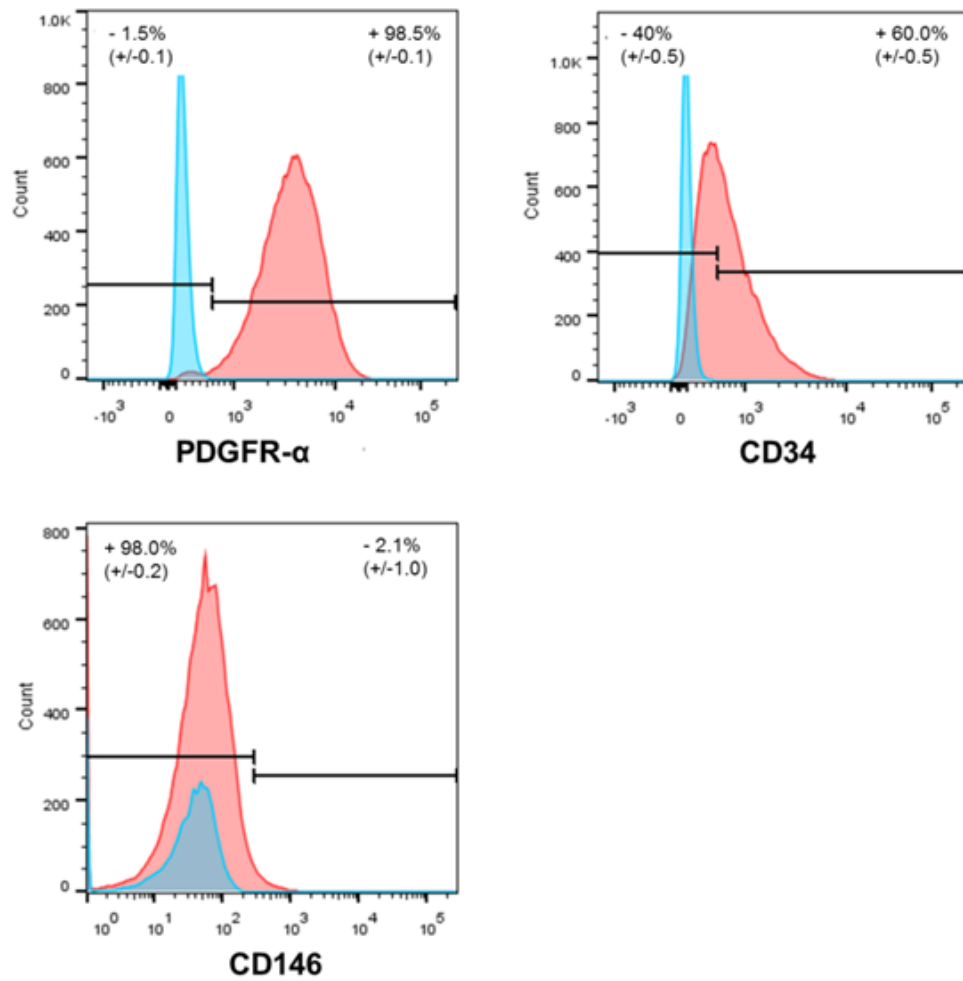


Figure 5-31 Representative flow cytometry histograms illustrating expression of PDGFR- α , CD34 and CD146 in a subsets of the cardiac fibroblast population isolated from the hearts of normal mTmG reporter mice. The majority of fibroblasts (approximately 99%) were PDGFR- α^+ whilst a major subset of approximately 60% were CD34 $^+$. No CD146 expression was present (n=3).

5.5.6 PDGFR- β perivascular cells appear to be pro-fibrotic *in-vitro*

To investigate whether PDGFR- β^+ perivascular cells have the potential to become pro-fibrotic *in vitro* eGFP $^+$ cells were isolated from mTmG reporter mice and cultured before being assessed for up-regulation of key pro-fibrotic genes and proteins.

Despite around 150,000 to 200,000 cells being obtained, RNA yields from freshly sorted cells were consistently low at around 50ng total. 260:280 ratios were generally good at between 1.8 and 2.0. 260:230 ratios were poor but considered unreliable due to the low concentration of the sample. None of the modifications tried to the technique significantly improved RNA quality or yield and consequently at the time of writing only a single good quality 0 day RNA sample was available for comparison. RNA yields were significantly better in the later cultured samples even after only a few days in culture. qRT-PCR analysis for pro-fibrotic markers showed evidence of increased relative expression of key markers collagen 1 alpha, vimentin and DDR2 over the 15 day culture period (Figure 5-32). However relative expression levels were not particularly high, never exceeding a 2.5 fold increase.

eGFP $^+$ cells sorted from three mTmG mice were cultured for 24 hours in EGM2 labelled with antibodies against α -SMA, FAP, DDR2 and vimentin. Strong expression of all four pro-fibrotic markers was evident in the majority of cells (Figure 5-33).

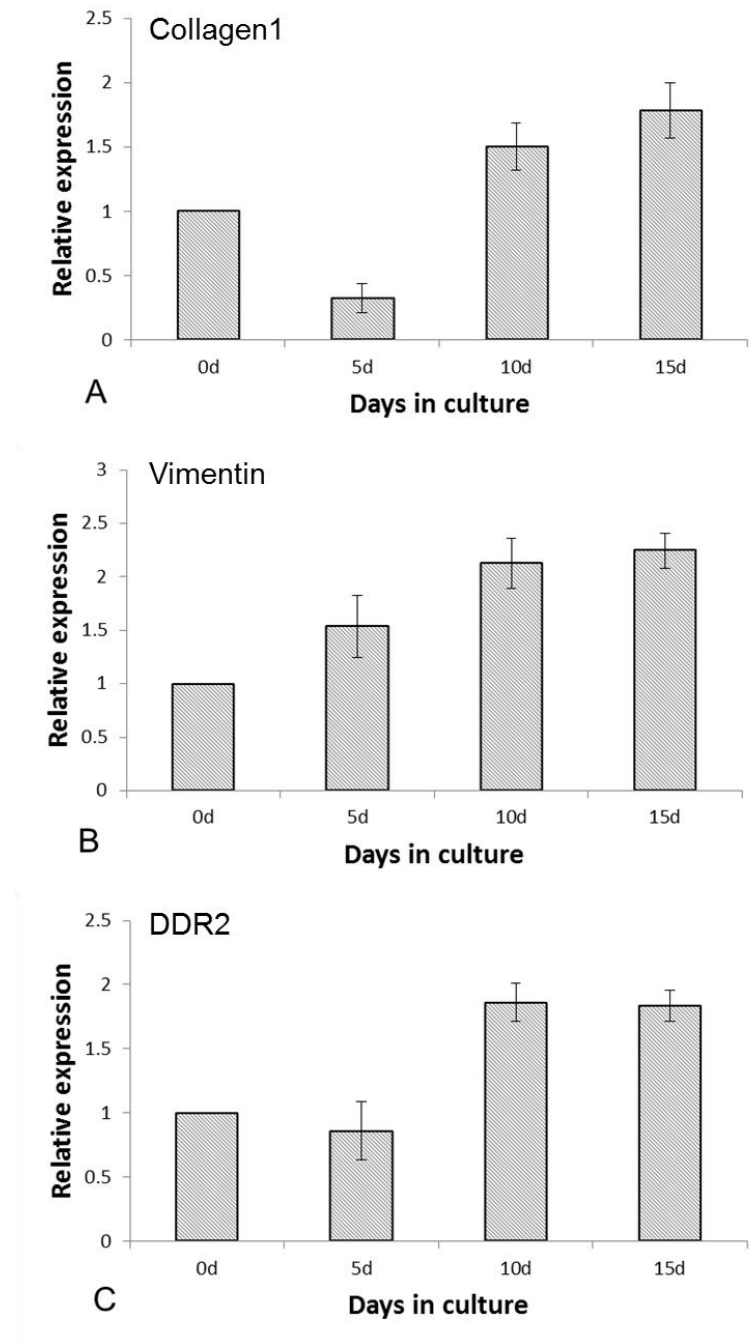


Figure 5-32 Relative expression of key pro-fibrotic genes by PDGFR- β^+ cells isolated from normal mTmG reporter mouse hearts and cultured for up to 15 days in EGM2 media. Minor fold increases were seen in the relative mRNA expression of: A - collagen 1; B – vimentin; C - DDR2. Columns represent mean $\pm$ SEM. 0d n=1, 5d n=2, 10d n= 3, 15d n= 3. No statistical analysis performed due to insufficient numbers in day 0 and 5 groups.

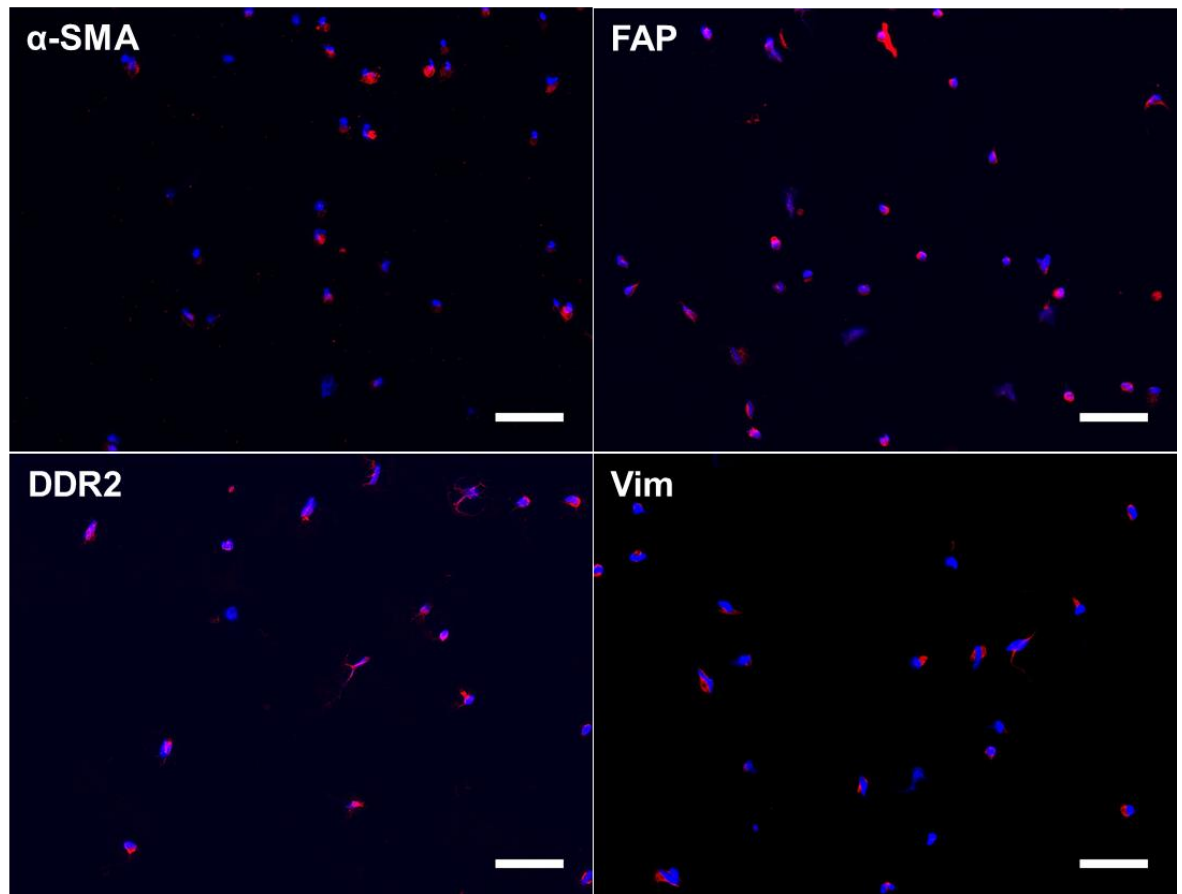


Figure 5-33 Representative wide field immunofluorescence images illustrating widespread expression of the pro-fibrotic proteins in PDGFR- β^+ cells isolated from mTmG reporter mice and cultured in EGM2 media for 24 hours. Alpha smooth muscle actin (α -SMA); fibroblast activated protein (FAP); discoid domain receptor 2 (DDR2); and vimentin (Vim) (n=3). DAPI nuclear staining in blue. All scale bars = 25 μ m.

5.5.7 Down regulation of α_v integrin does not appear to alter the expression of pro-fibrotic genes by PDGFR- β^+ cells *in-vitro*

5.5.7.1 Genetic deletion of α_v integrin on PDGFR- β^+ cells does not appear to reduce pro-fibrotic gene expression *in-vitro*

PDGFR- β^+ cells were isolated from the hearts of α_v integrin knockout (α_v integrin cre⁺) and wild type mice (α_v integrin cre⁻) and their expression of pro-fibrotic genes assessed after a period in culture.

One of the three α_v integrin cre⁺ samples failed to grow leaving two samples in the knockout group and three in the wild type group. Due to low sample numbers statistical analysis was not performed however no substantial differences in pro-fibrotic gene expression were noted between the cre⁺ knockout and cre⁻ wild type cells cultured in EGM2 for 2 days then 10% FBS DMEM 1% P/S for 4 days (Figure 5-34).

5.5.7.2 Small molecule inhibition of α_v integrin on PDGFR- β^+ cells does not appear to reduce pro-fibrotic gene expression *in-vitro*

PDGFR- β^+ cells were isolated from the hearts of mTmG mice and exposed to a small molecule α_v integrin inhibitor (C12) or control enantiomer (C96) and again assessed for expression of pro-fibrotic genes.

The results of the mRNA expression study in inhibitor treated cells were contradictory. No statistically significant differences were seen between inhibitor treated and control treated cells with regards the majority of pro-fibrotic genes. However, in inhibitor treated cells α -SMA expression was increased by approximately 25% and there was also a minor but statistically significant increase in PDGFR- β expression. DDR2 expression however was reduced by approximately 20% in this group (Figure 5-35).

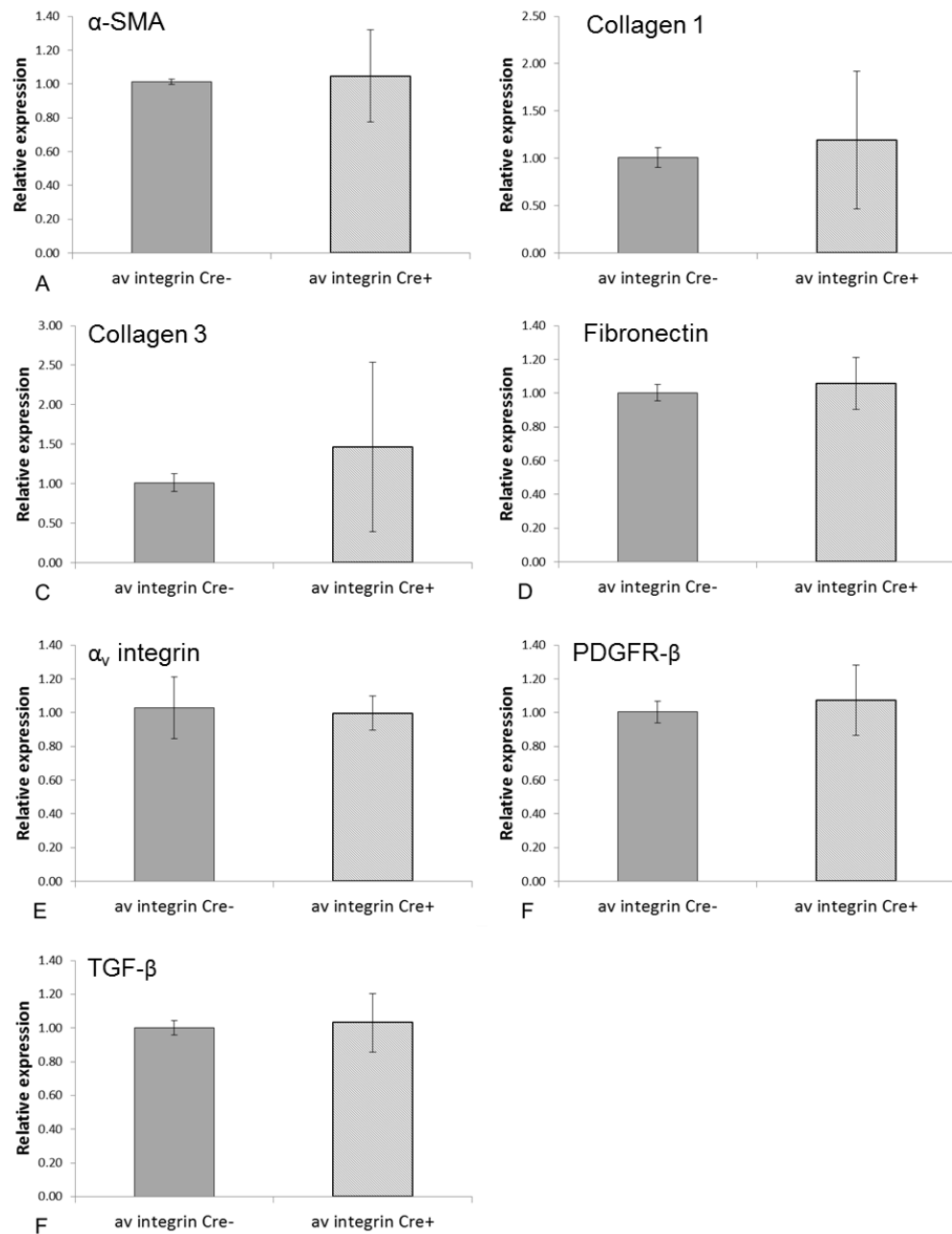


Figure 5-34 Relative expression of pro-fibrotic genes by PDGFR- β ⁺ cells isolated from the hearts of α_v integrin Cre⁺ knockout and α_v integrin Cre⁻ wild type mice and cultured for 2 days in EGM2 then 4 days in 10% FBS DMEM 1% P/S. No statistical analysis was performed due to low sample numbers however no obvious differences between groups were seen. Cre⁺ n=2, Cre⁻ n=3. Columns represent mean $\pm$ SEM.

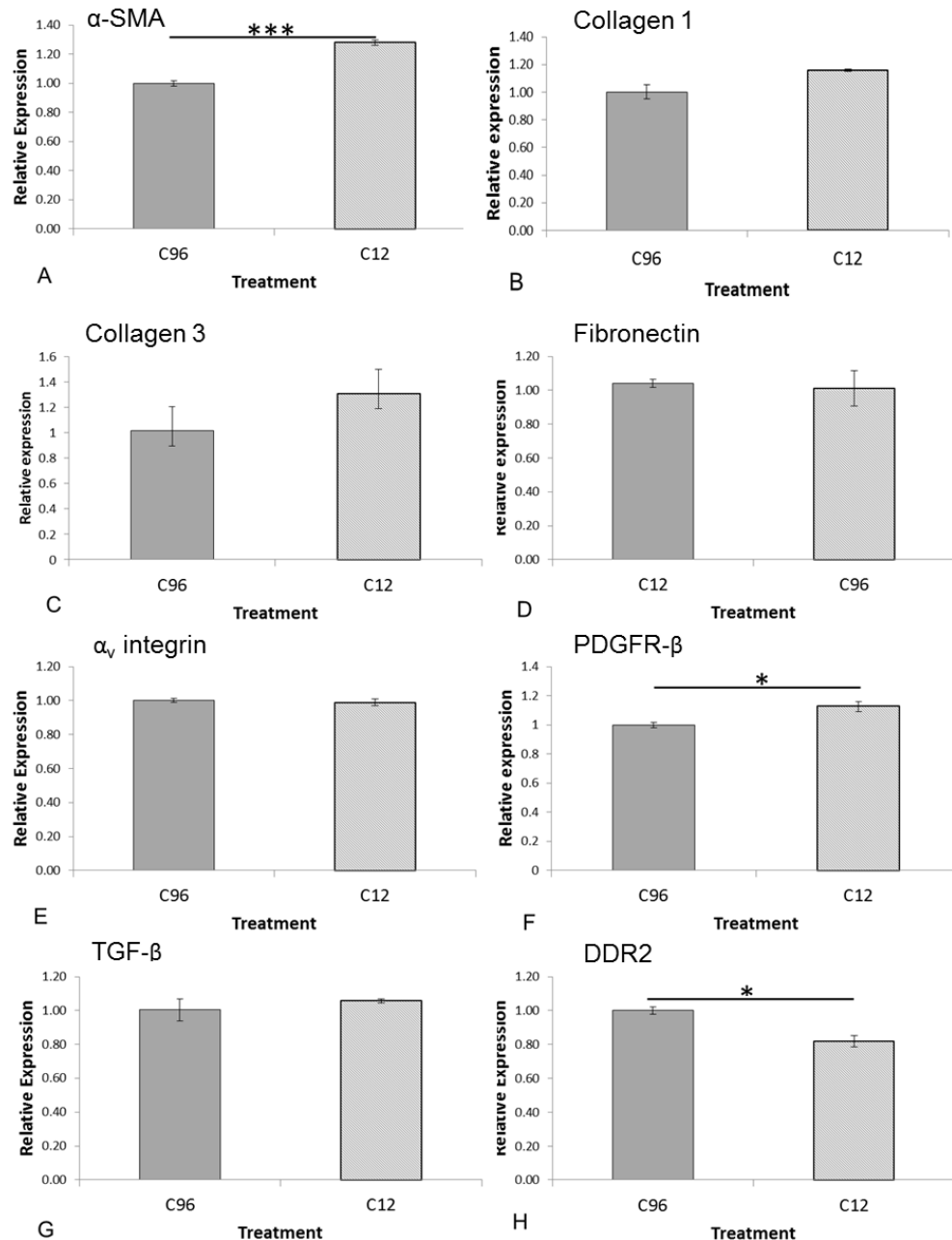


Figure 5-35. Relative expression of pro-fibrotic genes in PDGFR- β^+ cells isolated from the hearts of mTmG mice and cultured in EGM2 for 24hrs then treated with 0.01mM α_v -integrin inhibitor CWHM 12 (C12) or 0.01mM control molecule CWHM 96 (C96) for 3 days. A & F - Minor fold increases in α -SMA and PDGFR- β expression were apparent in inhibitor (C12) treated cells relative to enantiomer controls (C96). H - Minor fold reduction in DDR2 expression was seen in inhibitor treated cells relative to the control group. B, C, D, E & G - No differences in expression between groups (n = 3 per group). *p<0.001, **p<0.01, *p<0.05. Columns represent mean $\pm$ SEM. Independent t-test.**

5.5.8 α_v integrin expression by PDGFR- β expressing cells regulates the development of interstitial cardiac fibrosis *in-vivo*

5.5.8.1 14 day infusion of 200ng/kg/minute of angiotensin II results in the development of hypertension

No difference in systolic blood pressure was detected between untreated α_v integrin cre^+ knockout mice and α_v integrin cre^- wild type mice at the start of the angiotensin II treatment study. After 10 days of angiotensin II treatment of the wild type mice a significant elevation ($p<0.05$) in systolic blood pressure was apparent compared to vehicle (sterile water) treated cre^- wild type mice. This difference continued until the end of the study at day 14 ($p<0.001$). These mice also had elevated blood pressures compared to vehicle treated cre^+ knockout mice by 14 days ($p<0.001$). In the knockout mice group treated with angiotensin II an elevation of systolic blood pressure ($p<0.01$) compared vehicle treated knockout mice was apparent after 7 days and continued until the end of the study ($p<0.001$). After 3 days an elevation in blood pressure ($p<0.01$) was also noted in angiotensin II treated knockout mice compared to vehicle treated cre^- wild type controls and persisted until the end of the study ($p<0.001$). No differences were detected between genotypes subjected to the same treatment (Figure 5-36).

5.5.8.2 Exclusion of samples

Data from two mice were excluded from the angiotensin II treated cre^- wild type group on the basis of failure to develop hypertension (suggesting possible failure of the minipump). In these animals the change in blood pressure between day 0 and day 14 was +9 mmHg and +18 mmHg compared to a mean of +39 mmHg in the remaining animals. Data from one animal was omitted from the angiotensin II treated cre^+ knockout group as the blood pressure measurements oscillated dramatically over the 14 days with a final measurement of 114 mmHg compared to a mean of 152 mmHg for the rest of the group. Final experimental numbers were: 9 cre^+ angiotensin II treated mice; 8 cre^+ vehicle treated mice; 6 cre^- angiotensin II mice; 9 cre^- vehicle treated mice.

5.5.8.3 14 day infusion of 200ng/kg/minute of angiotensin II results increased heart weight

Mice treated with angiotensin II have increased heart weights as a percentage of body weight ($p < 0.01$) when compared to vehicle treated animals regardless of the genotype. No difference related to genotype was seen within treatment groups (Figure 5-37).

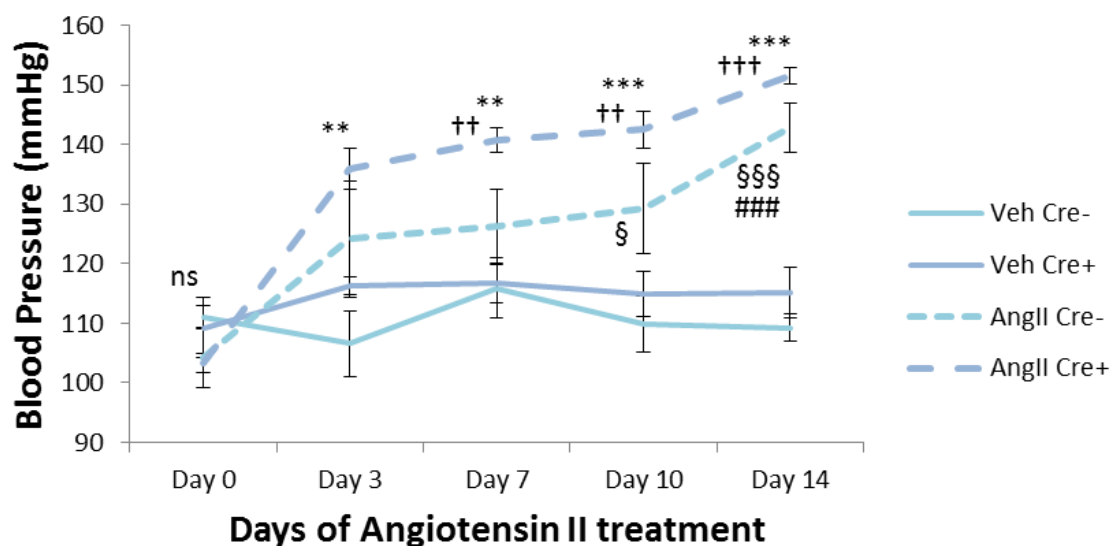


Figure 5-36 Systolic blood pressures of mice with PDGFR- β^+ cell specific α_v integrin deletion (Cre+) and wild type mice (Cre-) after 14 days administration of angiotensin II (AngII) at 200ng/kg/min or vehicle control (Veh). Treatment with angiotensin II results in elevated blood pressures after 10 days in cre- wild type mice compared to both wild type vehicle treated controls and vehicle treated cre+ knockout mice. Similarly elevated blood pressures were seen after 3 to 7 days of angiotensin II treatment in knockout mice compared to vehicle treated wild type controls and vehicle treated knockout mice. ns - no statistical significance; * vs veh cre- ; † vs veh cre+ ; § vs veh cre- ; # vs veh cre+. § p<0.05; **, †† p<0.01; §§§, *, †††, ### p<0.001. Veh Cre- n=9, Veh Cre+ n=8, AngII Cre- n=6, AngII Cre+ n=9. Data shown are mean $\pm$ SEM. Two way ANOVA with Tukey's post hoc analysis.**

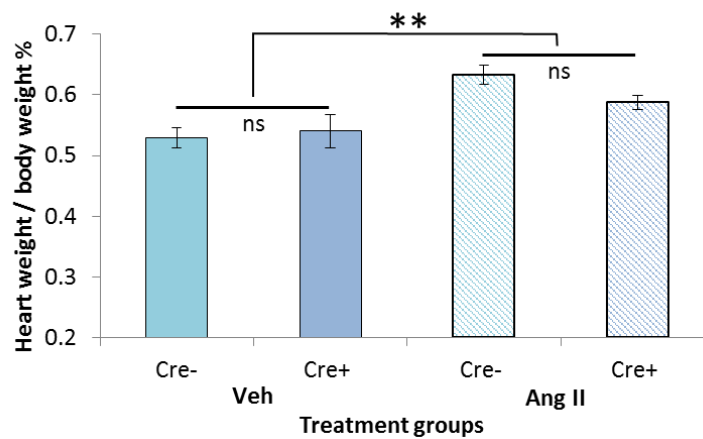


Figure 5-37 Heart weight as a percentage of body weight of mice with PDGFR β ⁺ cell specific α_v integrin deletion (Cre⁺) and wild type controls (Cre⁻) after 14 days administration of angiotensin II (AngII) at 200ng/kg/min or vehicle control (Veh). Regardless of genotype treatment with angiotensin II increased percentage heart weight. There was insufficient evidence to support a difference between effects of genotype. Cre⁻ veh n=9, Cre⁺ veh n=8, Cre⁻ ang II n=6, Cre⁺ ang II n=9. ** p<0.01; ns=non significant. Columns represent mean $\pm$ SEM. Two way ANOVA with t-test pairwise comparison of means.

5.5.8.4 14 day infusion of 200ng/kg/minute of angiotensin II results in increased endocardial area in cre⁻ wild type mice as measured by high frequency ultrasound

Following 14 days of 200ng/kg/minute angiotensin II treatment B-mode high frequency cardiac ultrasound examination revealed an increase ($p<0.05$) in the systolic left ventricular endocardial area in cre⁻ wild type mice compared to cre⁻ wild type mice vehicle treated controls. No differences were noted in endocardial area change, ejection fraction, fractional shortening or fractional area change (Figure 5-38).

5.5.8.5 Deletion of α_v integrin from PDGFR- β^+ cells results in a significant reduction in cardiac interstitial fibrosis in angiotensin II treated mice

Regardless of genotype Angiotensin II treatment resulted in a significant increase in interstitial collagen compared to treatment with the vehicle ($p<0.01$) as measured on transverse sections as a percentage of the total area of the left ventricular free wall, interventricular septum and right ventricular free wall. No difference was detected between genotypes in the vehicle treated group however within the angiotensin treated groups there was a significant reduction ($p<0.01$) of approximately 50% in interstitial collagen in the cre⁺ knockout animals compared to cre⁻ wild type mice (Figure 5-39).

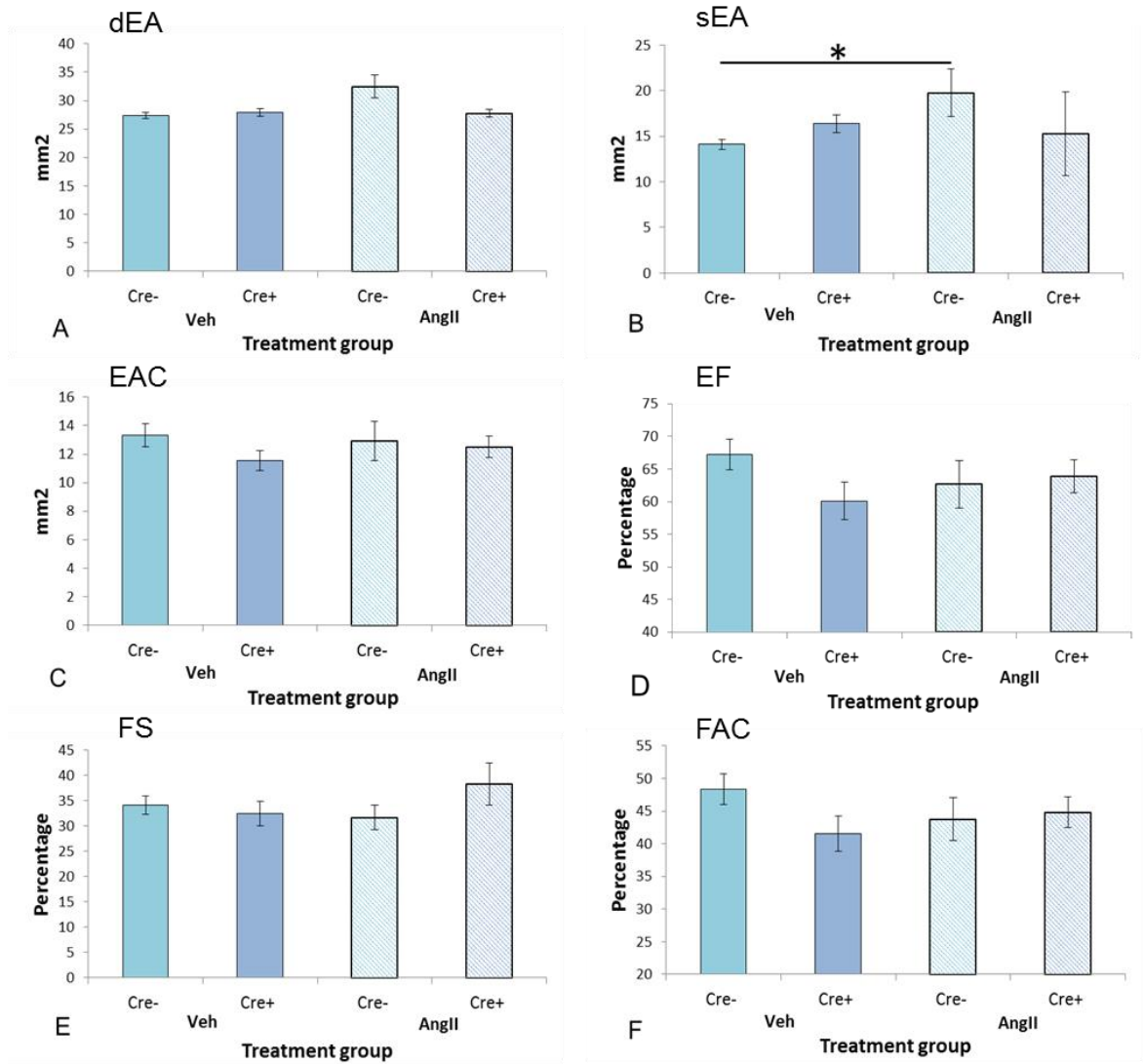


Figure 5-38 Cardiac ultrasound results of mice with PDGFR β ⁺ cell α_v integrin deletion (Cre⁺) and wild type controls (Cre⁻) treated with 200ng/kg/minute of angiotensin II (AngII) or vehicle control for 14 days (Veh). There was a significant increase in systolic endocardial area in angiotensin II treated cre- wild type mice compared to vehicle treated controls. No other differences were detected between groups. A - dEA = diastolic endocardial area; B - sEA = systolic endocardial area; C - EAC = endocardial area change; D - EF = ejection fraction; E - FS = fractional shortening; F - FAC = fractional area change. Cre⁻ veh n=9, Cre⁺ veh n=8, Cre⁻ ang II n=6, Cre⁺ ang II n=9. Columns represent mean $\pm$ SEM. * p<0.05. Two way ANOVA with Tukey's post hoc analysis.

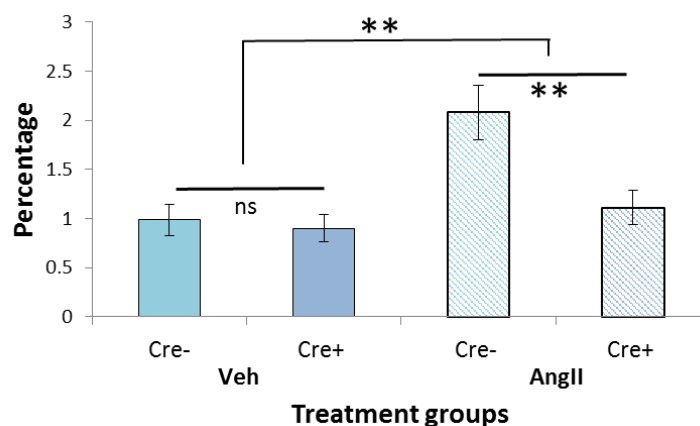


Figure 5-39 Cardiac interstitial collagen as a percentage of the total left and right ventricular cross sectional area in mice with PDGFR β ⁺ cell α_v integrin deletion (Cre⁺) and wild type controls (Cre⁻) treated for 14 days with 200ng/kg/minute of angiotensin II (AngII) or vehicle control (Veh). Regardless of genotype angiotensin II treatment resulted in an increase in interstitial collagen. Within the angiotensin II treated group α_v integrin deletion resulted in a significant reduction of approximately 50% in interstitial collagen. Cre⁻ veh n=9, Cre⁺ veh n=8, Cre⁻ ang II n=6, Cre⁺ ang II n=9. **p<0.01; ns=non-significant. Columns represent mean $\pm$ SEM. Two way ANOVA with Tukey's post hoc analysis.

5.5.8.6 14 day infusion of 200ng/kg/minute of angiotensin II results in an increase in the expression of selected pro-fibrotic genes in mice with PDGFR- β^+ cell α_v integrin deletion

Not all hearts yielded RNA of sufficient quality and as a result the final experimental numbers were: 9 cre⁺ angiotensin II treated mice; 6 cre⁺ vehicle treated mice; 6 cre⁻ angiotensin II mice; 9 cre⁻ vehicle treated mice.

Surprisingly 14 days of angiotensin II treatment resulted in minor increases in expression (fold changes of less than 1.5) of collagen 1 (p<0.05) and DDR2 (p<0.01) in the cre⁺ knockout group compared to vehicle treated knockout animals. DDR2 expression in this group was also elevated compared to both vehicle treated (p<0.05) and angiotensin II treated (p<0.05) cre⁻ wild type mice. No differences were seen between groups in expression of α_v -integrin, PDGFR- β , TGF- β or collagen 3, fibronectin or α -SMA (Figure 5-40).

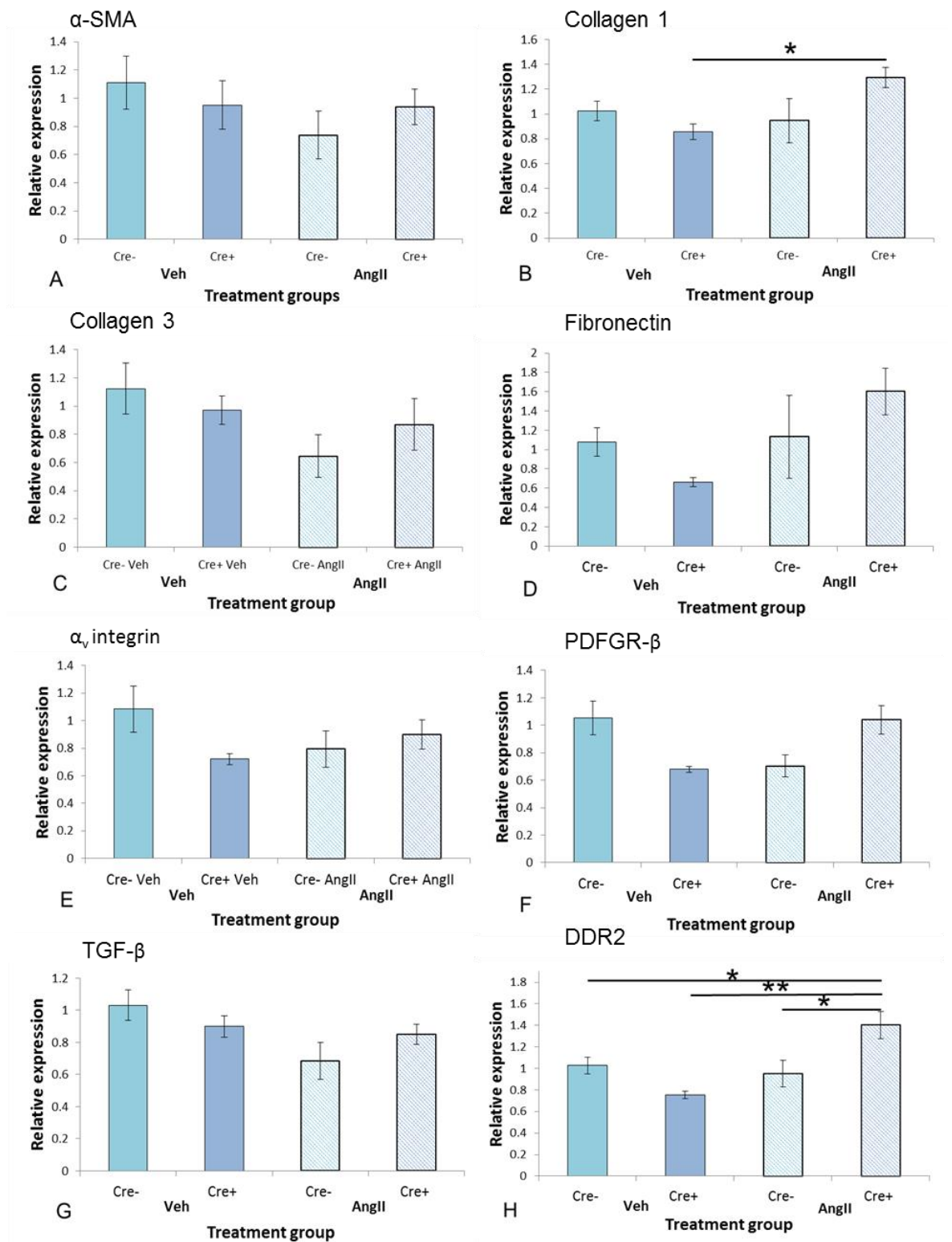


Figure 5-40 Relative expression of pro-fibrotic genes in PDGFR- β^+ cell α_v integrin knockout (Cre⁺) and wild type (Cre⁻) hearts treated with 14 days of 200ng/kg/minute angiotensin II (AngII) or vehicle control (Veh). Minor fold increases evident in collagen 1 and DDR2 expression in cre⁺ α_v integrin knockout mice after angiotensin treatment. Cre⁻ veh n=9, Cre⁺ veh n=6, Cre⁻ ang II n=6, Cre⁺ ang II n=9. * p<0.05, **p<0.01. Columns represent mean $\pm$ SEM. Two way ANOVA with Tukey's post hoc analysis.

5.6 Summary and discussion

The hypothesis on which the work in this chapter was based was that “Cardiac PDGFR- β^+ perivascular cells become fibroblasts following injury in a process mediated by α_v integrin expression.”

Although the results presented here do not fully prove this hypothesis they provide good evidence to support it.

5.6.1 Examination of mTmG mouse heart sections suggests that the fluorescence reporter signal accurately identifies PDGFR- β positive cells

The first aim of this study was to determine the accuracy of PDGFR- β reporting in mTmG reporter mice. It was clear from examination of tissue sections that reporting cells were predominantly to be found in a perivascular location around microvessels or in the subendothelial space in the endocardium. PDGFR- β is a well-recognised pericyte marker (Winkler et al., 2010) and thus this is the location expected for these cells. High levels (>90%) of eGFP $^+$ cells in the reporter tissue labelled with an anti-PDGFR- β antibody confirming good sensitivity of reporting and importantly the antibody only labelled a small proportion of cells (<5%) which did not express the reporter signal confirming good specificity. Interestingly PDGFR- β reporting could also be detected in rare cardiomyocytes. References to cardiomyocyte expression of PDGFR- β in the literature are scarce however in one paper increased expression was noted in cardiomyocytes following load induced stress (Chintalgattu et al., 2010). No increased frequency in cardiomyocyte expression of PDGFR- β was however seen in the current study when mTmG reporter mice were subject to angiotensin II induced hypertrophy and interstitial fibrosis. This suggests that, in this model at least, PDGFR- β is not up regulated in hypertrophic cardiomyocytes.

5.6.2 The identity of PDGFR- β^+ cells in normal murine hearts

Following confirmation of the accuracy of the genetic reporter it was important to identify which cells in the mouse heart express PDGFR- β under normal physiological conditions. In other studies PDGFR- β expression has been identified in

pericytes but not neurons, astrocytes or endothelial cells in the murine brain (Winkler et al., 2010). In skeletal muscle PDGFR- β expression has been seen in vascular smooth muscle cells in addition to pericytes (Hellstrom et al., 1999). In the mouse heart, in addition to pericytes, PDGFR- β also appears to be expressed by subsets of smooth muscle cells, fibroblasts and possibly leukocytes.

Smooth muscle cell expression of PDGFR- β was clearly seen in the media of coronary arteries in reporter mice. Interesting anti-PDGFR- β antibody staining labelled fewer smooth muscle cells than the reporter.

Murine fibroblasts can be identified by a variety of markers including vimentin, an intermediate filament. This protein is considered highly expressed by cardiac fibroblasts (Souders et al., 2009, Camelliti et al., 2005). It is also however expressed by endothelial cells and as a result of the scant interstitial stroma, high vascularity and intimate association of fibroblasts and endothelial cells in the heart staining must be interpreted cautiously. Careful examination using confocal microscopy revealed labelling of vimentin in a subpopulation of endothelial marker negative PDGFR- β^+ cells in the normal reporter mouse myocardium likely to be fibroblasts. CD34 expression has also been associated with perivascular progenitor cells: adventitial cells and fibroblasts (Diaz-Flores et al., 2014). CD34 expression by PDGFR- β^+ cells from normal hearts was seen in approximately 47% of the population on flow cytometry and tissue staining of reporter mouse myocardium confirmed CD34 labelling of reporting cells in a perivascular location. The presence of a significant mesenchymal progenitor/fibroblast subset within the PDGFR- β^+ population is further supported by positive staining for CD90.2 and PDGFR- α by 16% and 58% of the population respectively. In mice CD90.2 is associated with mesenchymal stem cell properties and expressed on cardiac fibroblasts but also peripheral T lymphocytes (Yusuf et al., 2013) whilst PDGFR- α has been used as a marker of mesenchymal progenitor cells in skeletal muscle (Oishi et al., 2013). Yet more evidence that PDGFR- β is expressed by a subpopulation of fibroblasts comes from the primary cardiac fibroblast cultures. Approximately 20% of cultured fibroblasts isolated from reporter mouse hearts using a standard technique expressed PDGFR- β on flow cytometry. Almost 100% of these cells were PDGFR- α^+ and 60% were CD34 $^+$.

Although these are not true lineage tracing models these findings strongly imply an association between these two markers with a pro-fibrotic phenotype and in fact other studies have indeed reported expression of PDGFR- α and PDGFR- β receptors on fibroblasts *in-vitro* (Simm et al., 1997, Gao et al., 2005).

The presence of a leukocyte subset within the PDGFR- β^+ population is suggested by the detection of CD45 expression by approximately 25% of PDGFR- β^+ cells on flow cytometry. This interesting finding warrants further investigation. PDGFR- β has not been reported to be expressed by leukocytes and CD45 has not been linked with the perivascular cell population. CD45 expression has however been reported in mouse fibroblasts which were also CD34 $^+$ (Haudek et al., 2010) and this may explain the finding.

CD45 however is also expressed by haematopoietic progenitor cells of which there is a circulating population in mammals (Kondo et al., 2003). CD34 in murine tissue, as in humans, has also been detected on the surface of haematopoietic progenitors, as well as endothelial cells (Lin et al., 1995). It is therefore possible therefore that some of the CD34 $^+$ and CD45 $^+$ /PDGFR- β^+ cells detected are circulating haematopoietic progenitors. Examination of blood from reporter mice via flow cytometry and immunofluorescence of smears may confirm PDGFR- β expression in circulating progenitors.

Expression CD146 was seen in a minor subset of the PDGFR- β^+ population with detection in 11% of the cells on flow cytometry. CD146 in mouse tissue is widely expressed by endothelial cells and is considered a poor marker of pericytes (Schrage et al., 2008). Given the close apposition of endothelial cells, pericytes and fibroblasts anatomically within the heart and the reported labelling of endothelial cells by CD146 and other markers such as CD34 there is question as to whether a subset of the PDGFR- β population are of endothelial origin. Confocal microscopy examination of reporter mouse myocardium stained with anti-CD146 antibody revealed close association but no overlap with the reporter signal suggesting that, despite tight singlet gating, the flow cytometry findings were likely a result of inadequate digestion and incomplete separation of closely apposed cells. Approximately 15% of

the PDGFR- β population were positive for the mature endothelial marker CD31 on flow cytometry however close examination using confocal microscopy of reporter mouse myocardium stained with CD31 revealed no overlap of staining and reporting. This again suggests that there is inadequate digestion and incomplete separation of closely apposed cells.

5.6.3 In injured myocardium PDGFR- β is expressed by a subset of the expanded interstitial fibroblast population

Following the identification of the PDGFR- β expressing population in normal mouse myocardium the next aim was to identify these cells in injured hypertrophied hearts. Angiotensin II treatment results in activation and expansion of the interstitial fibroblast population with deposition of extracellular collagen (Schnee and Hsueh, 2000). In angiotensin II treated reporter mice there was multifocal expansion of a vimentin positive interstitial cell population consistent with cardiac fibroblasts. Approximately half of this population also expressed PDGFR- β indicating that activated cardiac fibroblasts express this surface receptor. The cre in this model was constitutively expressed and therefore not inducible so it cannot be said with certainty whether the PDGFR- β^+ fibroblasts originated from the original PDGFR- β perivascular cell population or whether fibroblasts activated after injury switch on PDGFR- β . Interestingly none of these fibroblasts expressed α -SMA, one of the key markers of the myofibroblast (Hinz et al., 2007), suggesting that they had not developed the contractile myofibroblast phenotype. α -SMA expression was however seen in arterial smooth muscle positive internal control cells confirming that the antibody labels appropriately. The absence of interstitial myofibroblasts is likely to be a feature of the injury model as the overall cardiac fibrosis is relatively mild. α -SMA $^+$ myofibroblasts are commonly seen following myocardial infarction (van den Borne et al., 2010) and have been reported in angiotensin II induced myocardial hypertrophy and fibrosis but with a 10 times higher dose of (2.0 μ g/kg/min) angiotensin II (Sopel et al., 2011), likely to induce a more severe injury. Interestingly in the present study all the smooth muscle cells in the media of coronary arteries were α -SMA $^+$ but not all were PDGFR- β^+ with a subset failing to express the reporter signal. This may represent cre mosaicism in which cre is not active in every

target cell (Heffner et al., 2012) or it may be due to variability in PDGFR- β expression by smooth muscle cells.

5.6.4 Cardiac PDGFR- β^+ cells demonstrate a pro-fibrotic phenotype *in-vitro*

To confirm that cardiac PDGFR- β^+ cells are able to differentiate into fibroblasts/myofibroblasts *in-vitro* these cells were isolated from reporter mouse hearts, cultured over a 15 day period and the expression of pro-fibrotic genes was examined. In these studies experimental numbers were low despite the pooling of samples. This was due to the relatively poor yield of cells from cardiac tissue and the limited supply of animals available. Sufficient data however was obtained to draw general conclusions. Isolated cardiac PDGFR- β^+ cells demonstrated a trend towards increased expression of pro-fibrotic genes after 15 days culture in EGM2 and on a hard plastic matrix, however fold changes were only minor. The pro-fibrotic phenotype of isolated PDGFR- β cells in culture was also indicated by positive staining for α -SMA, FAP, DDR2 and vimentin in the majority of cells after 24 hours in EGM2. These staining results suggest that PDGFR- β^+ cells may up regulate the expression of pro-fibrotic markers rapidly after isolation or possibly even during the tissue digestion and isolation process. The failure to detect significant fold increases in profibrotic gene expression during prolonged culture may be due to near maximal levels of expression following isolation or, on the contrary, insufficient profibrotic stimulation perhaps due to inadequate levels of TGF- β in the media/serum (EGM2 contains only 2% FBS). Further studies in which the media is switched after 24 hours to a higher serum containing media (10% FBS DMEM 1% P/S) are required to determine whether significantly higher fold changes in pro-fibrotic gene expression are possible.

5.6.5 Reduced α_v integrin activity in PDGFR- β^+ cells does not appear to alter their pro-fibrotic phenotype *in-vitro*

As per the aims of the study experiments were carried out to investigate whether deletion or inhibition of α_v integrin reduces the pro-fibrotic potential of cardiac PDGFR- β^+ cells *in-vitro*. As with the above experiments the experimental numbers

were low due to poor cell yields and limited tissue supply. PDGFR- β^+ cells from cre⁻ wild type and cre⁺ α_v integrin knockout hearts were isolated and allowed to settle for 2 days in EGM2 then grown in 10% FBS DMEM 1% P/S for a further 4 days in an attempt to stimulate a more profound pro-fibrotic response than seen in the previous experiment. No significant differences were seen in gene expression between cre⁺ knockout or cre⁻ wild type mice. This may be due to cells from both populations responding equally rapidly to culture conditions and adopting a similar pro-fibrotic phenotype. Therefore a second experiment was carried out in which cells were collected directly into 10% FBS DMEM 1% P/S and RNA was collected after a shorter interval of 3 days in culture. Cells were seeded at twice the density to allow for poorer initial survival in this media. Again no significant differences were noted between groups. If this result is genuine then, unlike in the liver (Henderson et al., 2013), α_v integrin does not play a significant role in the development of a pro-fibrotic phenotype in cardiac PDGFR- β^+ cells. However it is equally plausible that culture conditions were inappropriate resulting in the development of an overwhelming pro-fibrotic phenotype, in both cre⁺ knockout and cre⁻ wild type populations, that was not modulated by deletion of α_v integrin. Starting with a much higher numbers of cells and collecting RNA after only 24hrs may be sufficient to detect a difference.

To double check the above findings, small molecule α_v integrin inhibition was also tested. The synthetic small molecule inhibitor, CWHM 12, is a RGD peptidomimetic antagonist consisting of a cyclic guanidino-substituted phenyl group as the arginine mimetic and a phenyl-substituted β amino acid as the aspartic acid mimetic, both linked by glycine (Henderson et al., 2013). It has been shown to strongly inhibit all of the five β -subunit binding partners of the α_v subunit (Henderson et al., 2013). The control molecule, CWHM 96, is the R enantiomer of CWHM 12 with only a difference in the orientation of a carboxyl (CO₂H) group. In *in-vitro* ligand binding assays CWHM 96 did not inhibit any of the β -subunit binding partners (Henderson et al., 2013). The inhibitor has been shown to attenuate fibrosis in the liver and lung of mice following administration via minipump (Henderson et al., 2013) and it was hoped that treatment of cultured cells would prevent them developing a pro-fibrotic phenotype.

PDGFR- β^+ cells were treated with CWHM 12, and the pro-fibrotic gene expression was compared with cells treated with CWHM 96. Cells were cultured in EGM2 for 24 hours before being switched to 10% FBS DMEM containing inhibitor or control molecule for a further 3 days. However no significant differences in pro-fibrotic gene expression were noted between groups. This could be a result of failure of the inhibitor to interact with cells in culture or, as for genetic deletion, could be due to inappropriate culture conditions which overwhelmingly promote fibrosis in both populations of cells.

5.6.6 Genetic deletion of α_v integrin on PDGFR- β^+ cells *in-vivo* however results in reduced cardiac fibrosis

The final aim of the study was to investigate the role α_v integrin expression by PDGFR- β^+ cells plays in cardiac fibrosis *in-vivo*. The model of cardiac fibrosis used was 14 day minipump administration of angiotensin II to induce cardiac hypertrophy and interstitial fibrosis. Endogenous angiotensin II is produced through activation of the renin-angiotensin-aldosterone system and results in an elevation in blood pressure via vasoconstriction and increased water retention in the kidney (Cotran et al., 1993). Untreated animals of both genotypes had similar day 0 basal systolic blood pressures whereas angiotensin II treated animals of both genotypes became significantly hypertensive after 14 days of treatment suggesting an appropriate response to treatment. Sterile water treated vehicle controls of both genotypes by contrast remained normotensive throughout the study period. As well resulting in an increase in blood pressure angiotensin II treatment of mice also results in elevated cardiac weight due to hypertrophy (Crowley et al., 2006). After 14 days treatment with angiotensin II a significant increase in heart weight as a percentage of body weight was noted indicating a suitable hypertrophic response to angiotensin II. No differences were noted between genotypes suggesting that genetic deletion of α_v integrin does not influence the hypertrophic response of the myocardium to angiotensin II.

High frequency B-mode ultrasound of the left ventricle revealed a significant increase in systolic endocardial area in cre⁻ wild type animals treated with angiotensin II for 14 days when compared to vehicle treated controls. This suggests a

degree of left ventricular remodelling however no difference was noted in endocardial area change or fractional shortening indicating that overall contractility is unaffected. The relatively limited impact of angiotensin II treatment on cardiac ultrasound parameters is not particularly surprising as the degree of fibrosis in angiotensin treated animals is probably insufficient to result in significant functional changes. Only a few studies using angiotensin II treatment report changes in cardiac ultrasound parameters. A statistically significant reduction in ejection fraction was noted in one study in which mice were treated with 1µg/kg/minute of angiotensin II (5 x dose used in current study) for 14 days with but this equated to only approximately 5% difference between groups (Huang et al., 2010). In another study mice were given 5 x the dose of angiotensin II used in the current study and no differences were noted on examination of ventricular systolic function. Doppler examination, which was not done in the current study, however revealed a statistically significant reduction in diastolic function (Mori et al., 2012).

More importantly however 14 days of angiotensin II treatment resulted in an increased deposition of cardiac interstitial collagen as measured morphologically and compared to vehicle treated animals. This is consistent with previous studies using angiotensin II to induce cardiac hypertrophy and fibrosis (Haudek et al., 2010). No differences were seen between vehicle control treated cre⁺ knockout mice and cre⁻ wild type mice suggesting that the genetic deletion does not alter baseline collagen levels. Genetic deletion of α_v integrin in cre⁺ mice however resulted in a reduction in cardiac interstitial collagen of approximately 50% compared to cre⁻ controls following angiotensin II treatment. The interstitial collagen component of hearts lacking α_v integrin treated with angiotensin II was in fact similar to that in the sterile water control treated groups.

A reduction in the level of α_v integrin in the hearts of cre⁺ animals will need to be confirmed via western blotting. However this finding suggests that despite the *in-vitro* findings the α_v integrin subunit on PDGFR- β^+ cells in the heart plays an important role in the development of cardiac fibrosis and extends the recently reported discovery of α_v integrin expression by PDGFR- β^+ cells as a key modulator of hepatic, pulmonary and renal fibrosis (Henderson et al., 2013) to the heart. In fact

individual integrins have been implicated in the development of cardiac fibrosis in several other studies. In mice lacking $\beta 3$ integrin, one of the β -subunit binding partners of the α_v subunit, and subjected to a 4 week pressure overload model a substantial reduction in the accumulation of interstitial collagen and fibronectin with reduced expansion of the interstitial fibroblast population was apparent (Balasubramanian et al., 2012). In another study $\alpha_v\beta 5$ and $\alpha_v\beta 3$ integrins were found to be up regulated in myofibroblast areas of cardiac fibrosis and differentiated human myofibroblasts in culture. When inhibited by function blocking peptides and antibodies both integrin were found to autonomously contribute to the activation of latent TGF- $\beta 1$ and the conversion of fibroblasts to a myofibroblast phenotype (Sarrazy et al., 2014). Integrins are important in the development of interstitial fibrosis not just in the heart but in other organs. In the lung deletion of $\alpha_v\beta 6$ integrin has been associated with a reduction in pulmonary fibrosis following bleomycin induced injury in mice and this correlated with a reduction in $\alpha_v\beta 6$ integrin expression in lung biopsies of human patients with fibrosing lung pathologies (Horan et al., 2008). $\alpha_v\beta 6$ integrin, again one of the integrins containing the α_v subunit, has also been associated renal fibrosis with overexpression detected in human renal pathologies. Antibody blocking of this integrin resulted in reduced interstitial fibrosis in a mouse model of glomerulonephritis (Hahm et al., 2007). Given that the α_v subunit is common to five β integrin subunits including $\alpha_v\beta 3$, $\alpha_v\beta 5$, and $\alpha_v\beta 6$ the results of these studies support the current finding of a reduced fibrosis *in-vivo* phenotype following α_v integrin deletion in the heart. The genetically modified model used in this study results in deletion of α_v integrin globally from PDGFR- β^+ cells. This includes possible bone marrow progenitor populations that have been proposed as contributors to cardiac fibrosis (Mollmann et al., 2006) providing a potential extra-cardiac pathway of action. It would be interesting therefore to compare the *in-vitro* pro-fibrotic potential of bone marrow versus cardiac PDGFR- β^+ cells from cre⁺ knockout and cre⁻ wild type animals.

When the expression of pro-fibrotic genes in the myocardium was compared surprisingly no significant differences were seen between groups. This contradicts the results of the interstitial collagen quantification however the latter result is based

on protein expression it is therefore likely to be the more reliable of the two. It is possible that this discrepancy between gene and protein expression may be due to differences in the pro-fibrotic potential of specific regions of the myocardium. Sections examined for collagen quantification were obtained from the basal heart as previous PSR staining of sections had shown this to be the region that develops the most marked interstitial fibrosis (personal communication Rachel Richardson). This region of myocardium is likely to undergo the greatest degree of stretch during ventricular filling and thus maybe the region in which fibroblast activation is most readily triggered. Alternatively the interstitium of the basal ventricular myocardium may contain a higher density of pro-fibrotic PDGFR- β^+ cells. These potential anatomical differences combined with the relatively poor fibrotic response in this model maybe the reason for lack of difference between groups.

Bromodeoxyuridine (BrdU) was administered to animals in the angiotensin II study however anti-BrdU staining of tissue sections could not be completed within the time scale of this thesis. It is hoped that this will be done in the near future to enable comparison of the levels of cell proliferation between treatment and genotype groups and identification the proliferative cell populations following angiotensin II treatment.

5.6.7 Conclusion

Time constraints and animal availability meant that it was not possible to fully complete all the aims of this part of the study however the data collected strongly supports the initial hypothesis that cardiac PDGFR- β^+ perivascular cells become fibroblasts following injury in a process mediated by α_v integrin expression. It is clear that in normal murine hearts PDGFR- β is expressed by a heterogeneous population of perivascular cells including a significant subpopulation of PDGFR- α^+ /CD34 $^+$ fibroblasts or fibroblast progenitors and this population appears to expand following cardiac injury. *In-vivo* deletion of the common α_v integrin subunit from the surface of cardiac PDGFR- β^+ cells results in the reduction of interstitial collagen deposition following induction of cardiac hypertrophy and fibrosis and suggests a critical role for one or more of the $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrin receptors in

cardiac fibrosis. Based on the current evidence what remains unclear however is whether α_v integrin expression by PDGFR- β^+ perivascular cells directly mediates their conversion into fibroblasts/myofibroblasts or whether α_v integrin expression by the PDGFR- β^+ cell population modulates proliferation and differentiation of an independent fibroblast/fibroblast progenitor population via the regulation of extracellular matrix active TGF- β concentrations. Optimisation of current experiments and the development of further studies will be required to answer this question.

Chapter 6: General Discussion

Stem cell therapies hold tremendous potential for the treatment of a wide range of conditions including ischaemic heart disease. Initial clinical cardiac stem cell therapies have utilised bone marrow stem cells (Janssens et al., 2006, Leistner et al., 2011). These adult stem cells are readily obtainable from patients, are unlikely to become tumourigenic and avoid immunological rejection and ethical complications as they are autologous. Clinical trials involving cardiac transplantation of bone marrow cells in humans for the treatment of myocardial infarction have, however, yielded only modest results. Meta-analyses of 50 studies revealed average improvements of 3.96 % ($\pm$ 1.06) in left ventricular ejection fraction and a reduction of 4.03% ($\pm$ 1.44) in scar size at 6 months post therapy (Jeevanantham et al., 2012). Meanwhile a recently published study examining 49 cardiac bone marrow stem cell trials found over 600 discrepancies in design, methods and baseline characteristics or results. Only 5 studies had no discrepancies and in these there was no detectable difference in ejection fraction whilst in the flawed studies improvement in ejection fraction increased with the numbers of discrepancies detected (Nowbar et al., 2014). The search continues therefore for an effective donor cell population for use in cardiac cell therapy. The right atrial appendage from by-pass surgeries or endocardial biopsies from patients undergoing coronary angioplasty provide a potential alternative to bone marrow as a source of cells. Within these tissues the perivascular progenitor cell populations are an attractive and possibly niche adapted donor cell population for use in regenerative therapies. In addition, these cells in other organs have been demonstrated to be the source of the MSC (Crisan et al., 2008b) and in skeletal muscle to contribute to myofibre regeneration (Dellavalle et al., 2007).

The work described in this thesis was designed to investigate the hypothesis that cardiac perivascular progenitor cells contribute positively to cardiac repair following ischaemic injury. The initial part of the study used human foetal heart to investigate the characteristics of these cells, their ability to differentiate into cardiomyocyte *in-*

vitro and their contribution to cardiac repair when transplanted *in-vivo*. Although not directly translational, it was hoped cells from this tissue would have a greater inherent plasticity and developmental potential that would enable establishment of proof of concept before investigation of the adult equivalent populations. As a result of the findings from this work an additional hypothesis was proposed and the latter part of the study was designed to investigate whether cardiac PDGFR- β^+ perivascular cells become fibroblasts following injury and whether this process is mediated by α_v integrin.

6.1 Cardiac pericytes and adventitial cells show differences but also marked similarities

The initial part of this study compared two populations of human cardiac perivascular cells *in-vitro* to determine whether clear differences could be seen between populations. Differences between pericytes and adventitial cells in surface marker expression in tissue, growth characteristics on isolation, response to cardiomyocyte differentiation studies and retention post transplantation were detected in this study (Chapter 3;Chapter 4:). This suggests the presence of two distinct cell populations within the cardiac perivascular compartment. Despite this, when cultured, CD146⁻/CD34⁺ adventitial cells spontaneously lost CD34 and up regulated CD146 whilst sharing homology of surface marker expression with pericytes (3.5.3.1). A similar finding has been reported in adipose adventitial cells following angiopoietin II treatment (Corselli et al., 2012) however it appears that cardiac adventitial cells spontaneously differentiate to pericytes in culture conditions and implies that these cells share related lineage differentiation pathways. The CD146⁻/CD34⁺ adventitial cell population from non-cardiac tissues has been used in cell transplantation studies and demonstrated regenerative capacity in mouse models of limb ischaemia and myocardial infarction (Katare et al., 2011, Campagnolo et al., 2010). It is noteworthy however that some investigators consider CD34⁺ stromal cells in humans to be a fibroblast/myofibroblast/MSC progenitor cell population more intimately involved in pathological processes than in regeneration (Diaz-Flores et al., 2014). This is supported by the discovery, in the current study, that adventitial

cells preferentially differentiate into fibroblasts upon transplantation into the injured myocardium

6.2 Cardiac perivascular progenitors share some overlap of markers with mesangioblasts

The overlap between pericytes, adventitial cells and mesoangioblasts is poorly defined. Like pericytes and adventitial cells, mesoangioblast-like cells can be isolated from adult murine and human tissues based on expression of a panel of surface markers. In humans these cells are generally negative for CD31, CD34, CD133 and CD45 and positive for NG2, CD56, CD90, CD44, PDGFR- α and PDGFR- β (Quattrocchi et al., 2012). As such they may be considered to be homologous with or to include pericytes, but not CD34 positive adventitial cells. Mesoangioblasts isolated from human cardiac tissue however have been reported to be positive for CD31, CD34, CD44, CD146, CD117 (c-kit), GATA-4, Nkx2.5, Mef2a, whilst being negative for CD45, Isl-1 and CD133 (Galvez et al., 2009). Cardiac pericytes by contrast are CD31 and Nkx2.5 negative and thus by definition are not be classified as mesangioblasts. In addition, unlike pericytes these cells were reported to differentiate to cardiomyocytes on treatment with 5-azacytidine or co-culture with neonatal rat cardiomyocytes and to readily differentiate into cardiomyocytes when transplanted into injured mouse hearts, although the evidence presented to substantiate this is not particularly convincing (Galvez et al., 2009).

6.3 Pro-cardiomyogenic progenitors are present in the perivascular compartment of the human heart

The original hypothesis underlying this study proposed that the perivascular compartment of the human foetal heart contains a progenitor cell population capable of positively contributing to repair following cardiac infarction. Furthermore it was hoped that these cells would be capable of differentiating into and replacing lost cardiomyocytes. *In-vitro* characterisation of these cells, in this study, demonstrated not only expression of pericyte and MSC markers (3.5.3, 3.5.1) but also cardiac transcription factors and c-kit (3.5.2), a marker that has been associated with cardiac progenitor potential (Tallini et al., 2009). Cells from both populations were able to

undergo adipogenic and osteogenic differentiation (3.5.3) further confirming their MSC phenotype and progenitor abilities (Caplan, 2007). Following demethylation and *in-vitro* co-culture with rat neonatal cardiomyocytes a very minor fraction of pericytes expressed the cardiac sarcomeric proteins troponin t and alpha actinin and also demonstrated spontaneous cytoplasmic calcium oscillations, although not convincing spontaneous contraction (3.5.4.1.5). Reports suggest that the efficiency of co-culture based cardiomyocyte differentiation techniques can vary greatly from low efficiencies, such as 0.8% of cardiosphere derived cells (Simpson et al., 2012), to high efficiencies, such as 32% using bone marrow stem cells (Xu et al., 2004). It should be remembered however, especially with regard with the higher figures, that in high cell density co-cultures it is common for cells to form multiple layers and therefore without critical interpretation of microscopic fields it is easy to mistake overlapping of the two cell types differentiation (Gruh et al., 2006), grossly overestimating the number present. The work presented here shows that, upon transplantation of cells into the border zone of ischaemically injured myocardium, low numbers of both pericytes (13%) and adventitial cells (3%) adopt a robust cardiomyocyte phenotype with single human nuclear antigen positive nuclei and distinct cross striations (4.5.10). Taken together the *in-vivo* and *in-vitro* data suggest that the cardiac perivascular compartment contains a minor subset of cells with cardiac progenitor capability able to differentiate into cardiomyocytes upon transplantation. However this was not a true lineage tracing study and therefore, although tempting, it cannot be assumed that endogenous perivascular cells are able to contribute to cardiomyocytes *in situ* following myocardial injury. Several lineage tracing studies, including two undertaken recently (Hsieh et al., 2007, Ellison et al., 2013, Malliaras et al., 2013), have concluded however that repair of the myocardium and regeneration of lost cardiomyocytes occurs via substantial contribution of cardiac progenitor cells and in particular the c-kit⁺ progenitor population. Future lineage tracing studies of perivascular cell populations will be complicated by both the lack of a single specific marker for pericytes and adventitial cells and differences in cell marker expression between rodents and humans. However the simultaneous comparison of several inducible cre models of common pericyte markers such as

CD146, NG2 and PDGFR- β and careful co-staining may help elucidate the role of these native cells post myocardial injury.

6.4 Improved purification of pro-cardiomyogenic progenitors from the cardiac perivascular cell population

Key to further investigation of the pro-cardiomyogenic potential of the perivascular cell population is better identification and enrichment of the pro-cardiogenic subsets within the CD146⁺ CD34⁻ and CD146⁻ CD34⁺ perivascular populations.

Unfortunately the range of predictive markers of cardiomyogenic progenitor potential is very limited and of those currently considered c-kit is probably the best investigated (Zaruba et al., 2010, Tallini et al., 2009, Bearzi et al., 2007). The cardiomyocyte progenitor potential of the c-kit⁺ population has however recently been challenged in a study using constitutive and inducible c-kit mouse reporter models that demonstrated that c-kit cells contribute minimally (<0.03%) to new cardiomyocytes but significantly to endothelial cells post infarction (van Berlo et al., 2014). In the current study it was discovered that c-kit is expressed in culture by a subpopulation of both cardiac pericytes and cardiac adventitial cells (3.5.2) and it would, nevertheless, make sense to purify these cells from the parent perivascular cell populations and examine whether they have enhanced cardiomyogenic potential in-vivo or in-vitro. Another marker that has been associated with cardiomyocyte progenitor potential in humans is Isl-1 (insulin gene enhance protein 1) (Cai et al., 2003, Bu et al., 2009). In the current study this marker was expressed in adventitial cells but only after a period of co-culture with neonatal rat cardiomyocytes (3.5.4.1.2) and given that it does not appear to be expressed natively in these cells Isl-1 may not prove useful in the identification of a pro-cardiomyogenic subset from the initial population. Finally antibodies against SIRPA (signal regulatory protein alpha) have been used in FACS to enrich for enhanced cardiomyocyte potential (Dubois et al., 2011). The starting populations in this case were embryonic stem cells, chemically induced to partially differentiate into cardiomyocytes, and SIRPA expression was detected after 6 to 7 days, at a similar time to nkx2.5 and around 2 days later than Isl-1 detection. It is not clear however whether SIRPA expression is

restricted to embryonic stem cell derived cardiomyocyte progenitors or whether it is also expressed by native precursors and a pilot study would be required to determine this.

6.5 Screening of potential perivascular cell candidate subpopulations for cardiomyogenic differentiation potential

One of the main issues in screening adult stem/progenitor cell populations for cardiomyogenic differentiation potential is (in contrast to adipogenic and osteogenic and chondrogenic differentiation) the lack of a simple *in-vitro* cardiomyogenic differentiation method. Co-culture with neonatal rat cardiomyocytes is inefficient, time consuming, technically complex and prone to variability and interpretation error (Ramkisoensing et al., 2012, Gruh et al., 2006). Cytokine and growth factor based techniques although promising are difficult to repeat in practice (Smits et al., 2009). Prior demethylation of cells is widely used in chemical based differentiation studies but it is debatable as to whether chemical demethylation in culture is representative of the native differentiation potential of cells in tissue. In addition most cardiomyocyte differentiation studies are carried out at atmospheric oxygen tension of 20% whilst physiological oxygen for most tissues is 1-7% meaning that standard culture conditions are in fact hyperoxic. It has been demonstrated that culturing cardiosphere stem cells in 5% oxygen results in a higher cell yields and better survival and engraftment following transplantation (Li et al., 2011). It is possible therefore that the efficiency and reliability of the above differentiation methods may be improved with culture at tissue representative oxygen tensions. Further work is clearly required to develop reliable cardiomyocyte *in-vitro* differentiation strategies for adult stem cells.

In the meantime, *in-vivo* studies involving the injection of cells into the border zone of myocardial infarcts have been used as the ultimate test of differentiation potential. As presented in chapter 3 this technique has significant limitations with regards high levels of animal mortality, variability in the infarct size and location and site of cell deposition. Transplantation of cells into normal uninjured myocardium in animals not undergoing coronary artery ligation would alleviate a lot of the problems

associated with mortality and loss of cells due to injection into non perfused tissue. However it is possible that without injury the local microenvironment may be insufficient to induce cardiomyocyte differentiation. An alternative to the coronary artery ligation infarct model used in recent cell therapy studies is the β adrenergic agonist (isoprenaline/isoproterenol) model of cardiac necrosis and fibrosis (Ellison et al., 2013). It is thought that isoproterenol in this model exerts its effects via the cardiac vessels inducing transient myocardial ischaemia and necrosis (Teerlink et al., 1994). This model requires less surgical skill and results in a more diffuse subendocardial injury which means deposition of cells is less critical. However it differs from the coronary artery ligation injury and less precisely models the human infarct lesion. As such it may result in a different tissue growth factor/cytokine environment. Regardless of which model is used it is important to note that they will all require the use of immuno-deficient animals and therefore be considerably more expensive than in-vitro techniques.

6.6 Enhancing cell survival and differentiation in transplantation studies

It was evident from the results of the transplantation study reported here that both cell retention/survival and cardiomyocyte differentiation were very low (4.5.10). This is a common finding in the majority of cardiac cell therapy studies both in animal models and human trials. In fact a recent paper reported that, following transplantation of 500,000 MSCs and bone marrow mononuclear cells by intra-myocardial injection in rats only between 97 and 1853 cells were retained 3 weeks later (van der Bogt et al., 2008). Another study in which neonatal rat cardiomyocytes were injected into adult rat myocardium revealed a reduction of cells from 57% (+/- 9) at 1 hour to 15% (+/-3) at 12 weeks (Muller-Ehmsen et al., 2002). Intra-myocardial injection of human cells in pigs has been shown to result in short term retention of more (11% +/-3) cells than intracoronary infusion (2.6% +/-0.3) although with greater variability (Hou et al., 2005). By contrast it has been estimated that an average size myocardial infarct in an adult human results in the loss of 1 billion cardiomyocytes (Robey et al., 2008). When this is considered in conjunction with the poor retention and poor survival of cells and the only limited cardiomyocyte

differentiation achieved in the various cell types studied so far it seems unlikely that direct transplantation of undifferentiated cells is likely to lead to regeneration of adequate myocardial mass. Much recent interest has therefore focused on methods to enhance transplanted cell survival or retention. Techniques to physically improve cell retention following transplantation have included the use of purse string sutures to compress the needle tract post injection and prevent retrograde cell leakage (Chong et al., 2014) and the use of biopolymers to increase adherence and integration of the injected cell suspension to surrounding tissue (Danoviz et al., 2010). An alternative method, used with ES and IPS cells, has been to induce partial differentiation of stem cells/progenitors into early cardiomyocytes prior to injection. This was used in a recent study in non-human primates in which embryonic stem cells were first differentiated into early cardiomyocytes prior to cardiac transplantation (Chong et al., 2014). Although the use of small molecules to induce partial differentiation has largely been used with multipotent stem cells populations (Ao et al., 2012) some groups have also been examining their effects on adult progenitors. In one study the authors identified a family of small molecules which up-regulate expression of the cardiac transcription factor *nkx2.5* in both embryonic stem cells and adult progenitors (Sadek et al., 2008). They then used these molecules to pre-condition peripheral blood mononuclear cells and demonstrated enhanced engraftment and improved cardiac function following myocardial infarction in a rat model.

6.7 Stimulation of endogenous cardiomyocyte progenitors *in-situ*

An alternative approach to transplantation of an exogenous cell population is to enhance the contribution of endogenous adult cardiac progenitor cells *in-situ* by using small molecules to stimulate their proliferation and differentiation. Proof of concept studies in this regard have been carried out with varying degrees of success. Stimulation of a *WT1*⁺ population of epicardial progenitors with the peptide thymosin β 4 resulted in enhanced cardiomyocyte differentiation in these cells *in-situ* following myocardial infarction (Smart et al., 2011). In another study treatment of notch activated epicardium derived cells with 3,5-disubstituted isoxazoles (Isx), stem cell-modulator small molecules, *in-vitro* resulted in the generation of cardiomyocyte

precursors (Russell et al., 2012). When used *in-vivo* in myocardial infarction models however the target cell population differentiated into fibroblasts and the small molecule treatment failed to prevent this. The cardiac perivascular cell population, as demonstrated in the current study, is both abundant and diffusely distributed throughout the myocardium. Given their anatomic location these cells are in close proximity to the circulation and also therefore systemically administered small molecules. Thus the identification of a small molecule therapy which targets and triggers proliferation and differentiation in the cardiomyocyte capable perivascular cell subset would be an elegant alternative to cell therapy.

6.8 Pro-fibrotic progenitors are present in the perivascular compartment of both human and mouse heart

Although the human cardiac perivascular compartment appears to contain a minor subset of cardiomyocyte progenitor cells it is evident from the work presented here that the majority of perivascular cells are pro-fibrotic progenitors. Following transplantation of pericytes and adventitial cells a fibroblast phenotype was detected in roughly 50% of each population although adventitial cell numbers were greatly increased due to the improved overall survival of this population (4.5.10). It was also evident that pro-fibrotic markers are widely expressed in cultured pericytes and adventitial cells (4.5.11), although not in all as some follow a cardiomyocyte differentiation pathway. Not only did perivascular cells readily exhibit a pro-fibrogenic phenotype they also had a negative impact on left ventricular function post transplantation (4.5.7). As mentioned previously no reports of fibroblast differentiation of a cardiac cell therapy population could be found in the literature and almost all studies have focused on cardiomyocyte (and occasionally endothelial) differentiation. In addition almost all studies report a beneficial effect following cell transplantation. Considering that the myocardium scars more readily compared to other tissues that act as sources of stem/progenitors cells, such as the bone marrow and adipose tissue it is not totally unexpected that MSC like progenitors from the heart more readily adopt a pro-fibrotic differentiation pathway. Studies have demonstrated both that fibroblast progenitors exist in the bone marrow and migrate

to cutaneous wounds to become fibroblasts (Opalenik and Davidson, 2005) and that connective tissue growth factor (CTGF) stimulated human bone marrow MSCs in culture to lose their MSC markers and become collagen producing fibroblasts (Lee et al., 2010). Similar findings have followed CTGF treatment of adipose MSC populations (Hu et al., 2014) suggesting a fibroblast progenitor role for MSCs from other tissues. It is thus possible that fibroblast differentiation occurs in cardiac cell therapy studies but because it is generally not expected nor desired it has not been investigated or reported. The discovery of a pro-fibrotic population in the human foetal perivascular compartment is further supported by the work presented here using PDGFR- β reporter mice. This protein is tightly restricted to cells located in the perivascular compartment, a proportion of the cells in which were also positive for fibroblast markers (5.5.5). PDGFR- β expression was found to be present in approximately 20% of fibroblasts isolated from reporter animal hearts and also in approximately 50% of the expanded interstitial fibroblast population following angiotensin II treatment (5.5.4). Importantly however these were constitutive cre reporter mice and thus not true lineage tracing models. The induction of PDGFR- β expression in fibroblasts as a response to tissue digestion or angiotensin II treatment rather than differentiation of PDGFR- β^+ perivascular progenitors into mature fibroblasts therefore cannot be ruled out. Inducible cre strains pulsed with tamoxifen to induce temporary cre expression would be required to accurately trace the differentiation pathways of the PDGFR- β^+ perivascular cell population and would be an important step in tracking the fate of perivascular cells following myocardial injury. Nonetheless these findings agree with previous attempts at pericyte lineage tracing carried out in a variety of other tissues. Lineage tracing mouse models in the kidney, spinal cord and the lung have implicated subsets of the pericyte population in tissue fibrosis. In the latter study NG2-CreER reporters were used to demonstrate NG2 $^+$ expression by cells in regions of pulmonary fibrosis following bleomycin injury (Rock et al., 2011). In the kidney a genetic fate mapping model was used to show that a subpopulation of Fox-D1 $^+$ /PDGFR- β^+ perivascular cells gives rise to α -SMA expressing interstitial myofibroblasts following unilateral ureteric obstruction . In the spinal cord glutamate-aspartate transporter (glast) is expressed by a subset of perivascular pericytes which are also PDGFR- β^+ and which proliferate in injured

tissue where they co-express pro-fibrotic markers α -SMA and fibronectin (Goritz et al., 2011). Common to these genetic fate mapping studies is not only a pro-fibrotic role for the perivascular cell population but also that only a subset of this population of cells is involved. This agrees with the results of the current study in both in the mouse model, in which only a proportion of PDGFR- β cells appear to contribute to the fibroblast population (Chapter 5:), and with the human perivascular cell study, in which both pro-fibrotic and pro-cardiomyogenic populations are present in the perivascular compartment (Chapter 4:).

6.9 Integrin receptor expression by pro-fibrotic perivascular cells appears to play a significant role in cardiac fibrosis

α_v integrin is a common subunit shared by five of the 24 transmembrane heterodimeric receptors and therefore genetic deletion of this subunit results in a reduction in function of all five receptors. In the current work deletion of this α_v integrin from the surface of PDGFR- β^+ perivascular cells resulted in a reduction in interstitial fibrosis of approximately 50% in the angiotensin II model of cardiac hypertrophy and fibrosis (5.5.8.5). This is in agreement with the findings of a recent study in which α_v integrin was deleted from PDGFR- β^+ hepatic stellate cells in the liver of mice protecting them from carbon tetra-chloride (CCL4) induced hepatic fibrosis (Henderson et al., 2013). Protection was found to be as a result of reduced levels of activated TGF- β in culture and a resultant reduction in the numbers of tissue myofibroblasts. Individual deletion of $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrin receptors by contrast was not protective. Individual integrin receptors in the myocardium however may play a role in cardiac fibrosis as demonstrated in recent work in which $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins were up-regulated in myofibroblast enriched fibrotic regions of a porcine model of right ventricular fibrosis and in human cardiac myofibroblasts in pro-fibrotic culture conditions (Sarrazy et al., 2014). Both integrins were found to autonomously contribute to latent TGF β -1 activation and fibroblast to myofibroblast differentiation in-vitro.

The exact mechanisms behind the α_v integrin mediated reduction in cardiac fibrosis are, as of yet, unclear. The model used in this current study is more complex than

CCL4 liver injury as angiotensin II acts in the myocardium in several ways. It promotes the development of hypertension by both increasing circulating blood volume and increasing peripheral vascular resistance (Cotran et al., 1993). Hypertension in turns leads to left ventricular overload and mechanical load on the cells of the myocardium including cardiomyocytes and fibroblasts. The surface integrins on the latter act as mechanoreceptors, transducing the load signal and up-regulating production of latent TGF β -1. This is activated by surface integrin receptors and feeds back on fibroblasts/myofibroblasts to stimulate the deposition of extracellular matrix (Diez, 2007, Bishop and Lindahl, 1999) and cardiomyocyte hypertrophy (Sadoshima and Izumo, 1993, Rosenkranz, 2004). Angiotensin II also acts directly on the angiotensin II type I receptors on cardiac fibroblasts to stimulate expression of TGF β -1 (Gray et al., 1998, Campbell and Katwa, 1997) (Figure 6-1). As expected TGF- β is induced and activated in the infarcted myocardium (Bujak and Frangogiannis, 2007). Interestingly TGF- β is thought to up regulate connective tissue growth factor (CTGF), the growth factor demonstrated to convert MSCs to fibroblasts (Leask and Abraham, 2004) and it is possible that this may have a direct effect on MSC like perivascular progenitors.

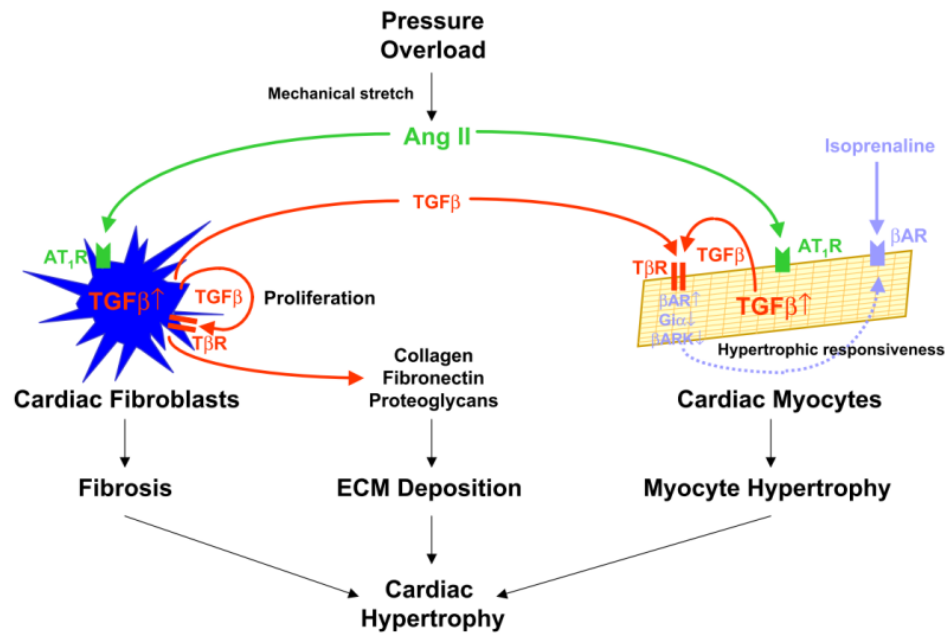


Figure 6-1 Schematic taken from Rosenkranz, 2004 depicting the relationship between cardiomyocytes, fibroblasts, angiotensin II and TGF-β1 in relation to interstitial cardiac fibrosis and hypertrophy.

6.10 General limitations of the study

Perhaps the single biggest limitation of this study is that a single population of human perivascular cells was transplanted. This was principally due to the high purchase and housing costs of NOD/SCID mice. As a result it could be argued that the pro-fibrotic response of these cells was an anomaly specific only to this donor. Counter to this suggestion however is the in-vitro expression of pro-fibrotic markers detected in perivascular cell populations from three separate donors. The pooling of cells from several donors for injection was considered but discounted on the basis that should an effect be seen further identification of the specific donor of origin would not be possible. Another limitation is that the transplantation study data was acquired at a single post-operative time-point. 21 days was selected, based on previous studies, as the point at which cells were still likely to be retained and at which differences in cardiac function were likely to have developed. Ideally an earlier and a later end-point would also have been included but this was out-with the budget of the study. From a translational point of view a limitation is the fact that foetal cells rather than adult cells were used. Foetal donors were chosen over adult because it was anticipated that foetal cells would have an increased likelihood of enhanced plasticity compared to their adult counterparts. Further work is required to determine whether the limited cardiomyocyte regenerative capacity and pro-fibrotic tendencies of foetal perivascular cells is shared by adult cardiac perivascular cells. Finally it is clear from the quantitative gene expression and flow cytometry data that there is variability between populations of cells of the same type. The use of five antibodies to widely expressed surface receptors, although a significant improvement on standard adherence based MSC isolation techniques, still results in selection of a heterogeneous target population. The development of further more specific marker panels is required to better identify the cells of interest whilst still obtaining sufficient numbers for use in downstream experiments/therapy.

6.11 Future studies to investigate the role of perivascular cells in cardiac fibrosis

The current findings suggest that α_v integrin expression by PDGFR- β^+ cells is important in the development of cardiac fibrosis *in-vivo* however it is unclear

whether this is a result of reduced activation of latent TGF- β or through inhibition of the cells ability to differentiate into fibroblasts/myofibroblasts or a combination of both. *In-vitro* experiments to clarify this have thus far been inconclusive and these need to be repeated in optimised culture conditions to determine whether genetic deletion of α_v integrin prevents differentiation of PDGFR- β^+ cells into fibroblasts/myofibroblasts. It will also be important to confirm deletion of α_v integrin from the PDGFR- β^+ cells isolated on FACS via western blot. Immunostaining and primary fibroblast flow cytometry results suggest that within the PDGFR- β^+ population only a subset of cells are able to become fibroblasts/myofibroblasts. To investigate this potential pro-fibrotic subset the PDGFR- β^+ population could be further subdivided by FACS for expression of potential markers of fibrotic progenitors such as PDGFR- α or CD34. Expression of profibrotic markers in cultured PDGFR- β^+ /PDGFR- α^- versus PDGFR- β^+ /PDGFR- α^+ populations for example could then be compared. This could be checked by isolating the above subsets of cells from C57/Bl6 mice and treating them with the small molecule inhibitor or the enantiomer. Should a difference in pro-fibrotic potential be confirmed between groups then the level of active versus inactive TGF- β in media from cultures of PDGFR- β^+ α_v integrin knockout and α_v integrin wild type cells could be measured to confirm a role for this growth factor in development of a pro-fibrotic phenotype (Abe et al., 1994). It would be interesting to compare the role of α_v integrin expression by PDGFR- β^+ cells in the angiotensin II model with other models of cardiac injury such as the trans-aortic constriction pressure overload model, the myocardial infarction ischaemia model and the β adrenergic agonist induced ischaemia model. These quite different models will result in different mechanisms of myocardial injury and it is possible quite different fibrotic responses will result. Also of interest would be whether a similar proportion of interstitial fibroblasts are PDGFR- β^+ and which subsets of the PDGFR- β^+ population are most pro-fibrotic.

6.12 Conclusion

The original hypothesis for this thesis was that “Human cardiac perivascular progenitor cells contribute positively to cardiac repair following ischaemic injury.”

The work described here in part confirms this hypothesis by demonstrating that a subset, albeit minor, of these MSC-like cells appear able to differentiate into cardiomyocytes both *in-vitro* and *in-vivo*. The majority of perivascular cells however appear to contribute to the pool of cardiac fibroblasts in the injured myocardium and additional investigation using genetically modified mouse models revealed an important role for α_v integrin by perivascular cells in the cardiac fibrotic process. These findings expand the current knowledge of cardiac perivascular cells and add weight to the increasingly popular suggestion that these cells play a key role in cardiac fibrosis. They also raise questions regarding the profibrotic potential of other MSC populations used for cell therapy and emphasize the importance of fully characterising donor cell populations before their use in therapeutic studies.

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Appendix: Publications, oral presentations and poster presentations

Publications directly relating to the PhD

Human Myocardial Pericytes: Multipotent Mesodermal Precursors Exhibiting Cardiac Specificity.

William C.W. Chen^{*}, James E. Baily^{*}, Mirko Corselli, Mary Diaz, Bin Sun, Guosheng Xiang, Gillian Gray, Johnny Huard, Bruno Péault. ^{*} contributed equally to this work.

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Oral presentations

British Heart Foundation Centre of Research Excellence external assessor visitation, Centre for Cardiovascular Sciences, Edinburgh. "The role of pericytes in cardiac repair." June 2014

Physiologic Society H3 Symposium, Cellular approaches for cardiac repair, London. "Perivascular progenitors: cardiac progenitor cells or contributors to cardiac fibrosis." March 2014.

Poster presentations

European Society of Veterinary Pathologists, Cutting Edge Pathology, Berlin.

"Perivascular progenitors: cardiac progenitor cells or contributors to cardiac fibrosis." August 2014

Physiologic Society H3 Symposium, Cellular approaches for cardiac repair, London.

"Perivascular progenitors: cardiac progenitor cells or contributors to cardiac fibrosis." March 2014

BHF core synergy meeting, Oxford University. "Perivascular progenitors and their role in myocardial repair." February 2013