Characterisation of resident multipotent vascular stem cell derived smooth muscle cells in culture

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of ..... is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abbreviations

APS – Ammonium Per Sulfate

BCA - Bicinchoninic Acid

bFGF – Fibroblast Growth Factor

BMP – Bone Morphogenic Protein

BMS – Bare Metal Stents

BSA – Bovine Serum Albumin

CCD – Charged Coupled Device

CD – Cluster of Differentiation

CNN – Calponin

CNS – Central Nervous System

CSO – Central Statistics Office

Cu^{2+}- Copper

CVD – Cardio Vascular Disease

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

DEPC – Diethylpyrocarbonate

DES – Drug Eluting Stents

DMEM – Dulbecco's Modified Essential Medium

DMSO - Dimethyl Sulfoxide

DNA – Deoxyribose Nucleic Acid

EC – Endothelial Cell

ECM – Extra Cellular Matrix

EMEM – Eagle's Minimum Essential Medium

FACS - Fluorescent Activated Cell Sorting

FBS – Fetal Bovine Serum

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GFP – Green Fluorescent Protein

HMG – High Mobility Group

iPSC – induced Pluripotent Stem Cells

LIF – Leukemia Inhibitory Factor

MSC – Mesenchymal Stem Cell

MVSC – Multipotent Vascular Stem Cell

NCSC – Neural Crest Stem Cell

P/S – Penicillin-Streptomycin

PBS – Phosphate Buffer Saline

PCR – Polymerase Chain Reaction

PDGF – Platelet Derived Growth Factor

PNS – Peripheral Nervous System

RIPA - Radioimmunoprecipitation Assay

rmFGF – recombinant mouse Fibroblast Growth Factor

rMSC – rat Mesenchymal Stem Cell

RT – Reverse transcriptase

RT – Room Temperature

S100β - *S100* calcium binding protein B

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

shRNA – short hairpin Ribo Nucleic Acid

SMA – Smooth Muscle Actin

SMAD(2/3) - Mothers against decapentaplegic homolog (2/3)

SMC – Smooth Muscle Cell

SM-MHCII/SM-2 – Smooth Muscle Myosin Heavy Chain II

Sox (2/10/17) - SRY (sex determining region Y)-box (2/10/17)

TEMED- Tetramethylethylenediamine

TGFβ – Transforming Growth Factor Beta

TMB - 3,3',5,5' Tetramethylbenzidine

VEGF – Vascular Endothelial Growth Factor

vSMC – vascular Smooth Muscle Cell

αMEM – Minimal Essential Medium

Units

cm - centimetres

cm^2 – centimetres squared

°C – Degrees Celsius

g - grams

h - hours

kDa – kilodaltons

L - Litre

M - Mole

min - minutes

ml – millilitres

mm - millimetres

mM - millimole

ng - nanogram

OD – Optical Density

Rpm – revolutions per minute

Sec - seconds

µg - microgram

µl - microlitre

µm - micrometer

µM - micromolar

V/v – volume per volume

V – Volts

x g – gravity

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Abstract

Author: Eimear Kennedy

Thesis Title: Characterisation of resident multipotent vascular stem cell derived smooth muscle cells in culture

The origin of the vascular Smooth Muscle Cell (SMC) involved with vascular remodelling is very controversial. The theory that SMCs can dedifferentiate is long standing. However, in more recent years this idea has been challenged with the emergence of resident progenitor stem cells in the vascular wall. Here, a population of primary Multipotent Vascular Stem Cells (MVSCs) were isolated using explant culture from the medial layer of rat aortic tissue. MVSCs were characterised for multipotency based on expression of neural crest markers Sox10, Sox17 and glia cell marker S100β. The cells were also characterised for their mesenchymal stem cell (MSC) like properties through their ability to differentiate into adipocytes and osteocytes and expression of markers CD44 and CD29. In maintenance media, the cells displayed a $SMA^{+}/CNN^{+}/SM\text{-}MHCII^{-}$ phenotype. After TGFβ1 and PDGF-BB stimulation the cells presented a $SMA^{+}/CNN^{+}/SM\text{-}MHCII^{+}$ phenotype demonstrating their transition to SMCs. A10 and A7r5 cell lines are derived from the thoracic aorta of rat embryos. They are used widely as a model for non-differentiated neonatal and neointimal vascular SMCs. The discovery of resident MVSCs in the cell wall prompted the evaluation of these embryonic lines in relation to MVSC markers [Sox10, Sox17, S100β]. These cells expressed all three MVSC markers while concomitantly expressing SMA, CNN1 and SM-MHCII. Upon serum deprivation both cell lines had increased SMA and CNN1 expression. Both cell lines showed some potential to differentiate into adipocytes. Adult SMC lines from three different sources (bovine, rat and murine) were screened for MVSC markers and were found to express all three markers along with SMC markers SMA, CNN1 and SM-MHCII. However, these cell lines did not give rise to adipocytes or osteocytes after stimulation with induction media. These results indicate that a population of resident progenitors do exist in the vessel wall and may contribute to vascular remodelling after injury. The expression of MVSC markers in A10, A7r5 and various SMC lines indicates that these cells still retain some of their stem cell like properties and may not be terminally differentiated as previously described. NE4C neural stem cells were analysed for their SMC differentiation capabilities. The cells show some potential as a model for MVSC to SMC after TGFβ1 stimulation but further experiments are required to conclude these results.

Publications

Embryonic rat vascular smooth muscle cells revisited - a model for neonatal, neointimal SMC or differentiated vascular stem cells?

***Eimear Kennedy**, Roya Hakimjavadi, Chris Greene, Ciaran J Mooney, Emma Fitzpatrick, Laura E Collins, Christine E Loscher, Shaunta Guha, David Morrow, Eileen M Redmond, and Paul A Cahill*

Journal: Vascular Cell 2014, 6(1):6

Adult vascular smooth muscle cells in culture express neural stem cell markers typical of resident multipotent vascular stem cells.

***Eimear Kennedy**, Ciaran J Mooney, Roya Hakimjavadi, Emma Fitzpatrick, Shaunta Guha, Laura E Collins, Christine E Loscher, David Morrow, Eileen M Redmond, and Paul A Cahill*

Journal: Cell tissue Research 2014, 358(1):203-16

Chapter 1:

Introduction

1.0 General Overview

The main objective of this study was to discover if resident progenitor stem cells exist in the vessel wall. Aortic explant cultures were prepared from freshly isolated rat aortic tissue. These cells were grown up and characterised for MVSC markers Sox10, Sox17, S100β. The cells were then analysed for multipotency capabilities following treatment of cells with induction media for osteocyte and adipocyte differentiation. MVSCs were treated with TGFβ1 to induce smooth muscle cell differentiation. Two commercial cell lines, A10 and A7r5, both embyronic rat smooth muscle cell lines, were evaluated for stem cell properties by investigating their MVSC marker expression and their potential to differentiate to adipocytes. Commercially available adult smooth muscle cell lines from three different animal sources were evaluated for the expression of MVSC markers and potential to differentiate to adipocytes and osteocytes. The cell lines were also analysed for MSC marker expression including CD29, CD44 and CD146. The immortalised neuroectodermal derived stem cell line, NE4C was evaluated as a model for MVSCs. NE4Cs were analysed as MVSCs and the other cell lines were for MVSC markers and their ability to differentiate to adipocytes and osteocytes. The cells were also stimulated with TGFβ1 to induce smooth muscle cell differentiation.

1.1 Cardiovascular Disease

1.1.1 The Vasculature

The vascular system consists of a network of blood vessels, macro and microvascular systems. The larger arteries supplying blood around the body to target organs are arteries and veins. The capillaries (venules and arterioles) of the micro-system serve to supply the blood within organs and to smaller areas of the body. The primary function of these structures is to transport blood around the body. It is a vital system ensuring that oxygen and other fundamental nutrients reach the organs where they are needed. Arteries pump oxygenated blood from the heart to target organs. Blood passes through organs via capillaries. The small size and diameter of a capillary wall allows for the diffusion of nutrients and oxygen within the organ. The deoxygenated blood containing waste material then passes into the veins which carry

the blood back to the lungs where it can be expelled. The cycle continues in the lungs, where the blood is oxygenated and brought back to the heart.

1.1.2 Vessel Structure

Arteries and veins are composed of three different layers which can be seen in *Figure 1.0.*

The tunica intima/endothelium; this is the thinnest innermost layer which consists of a monolayer of endothelial cells and plays a fundamental role in vessel homeostasis (Gimbrone et al 2000). These cells are connected to a basal lamina which is an extracellular membrane layer consisting of collagen, proteoglycans and glycans. Properties of the endothelium are very important for blood flow. The cells naturally promote an anti-thrombotic/anti-inflammatory surface (Gimbrone et al 2000). The flat alignment of the cells prevents friction and reduces turbulence allowing blood to pass smoothly and be pumped further distances around the body. There is an internal elastic membrane which contains pore like structures permitting the passage of material through the vessel wall. In veins, small valves connected to the endothelium are in place to prevent backflow of blood (Bonow et al 2011).

The tunica media; this is the middle layer of the vessel and has elastic properties. It is composed of vascular smooth muscle cells, pericytes, elastin and proteoglycans. The cells and proteins are arranged in a fibrous manner contributing to the tough structure of the medial layer. The cell primarily attributed to the tunica media is the vascular smooth muscle cell, which is not terminally differentiated and thought to be capable of undergoing “phenotypic modulation”.

The tunica adventitia; this is separated from the tunica media by a thin layer of elastin. It is the outer layer and consists of connective tissue and fibroblasts. It contains collagenous tissue and some elastic fibres. This layer is less rigid than its neighbouring medial layer. The radius of this vessel wall will vary between arties and veins, being slightly thicker in veins (Bonow et al 2011).

Recent evidence has suggested the existence of resident stem cell progenitors within the vessel wall (Tang et al 2012, Klein et al 2011, Hu et al 2002). The three layers of the vessel wall are thought to be home to some populations of stem cells,

varying in stemness at each layer, for example progenitor adventitial cells display Sca-1 and this marker is lost in the medial progenitor cells isolated by Tang et al 2012. It is very difficult to pinpoint an exact phenotype or cell marker that can describe/identify a typical stem cell. Nevertheless, the vessel wall is very dynamic by nature and as a result different types of stem cells may exist to assist in the regeneration and remodelling following injury Tang et al 2012, Gomez and Owens 2012, Xu et al 2010). These researchers, along with others have shown that stem cell populations exist within the two different layers but different conclusions relating to the phenotypic markers have been reached.

Many different populations of stem cell have been identified displaying different markers of stemness (Naito et al. 2012, Wang et al. 2012a, Sainz et al. 2006). Research has shown that the adventitia is one major source of progenitor cells that contribute to vessel structure and to disease (Majesky et al. 2012), (Klein et al. 2011), (Tigges & Stallcup 2013)). The endothelium also harbours stem cells. The role of endothelial progenitor stem cells and their contribution to pulmonary hypertension has recently been proposed (Doerschuk 2005). The increase of SMA found in lesions and other forms of pulmonary hypertension had been attributed to an increase in SMC number (Arciniegas et al. 2007). More recent research suggests that endothelial cells undergo a transition to SMA expressing mesenchymal stem cells and contribute to disease (Arciniegas et al. 2007). It has also been suggested that this endothelial to mesenchymal transition [EMT] can cause an increased production of extra cellular matrix and lead to the development and progression of pathological fibrosis (Piera-Velazquez et al. 2011). This transition is also thought to be induced in part by the TGFβ1 pathway (Chen et al. 2012).

The most recent studies have indicated that populations of stem cells exists within the tunica media layer (Tang et al 2012, Sainz et al 2006, Tintut et al 2003, Hu et al 2002) The cells isolated have been shown to harbour multipotent potential similar to that of mesenchymal stem cells (Tintut et al 2003).

Tang et al 2012 have isolated a population of progenitor cells displaying phenotypic markers associated with stem cells, Sox10, Sox17 and S100β. They show that these cells can differentiate into SMCs upon injury and contribute to intimal thickening of the artery and may be involved with disease. It is suggested that these

multipotent progenitor cells are the source of vascular disease ie restenosis and that the "de-differentiated" SMC is not involved in the process.

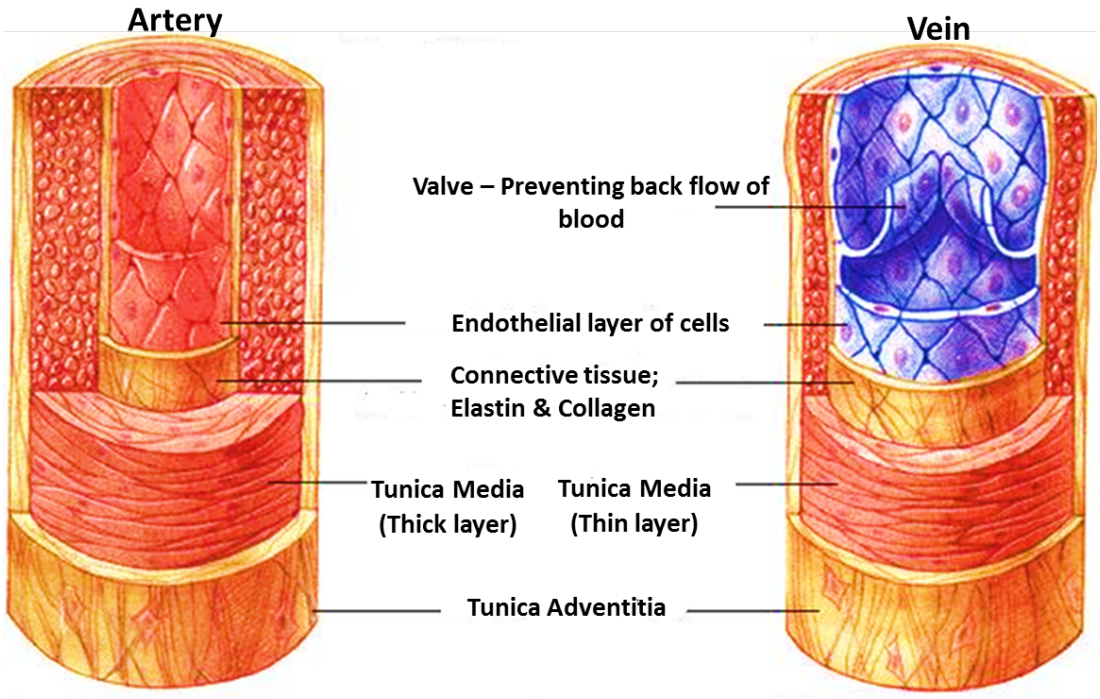


Figure 1.0 *Representative image of the layers of arteries and veins in cardiovascular system. Image adapted from Shier et al. 1996.*

1.1.3 Vascular Disease

Cardiovascular Disease (CVD) is a leading cause of death in the United States, with recent reports of one CVD related death in every 40 seconds (American Heart Association, 2013 update). In Europe CVD is responsible for 47% of deaths and 40% of deaths within the EU (European Society of Cardiology 2012). Although CVD mortality rates have declined since the 1970's in Ireland, statistics still show that CVD is the greatest cause of death, accounting for 31.8% of deaths in the first quarter of 2012 (CSO). 13% of premature deaths (under the age of 65) are from cardiovascular disease (Irish Heart Foundation). Different forms of cardiovascular disease exist.

1.1.4 Cardiovascular Disease

As previously described, the function of the vascular system is to supply blood, oxygen and nutrients to the body's vital organs. A lack of nutrients and oxygen to any organ can cause severe damage to the restricted organ and will have counter effects on connected organs. Some examples include: aneurysm, stroke, hypertension, arteriosclerosis and atherosclerosis. The primary cause of vascular disease is as a result of the blockage of vessels. This can happen in different ways such as a blood clot (thrombus), plaque formation or inward vessel remodelling (arteriosclerosis). A lot of vascular disease stems from arteriosclersosis (and atherosclerosis) whereby the vessel becomes obstructed and blood flow is restricted. The cells that are responsible for this obstruction has generated much debate and controversy in the context of the purported contributory role of resident vascular stem cells (Tang et al 2012, Nguyen et al. 2013).

1.1.5 Etiology of vascular disease

There are many factors associated with CVD (Jiang et al 2000). In more recent years there has been a lot of research into CVD and facts continually emerge. There has been a subsequent increase in media coverage to educate the general population about the causes of CVD disease. Most of the risks are interlinked and one can affect another. The main causes are obesity and smoking with counter side effects including activity level, stress, drinking habits, diet and high cholesterol. In the United States, the alarm was first raised in 1994 linking obesity to heart disease. The National Centre for Health Statistics reported that between 1988-1994 and 1999-2000 that the prevalence of overweight adults increased from 55.9% to 64.5% and an increase in obesity from 22.9% to 30.5% (Flegal et al. 2010). Obesity is an independent factor of CVD (Poirier et al. 2006), and as a result a lot of publicity focuses on this. Another factor associated with CVD is smoking, early studies have shown that the rate of CVD has been shown to triple in those who smoke 20 cigarettes a day (Doyle et al. 1964). Even passive smoking has shown to increase CV risk by 1.25% (He at al 1999). Genome Wide Association Studies have been undertaken to identify the genes linked to cardiovascular disease. Interestingly, the

TCTN1 gene has been linked to CVD in these studies. This gene has a role in hedgehog signalling during embryonic development (Erbilgin et al 2013).

1.2 Arteriosclerosis

Arteriosclerosis is a form of cardiovascular disease. It is defined by a symmetrical thickening of the vessel wall. It is primarily attributed to hypertension and elevated blood pressure. Hardening of the muscular tissue in vessel wall occurs and this results in a loss of elasticity and rigidity of the vessel wall (Fishbein and Fishbein 2009). The process can occur over a long period of time without diagnosis. The disease becomes a problem when it restricts blood to organs, especially where the coronary artery is concerned and a lack of blood flow to the heart results. The disease can progress onto atherosclerosis (a sub form of arteriosclerosis), which can be a result of many factors such as fat build up, cholesterol and plaques formations (Fishbein and Fishbein 2009).

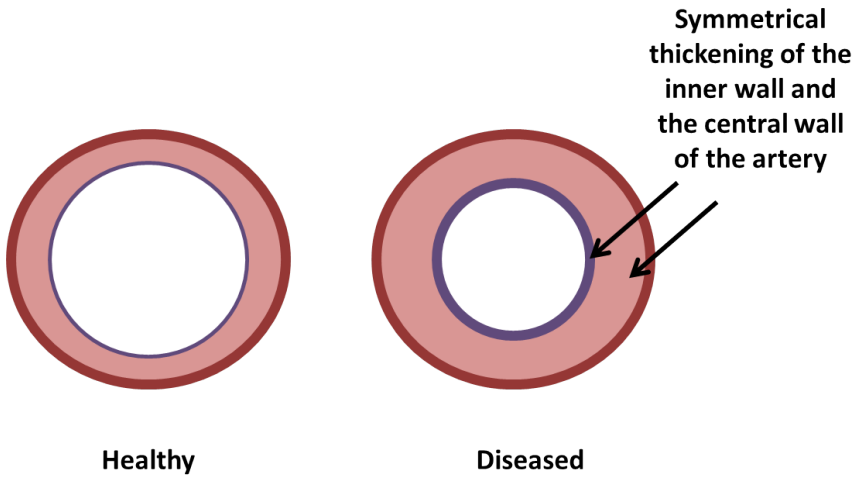


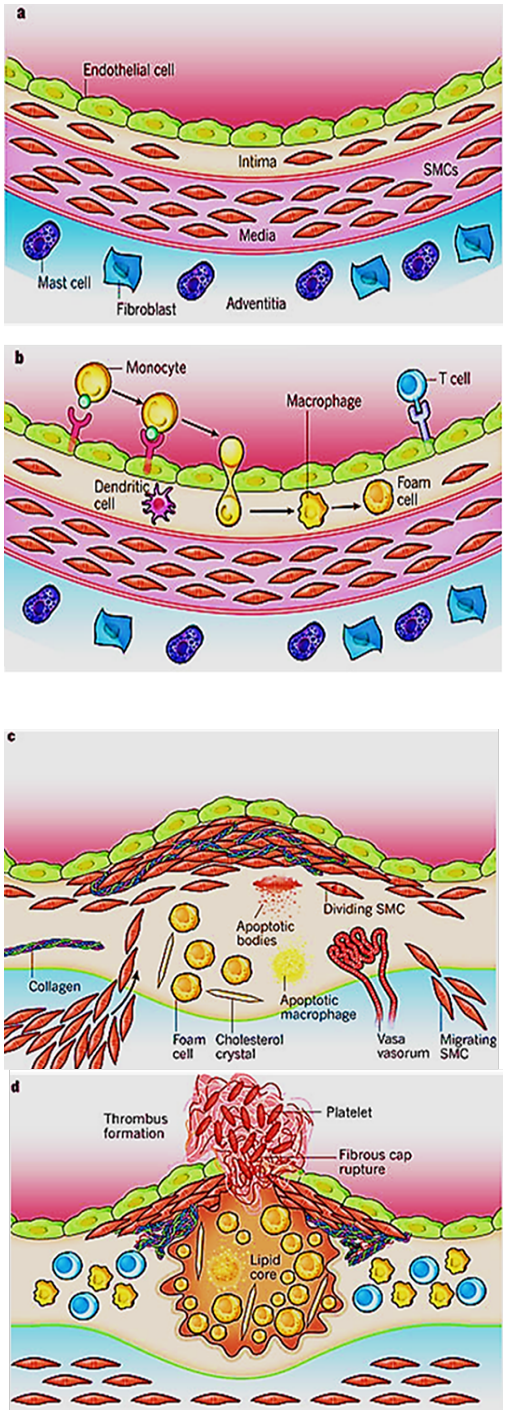
Figure 1.1 *Arterioslcerosis. Symmetrical thickening of the tunica media layer occurs.*

1.3 Atherosclerosis

Atherosclerosis is initiated by EC dysfunction (Ross et al. 1977), Quinn, et al. 1987). It is a form of asymmetrical vascular disease. NO depletion can occur and cause the

endothelial "activation" which leads to an inflammatory response activation initiating thrombosis (Cunningham & Gotlieb 2005a). Plaque builds up in regions of the vascular system causing the narrowing of the lumen of vessels. It can be distinguished by interactions between "a fibrous cap of smooth muscle and extracellular matrix that encloses a lipid rich core". In the literature the plaque is described as being composed of lipids, cholesterol, Ca^{2+}, SMCs which have migrated and other molecules located in the blood. More recently it is believed that these cells found in the plaque may be newly differentiated SMCs rather than proliferating or "de-differentiated" SMCs.

There are different stages of atherosclerosis *(Figure 1.2),* some of which are completely silent until the build-up becomes too much pressure for the vessel to handle. It initially begins when the surface of the endothelium becomes activated. Circulating leukocytes adhere to the endothelium and migrate into the intima. Monocytes mature into macrophages (Kume et al. 1992), and uptake lipids which results in the formation of foam cells *(Figure 1.2b).* After this stage, the lesion can progress and SMCs/progenitor cells migrate/proliferate from the tunica media into the intimal layer with encouragement from the macrophages. The presence of these cells increases the amount of extracellular matrix molecules gathered in the lesion, including collagen, elastin and proteoglycans. SMCs and macrophages frequently die within the lesion but the lipids derived from these cells can continue to accumulate in the region of the plaque, creating a lipid core *(Figure 1.2c).* The ultimate stage of atherosclerosis is thrombosis (Libby et al. 2011). This is initiated when rupture or erosion of the lesion occurs. It is induced by changes in the anticoagulant state of the endothelium, overlying the plaque. The coagulation process is activated and tissue factor located in the inner core of the lesion is released due to the rupture. There is a decrease of antithrombotic properties on the endothelium (Landmesser et al. 2004), which increases pro thrombotic properties of the vascular wall, promotes coagulation and initiates thrombus formation (Laszik et al. 2001). The accumulation of blood around the plaque increases the clot diameter, and as a result of this blood flow is restricted *(Figure 1.2d).* Other environmental factors including hemodynamic factors such as cyclic strain and shear stress are also thought to contribute to atherosclerotic lesions (Jiang et al. 2000), (Cunningham & Gotlieb 2005b).



A) Resting endothelium (unactivated)

B) Immune system activation

C) Accumulation of components creating a lipid core

D) Rupture of Lipid Core and Thrombus formation

Figure 1.2 *The onset and development of atherosclerosis, adapted from* Libby et al. 2011.

1.4 Current therapies for cardiovascular disease

Basic treatment for cardiovascular disease can include techniques as simple as diet change and exercise. This type of treatment would be used for patients that are at the

very early stages and also as a prevention mechanism for at risk patients. More serious treatments include medicines and then after this surgery could be necessary. Medicines are prescribed to reduce strain on the heart and to relieve symptoms (Ara 2004). There are different types of medicines available. Drugs called statins are used as cholesterol lowering drugs, antiplatelet treatment and anti-clotting/coagulant treatment uses Clopidogrel and Warfarin and medication known as Pentoxifylline (Trental) can be prescribed to increase blood supply to the extremities. Futher to this is, surgical treatment is needed the procedures that are carried out mainly include by-pass surgery and stenting. By-pass surgery is a process whereby a vein or artery from another part of the body is removed and used to by-pass blockages in the heart. Stenting is the main application of this work and is discussed further below.

1.5 Treatment of CVD using Stents

If CVD has been identified in a patient there are certain treatments which can be undertaken. For the purpose of this project stented arteries/veins will be discussed. A treatment currently used is Angioplasty and Percutaneous Transluminal Coronary Angioplasty (PTCA) whereby a stent is introduced into the vessel. This process assists in shrinking the atherosclerotic plaque and widens the artery reducing the obstruction of blood flow *(Figure 1.3)*.

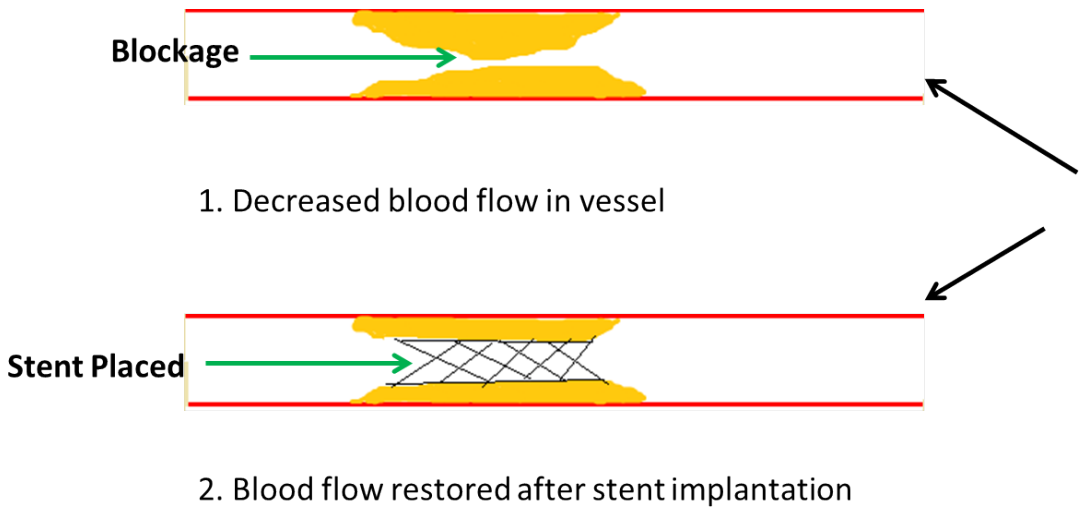


Figure 1.3 *Stented Artery restores blood flow*

A stent is a small metal mesh tube that serves to support the mechanical structure of the vessel in which it is placed. Stents are usually about 18mm in size and 3-3.5mm in diameter. The first stent to be used to treat a human patient was in 1986 by Peul and Sigwart. Stents are inserted into the target vessel by a surgeon using an angioplasty balloon method. Although stents have been shown to reduce the rate of angiographic and clinical restenosis when compared with the PTCA procedure, failure rates as high as 70% have been reported in plain stents (Bare Metal Stents (BMS)) (Haery et al. 2004). BMS were originally used, but developments have led to the discovery of Drug Eluting Stents (DES) which have proven to be superior and have reduced failure rates. The two main types of stent which currently used are stents coated with either Sirolimus or Paclitaxel drugs. Patients may receive Clopidogrel treatment at the same time (antiplatelet drug). Many trials have been undertaken to determine which stent is more appropriate in the RAVEL and SIRIUS trials. The cost of a stent is usually around $2500-2700 (Bhatia et al. 2004).

1.6 In-stent restenosis

Ultimately, in-stent restenosis is when the stent stops doing its job. Stent failure is due to the re-occlusion (restenosis) of the vessel in the area where the stent has been implanted, whereby the gradual re-narrowing of the vessel can be observed (Nikolsky et al 2003). When stents are placed in vessels the metal can cut into the endothelial layer disrupting it and exposing the medial layer. Inflammatory cascades leading to an immune response is initiated similar to any other wound healing process that occurs in the body (Nikolsky et al 2003). This activates macrophages and platelets, signalling them to home in on the site of injury.

These cells then signal proliferation of vascular smooth muscle and inflammatory cells. Cytokines and growth factors, recruited by monocytes, both accumulate at the site of injury at the endothelium. The presence of these factors causes vascular smooth muscle cell infiltration to the intimal layer (Marx et al. 2011). In this context, it is proposed that the cytokines involved are directly involved with ‘SMC proliferation’ and that they prompt either the growth of de-differented

SMCs and/or the differentiation of resident progenitors to SMCs before they migrate to the site of the stent in the intimal layer and contribute to in stent restenosis (Nikol et al 1992).

The mechanism by which restenosis occurs may be different from those that produce the primary atherosclerosis. Primary atherosclerosis plaque is usually hypocellular with collagen and lipid components, whereas secondary atherosclerosis is usually hypercellular containing extra cellular matrix (ECM). Smad is upregulated in injured arteries, this increase in Smad is associated with an increase in SMC proliferation and is found exclusively in restenoic lesions (pro SMCs) (Edlin et al. 2009). Smad proteins are transducers of TGFβ. They are intracellular proteins that receive the extracellular signals from TGFBβ and act via the nucleus to influence gene transcription (Ikedo et al 2003).

Once in-stent restenosis has occurred the chances of it re-occuring are very likely with rates from 30-70% (Haery et al. 2004). A gradual re-narrowing of the stent occurs between 3 and 12 months after stent placement. This phenomenon led to the development of DES which have proven more successful in delaying the process.

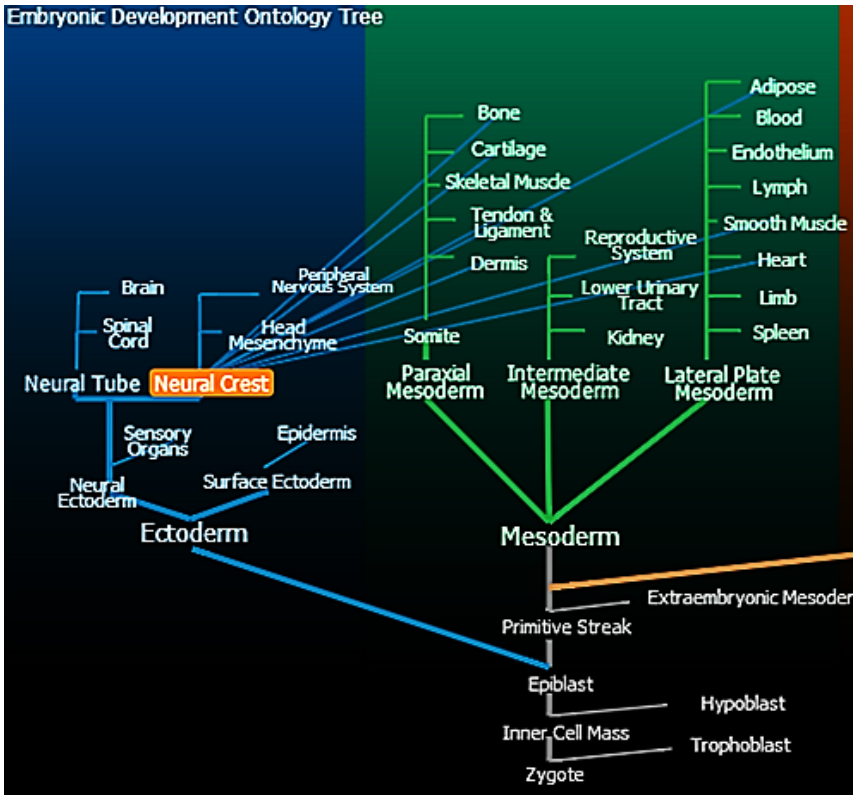


Figure 1.4 *Developmental origin of the smooth muscle cell* (adapted from Life Map Sciences, 2012)

1.7 Smooth Muscle Cell Origin

The recent developments suggesting that resident progenitor cells derived SMCs contribute to vascular remodelling has brought about great interest regarding smooth muscle cell development and origin. If the mechanism(s) responsible for stem cell derived SMC differentiation are elucidated, the development of specific therapeutics that can directly target these populations will follow. Targeting specific cell populations is very important in disease to minimise the side effects of a given drug. The origin of the smooth muscle cell is diverse and the embryological origins can differ. Vascular SMCs arise from approximately five different sources during embryogenesis (see Figure 1.4 for example). These include; neural crest, proepicardium, serosal mesothelium, secondary heart field and somites (Xie et al. 2011a). This means that the origin of SMCs can vary depending on blood vessel and even within certain vessels there exists a difference in SMC origin for example, the aorta (Seidelmann et al. 2014), whereby there is a mixed population, the neural crest cells of the ectoderm give rise to the SMCs of the aortic arch and the mesoderm

gives rise to the ascending aorta. Similarly, the carotid is of neuroectoderm origin but the coronary artery is of epicardial origin.

1.8 Smooth Muscle Cells in Culture

The first reports identifying vascular smooth muscle cells in culture were by Champy, who described the cells as elongated cells with fibrillae and noted the loss of these characteristics after many passages (Lewis & Lewis 1914) who described the mitochondrial staining in smooth muscle cells and by Laquer who noted that contractility was present in explant SMCs. It is well accepted that SMC phenotype can be modulated in culture. This involves a process whereby the expression of certain markers can be difficult to identify and sometimes the expression of SMC differentiation markers can be lost in culture. SMCs respond differently to serum and this can affect their growth pattern, for example when SMCs are serum deprived there is a significant increase in SMC differentiation markers (Metz et al. 2012).

1.9 Phenotypic Modulation/Switching

This idea of "phenotypic modulation" of SMCs has been used to explain the proliferation and de-differentiation of SMCs both in culture and playing a role in vascular remodelling *in vivo* The theory that SMCs are not fully differentiated and can undergo phenotypic modulation/switching is widely accepted. In a review from (Gomez & Owens 2012) this concept is discussed. The author makes an important point that although this theory is accepted there have not been in depth studies into defining how SMCs acquire the alternative phenotype and contribute to vascular remodelling. Lineage tracing studies are very important for identifying the exact cells involved in vascular remodelling and much research to date lacks this system. This means that is difficult to define the origin of the SMCs involved in injury. More recently Tang et al 2012 have used a lineage tracing method to show that MVSCs are resident in vascular tissue and can be found in the lesion tissue after vascular injury.

A previous study undertaken by (Rong et al. 2003) demonstrated how macrophage markers may be increased in SMCs in the lesion – meaning that some of the macrophage positive markers found may be of SMC origin, indicating that SMCs have great scope for modulation during vascular remodelling (Gomez & Owens

2012). In another study, (Bentzon et al. 2006) found that some of the macrophages associated with the lesion are not of bone marrow origin. There is a lot of different research suggesting different sources of the SMCs, yet there is a lack of knowledge about the exact origin of SMCs involved in lesions.

1.10 Contractile versus Synthetic SMCS

The dedifferentiation theory has led to the concept that two different smooth muscle cell types exist: contractile and synthetic. Contractile SMCs are recognised as being "mature" SMCs. Typically, they are elongated and spindle shaped cells. They produce extra cellular matrix proteins such as elastin and they display the three markers associated with early (SMA), middle (CNN1) and late (SM-MHCII) stage SMCs. Their proliferation and migration rates are much lower as the cells are in a state of quiescence in the vessel wall. They support the vessel wall due to their contractility and contribute to structural hemodynamics regarding cyclic strain and shear stress (Cunningham and Gotlieb 2005). In comparison SMCs with a synthetic phenotype are shorter and less elongated, they have a "cobblestone" morphology. There are more organelles present in synthetic SMCs as they play a role in protein synthesis and vessel development. Contractile SMCs have contractile filaments in place of these organelles. Synthetic SMCs show higher growth and migratory rates along with greater proliferation activity. Contractile cells in comparison remain in a more "dormant" state (Wanjare et al. 2013; Rensen et al. 2007).

It is these "activated" synthetic cells which are thought to be the cells which have migrated from the tunica media to the intimal layer in in-stent restenosis. The source of the SMCs was previously attributed to the dedifferentiation of a SMC population from the vessel wall whereby a resident contractile SMC becomes activated under certain environmental conditions and dedifferentiates to a synthetic/proliferative phenotype (Rensen et al. 2007). The dispute continues as to whether or not these cells are directly involved or if a population of resident stem cells could be the cause. Currently, there is a lot of conflicting information regarding the origin of these SMCs within the lesion. It has only more recently become accepted that the accumulation of these cells may be sourced from progenitor resident stem cells (mainly Mesenchymal Stem Cells) present in the vessel wall

(Tang et al. 2012b; Klein et al. 2011; Naito et al. 2012). A precise location of the stem cell niche has yet to be identified, unlike in other organs such as the intestine whereby the exact mechanisms are understood (Barker et al. 2008).

1.11 Smooth Muscle Myosin Heavy Chain II expression in Culture

The lack of expression of SM-MHCII in culture is associated with proliferating/synthetic SMCs (Fukuda & Aikawa 2010a; Aikawa et al. 1997). Up until recently it was widely accepted that SM-MHC negative (SM-MHC$^-$) cells in culture were of a synthetic and/or proliferative phenotype (Orlandi et al. 1994; Nakajima et al. 1993). It has been thought that as SMCs are grown in culture that they lose their SM-MHCII expression, with this loss being attributed to the transition of contractile SMCs to a synthetic phenotype.

1.12 Evidence of Smooth muscle progenitor cells

1.12.1 Resident Vascular Stem Cells

From as early as 1996 canine studies researchers have proposed the idea that resident progenitor cells exists in the vasculature (Holifield et al. 1996). Hu et al 2004 suggests a role for MSCs in vascular disease, whereby they differentiate into SMCs and contribute to an atherosclerotic plaque. Originally it was thought that the cells involved accumulated as a result of PDGF being released by injured ECs and platelets (Ross et al. 1977). In transplant studies, Hu et al. have shown that about 60% of the SMCs in atherosclerotic lesions have been derived from the donor vessel and the remaining 40% has been attributed to the recipients, which could be from circulating progenitors in the blood. These researchers showed that progenitor cells were present in the adventitia and could differentiate into SMCs in vitro and in vivo using ApoE deficient mice (Hu et al 2002).

In another case, Tang et al. 2012 have identified a population of cells within the tunica media that express unique markers associated with stem cells/neural crest cells, Sox10, Sox17 and astrocyte marker S100β. They show that an isolated pool of Mulitpotent Vascular Stem Cells (MVSCs) can differentiate into SMCs *in vivo* and

in vitro, using SM-MHC and a GFP (Green Fluorescent Protein) lineage tracing system. Their research shows that in response to vascular injury these specific MVSCs become proliferative and differentiate into SMCs and it is these newly differentiated SMCs which contribute to vascular remodelling (neointimal hyperplasia).

In a similar study from 2011, Klein et al published a paper whereby vascular wall-resident Multipotent stem cells give rise to pericytes and SMCs which contribute to new vessel maturation. The cells identified by this team are $CD44^+$ and are isolated from the adventitia. It is also agreed here that vessel formation (SMC differentiation) both physiological and pathological begins in the vasculogenic zone/niche.

A recent study (Tsai et al. 2012) has demonstrated that the cells repopulating a de-cellularized graft arise from a progenitor cell type, with markers for ECs, SMCs and stem cells found within a newly formed neointima. Repopulated cells were confirmed not to be bone marrow derived by use of a chimeric mouse model whereby a SM22-LacZ bone marrow was transplanted into a wild type mouse, then use of βgal staining.

Overall, there is a lack of knowledge regarding the exact phenotype of resident progenitor stem cells as no "standard" progenitor cell has been identified. To some extent this topic is under-developed, which is acceptable because the idea that of resident progenitors exists is relatively new and not widely accepted. However, in order for model to be conducted that will have an impact on human health a considerable amount of research is required in this area.

1.12.2 Circulating progenitors and Bone Marrow derived stem cells

Circulating stem cells and those derived from the bone marrow have been reported to play a role in the re-endothelialisation of vessels after injury and to contribute to neointimal lesions using a GFP murine model (Tanaka et al. 2008). Notch signalling has also been shown to regulate the differentiation of bone marrow derived cells into cells with smooth muscle cell characteristics (increased expression of SMA) during arterial lesion formations (Doi et al. 2009). Other studies from (Saiura et al. 2001)

have recognised that these circulating stem cells can contribute to atherosclerosis. Interestingly, (Sata et al. 2002) found that hematopoetic stem cells could differentiate into vascular cells that then contribute to atherosclerosis. However, some researchers have dismissed this idea and

It is possible that a combination of circulating progenitor cells and bone marrow derived cells are involved with contribution to vascular remodelling, depending on the degree of damage or measurement of strain acting on the vessel wall, with the possibility that progenitors may only be recruited from the bone marrow if the injury is of a high degree to help with SMC differentiation.

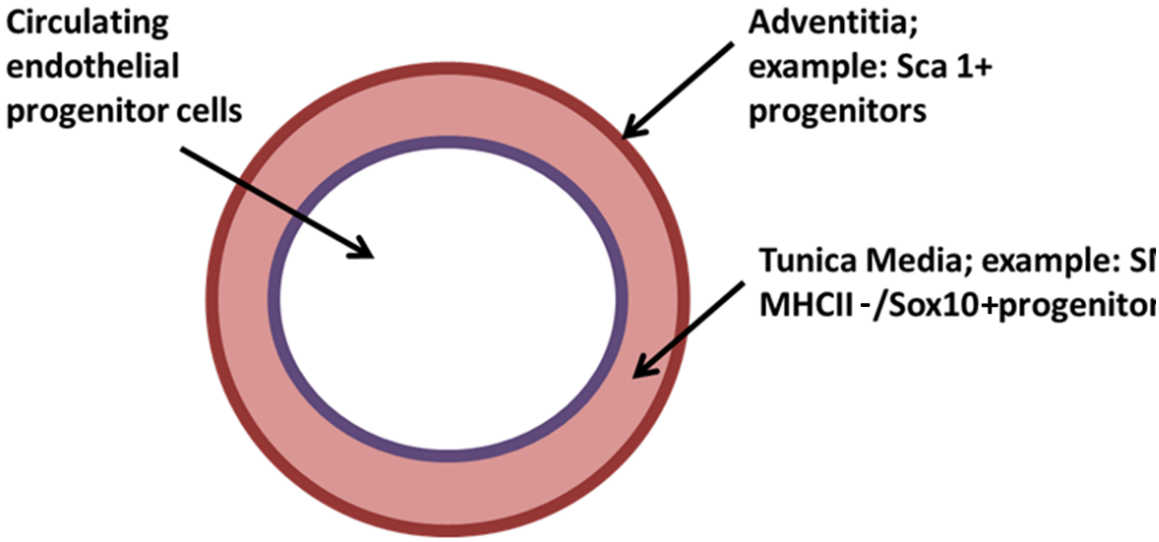


Figure 1.5 *The location of progenitor cells associated with the vasculature.*

1.12.3 Mesenchymal Stem Cells

Mesenhcymal stem cells were originally identified in the bone marrow and as research progressed, other tissues were revealed as a source for mesenchymal stem cells, one of which includes vascular tissue (Crisan et al. 2008). MSCs are mainly adult stem cells that have the capacity for self-renewal and differentiation. They can contribute to mesoderm and non-mesoderm lineages (Williams & Hare 2011). Due to their diversity, MSCs could possibly serve as a good target for tissue regeneration. MSCs have been transplanted into animal models of myocardial infarction and have shown to differentiate into cardiomyocytes and vascular cells (Shake et al. 2002);

(Toma et al. 2002). MSCs are defined by their capability to differentiate into adipocytes, chondrocytes and osteocytes *(Figure 1.6)* (Dominici et al. 2006). It is difficult to completely define the profile of an MSC as the range of markers associated with the cells is quite diverse and there are many cross overs with other cell types. Cluster of Differentiation (CD) markers are present on the surface of MSCs, and these markers are mainly used to identify the cells. However, many CDs have been linked to MSCs. The most common surface markers used to define an MSC in the literature include; CD146, CD44, CD29, CD105 and CD90 along with others (Lv et al. 2014). Other MSCs with a more immature phenotype have also been identified and express Sox2 and Oct-4, markers associated with embryonic stem cells (Iwata et al. 2010); (Kuroda et al. 2010). Differences in murine and human MSCs have been identified by (Peister et al. 2004). This limits the amount of work that can be done on animal models. More work is needed to identify markers specific to MSCs to give the cells more scope for use in regenerative medicine.

In relation to cardiovascular disease, MSC progenitors have been identified in the artery wall (Abedin et al. 2004), and have been shown to participate in the restenoic response (Tigges & Stallcup 2013). Interestingly, in 1967 Robert Wissler described smooth muscle cells as "multifunctional mesenchymal stem cells", foreshadowing the more recent findings for multipotent mesenchymal stem cells in the artery wall. Atherosclerotic lesions have been shown to contain mature bone tissue (Seemayer et al. 1973), and also cartilage (Christian & Fitzpatrick 1999). Evidence of MSC derived cells in atherosclerotic lesions indicates the presence of MSC progenitor cells in the artery. It also presents the argument that MSC differentiation is involved in vascular disease. MSCs have been shown to differentiate to SMCs via Notch and TGFβ signalling (Kurpinski et al. 2010). (Bajpai et al. 2012a), have shown that human iPSC derived MSCs can differentiate into SMCs by use of TGFβ. Research into the types of MSC involved in vascular remodelling has changed in recent years, with a bigger focus on multipotent progenitor stem cells resident in the vessel walls (Wang et al. 2012b).

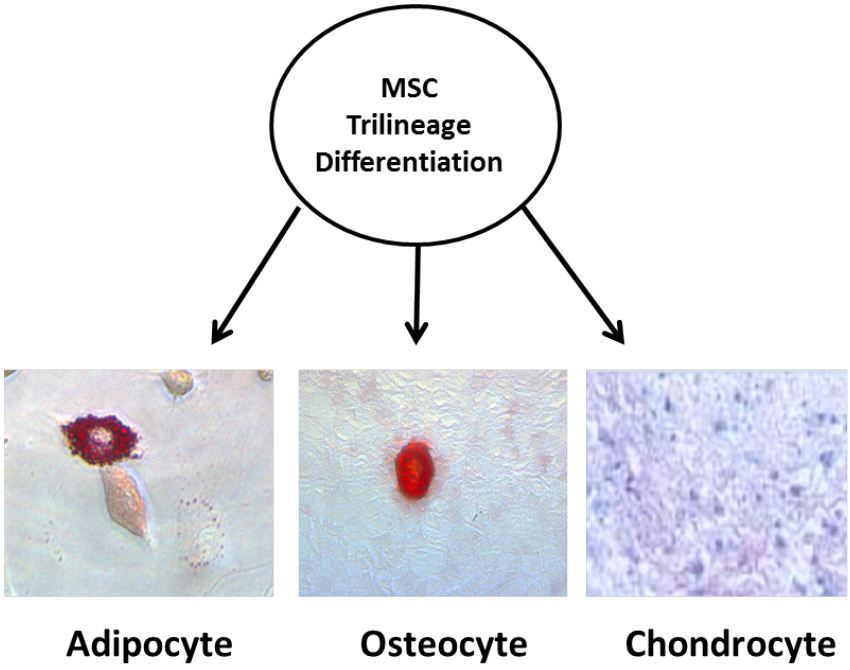


Figure 1.6 *Mesenchymal trilineage transition; adipocyte, osteocyte and chondrocyte. (Chondrocyte image, John Hopkins Medicine).*

1.13 Smooth Muscle Cells

As described previously, vascular smooth muscle cells are found in the medial layer of arteries and veins and control tone and contractility in the vessel. Common markers associated with smooth muscle cells include smooth muscle alpha actin (SMA), Calponin 1 (CNN1), smooth muscle heavy chain myosin II (SM-MHCII/Sm-2/Myh11), SM22 and Smoothelin. SMA is often used in research to identify SMCs as an early stage marker for SMC differentiation. However, this marker is an early non-specific SMC marker, with SMA being found in other cell lines such as endothelial cells (Azuma et al 2009). Moreover, sub endothelial cells in atherosclerotic lesion have been found to express both SMA and macrophage marker CD68 which can make it difficult to identify the cells involved in lesions with the marker cross over (Andreeva et al. 1997). More recently, monocytes have been shown to express SMA (Ludin et al. 2012).CNN1 is associated with being a middle stage marker, SM-MHCII is recognised as being a late stage marker for contractile SMC differentiation (Rensen et al. 2007).

1.14 Models for Vascular Cells in Culture

1.14.1 A10 and A7r5 Embryonic Stem Cell lines

A10 and A7r5 cell lines are embryonic vascular smooth muscle cell lines. They are rat cell lines which have been derived from the thoracic aorta of 14-17 day old embryonic BD1X rats. These cell lines have been used by research groups as models for vascular smooth muscle cells, representing a non-differentiated, neonatal and neointimal cell population (Rao et al. 1997). The cells have also been described as non-differentiated SMCS (Rao et al. 1997). Due to this and their embryonic origin, it was of great interest to re-examine these cell lines with regard to their stem cell marker expression in culture.

1.14.2 NE-4C Neural Crest Stem Cells as a Model for SMCs tranition form Neural progenitors

Neural crest stem cells are a type of stem cell that give rise to many neural crest derivatives (Teng & Labosky 2006). These include neurons, glial cells in the peripheral nervous system and skin melanocytes, these types are deemed “neural deriviatives”. However the neural crest can also give rise to non-neural cell types including mesenchymal associated cell types such as chondrocytes and adipocytes (Le Douarin et al. 2008). One of the purposes of this study was to investigate if an immortalised neural crest stem cell line like NE4C could be used as a model for neuroectodermal differentiation to SMC, typical of MVSCs. Therefore the potential of NE4C differentiation to SMC was investigated.

1.15 Neural Crest Stem Cell fate

The fate of neural crest stem cells (NCSC) like many other progenitor cell types, depends on how they are cultured and the components of their growth and maintenance media (Le Dourain et al 2008). When NCSCs are cultured in FBS they can easily differentiate due to the undefined nature of FBS. Rat NCSC have been shown capable of differentiation to a variety of neural crest lineages by use of BMP6, BMP7, GDF5 and GDF6. For examples BMP7 in particular was shown to induce smooth muscle and peripheral glial differentiation (Gajavelli et al. 2004).

NCSCs have also differentiated to astrocytes using retenoic acid in the presence of LIF (Asano et al. 2009). Specifically speaking, NE4C cells have been shown to produce neurons upon co-culture with with astroglia cells. Interestingly, it was also suggested that the co-culture of astroglia cells with embryonic stem cells did not result in neuronal differentiation (Kornyei et al 2005). In additon, induction with media containing Neuregulin I, glial differentiation was observed (Adlkofer & Lai 2000).

1.16 Neural Crest Stem Cells and Smooth Muscle Cells

The Neural Crest is a source for different types of vascular smooth muscle cells. Some studies have propsosed that TGFβ1 has the ability to differentiate NCSC to a smooth muscle cell fate. Bergwerff et al 1995 conducted lineage tracing studies using quail-chick chimera techniques and found that NCSCs differentiated into SMCs and also into non musclular cells of the tunica intima and tunica media in the aortic arch. However, coronary and pulmonary arteries and the descending aorta showed no sign of SMC derived NCSCs (Bergwerff et al 1995). Neural crest cell importance to the vasculature is demonstrated by the fact that when a congenitatl malformation occurs involving the neural crest presents itself in severe cardiocascular abnormalities. It was demonstrated that the removal of the cardiac crest in chick embryos or the deletion of retenoic acid receptor genes cause severe malformations in embryological development such as, ventrical septal defects, common arterial trunk and aortic arch interuptions (Bockman et al. 1989; Mendelsohn et al. 1994).

Another example that emphasises a neural crest origin for vascular SMCs comes from a genetic study carried out on mice by (Epstein 2001). The failure to orchestrate neural crest development produced a phenotype that was reminiscnet of DiGeorge Syndrome, with symptoms including outlfow tract abnormalities and a reduction of smooth muscle in the proximal great vessels.

1.17 Expression markers associated with stem cells

1.17.1 Multipotency markers associated with MVSCs

Cells continually make decisions that determine their fate, whether it is migration, proliferation, apoptosis or self-renewal (in the case of stem cells). This fate is monitored by both intrinsic and extrinsic factors. Cell specific transcription factors have great influence over these decisions and serve to repress or activate different genes. Many different groups of factors have been identified, one of which includes the Sox proteins. Nanog, Oct4 and Sox2 pluripotency markers are well recognised as being associated with embryonic stem cells (Kashyap et al. 2009). However, markers associated with progenitor cells are less established. The panel of markers chosen to identify the MVSCs is a combination of Sox10, Sox17 and S100β.

Sox proteins are a group of transcription factors found in all metazoan species (Wilson & Koopman 2002). These proteins hold various functions, playing a role in early development, organ formation and are also important for general maintenance within the cell, including self-renewal (Lefebvre et al. 2007). The Sox proteins regulate the transcription of certain genes by coordinating with other partner proteins; resulting in both gene-repression and gene-activation, depending on the type of interaction which occurs (Wilson & Koopman 2002).

The first Sox gene to be identified was *Sry* which was discovered almost 23 years ago. It is located on the Y chromosome and is responsible for determination of the male sex. It contains a conserved High Mobility Group (HMG) box domain. This domain is composed of 80 amino acids and has an affinity to 5'(A/T)AACAA(T/A)3' (Knower et al. 2003). Other proteins identified whose HMG domain have 50% or higher amino acid similarity to Sry are referred to as Sox (Sry related HMG Box) proteins, with corresponding genes termed "sox genes" (Lefebvre et al. 2007). Transcription can be activated by the HMG box via DNA binding activity but the non HMG domains can also play a role in partner protein selection and/or binding stability (Wilson & Koopman 2002). They can bind to all the minor grooves of DNA recognising the common motif sequence, it can also bend DNA and has therefore been linked to a role in assembly of transcriptional enhanceosomes. The Sox proteins are divided up into different groups (A-H) depending on their function *(Table 1)*. Functions of each group may overlap. Within each group 70% of

the amino acids are common to one another. In contrast, those in different groups share very little similarity outside of their HMG domain (Lefebvre et al. 2007).

Group	Member(s)
SoxA	Sry
SoxB2	Sox1, Sox2, Sox3
SoxB2	Sox4, Sox11, Sox12
SoxC	Sox5, Sox6, Sox13
SoxD	Sox8, Sox9, Sox10
SoxE	Sox17, Sox7, Sox18
SoxF	Sox15
SoxG	Sox30
SoxH	Sox14, Sox21

Table 1.0 *Summary of Sox Proteins*

Sox activity varies from cell type to cell type and is also promoter dependent. Darby et al 2001 describe how Sox18 functions during embryonic development for endothelial differentiation and similarly holds a function by inducing angiogenesis during wound healing and tissue repair. Another example showing the variability of the Sox proteins is during neural crest development, the stages which Sox10, Sox9 and Sox8 are expressed in mice, frog and chick embryos all differ from species to species (Hong & Saint-Jeannet 2005).

The Sox proteins of interest here are Sox10 and Sox17, both of which are neural crest associated. Sox10 plays a role in neural crest development but has also found to be fundamental to neural crest survival (Paratore et al. 2001). It is suggested that Sox10 maintains multipotency and can inhibit differentiation of NCSC to a neuronal lineage (Kim et al. 2003). A study from (John et al. 2011), demonstrated that TGFβ1 mediated suppression of Sox10 induced a switch in neural potential favouring mesenchymal differentiation. It is suggested by (Corada et al. 2013) that Sox17 expression is exclusive to arteries and not found in veins. The expression of Sox17 is high in the mouse embryo and new borns with the expression being

retained in adult arteries. They also demonstrated that Sox17 acts upstream of Notch and downstream of the Wnt system. Abnormal pulmonary vascular morphogenesis which is linked to malfunctions within the postnatal cardiac phenotype, has been partly attributed to the deletion of Sox17 (Lange et al. 2014). Sox17 has also shown to be a fundamental component for the formation of the mesoderm in embryonic stem cells. RNA interference using short hairpin RNA (shRNA) inhibited the differentiation to cardiac counterparts in the primitive mesoderm (Liu et al. 2007).

S100β is a glial specific calcium binding protein with both intracellular and extracellular functions. Its expression is associated with the brain and cells such as astrocytes, oligodendrocytes and schwaan cells. It has also shown to play a role in the cardio vascular system, whereby it contributes to the left ventricle remodelling following myocardial infarction in mice (Tsoporis et al. 2005). The role of S100β in smooth muscle differentiation is not well understood.

1.17.2 C-kit, Flt-1 and CD133 expression

C-kit is a cytokine receptor that is also known as Cluster of Differentiation 117 (CD 117). It is used to identify hematopoietic progenitor cells in the bone marrow (Fazel et al. 2006). It binds to stem cell factor also known as c-kit ligand and henceforth contributes to cell signalling by playing a role in cell survival, proliferation and differentiation (Matsui et al. 2004). Interestingly, (Hollenbeck et al. 2004) found there was an increase in c-kit expression in vascular lesions in rat balloon injury models.

Flt-1 is a receptor for Vascular Endothelial Growth Factor (VEGF). VEGF is important for vascular development and angiogenesis. It is normally associated with endothelial cells (Drake et al. 2000). It has been shown by (Orlandi & Bennett 2010) that blocking Flt-1 has an influence over several SMC functions including apoptotic susceptibility. Apoptosis was down regulated in balloon injury models which positively contributes to homeostasis within the vessel. Flt-1 expression was also found in human atherosclerotic lesions and restenoic lesions as well as in post injury rat aortic intimal thickening. However, there was no expression found in the normal tunica media. This might suggest that MVSCs express Flt1, but not adult SMCs.

CD133 is also known as Prominin. The function of this surface protein is not fully elucidated. CD133 is expressed in hematopoetic stem cells, endothelial

progenitors as well as neuronal and glial stem cells. In a study undertaken by (Adini et al. 2013), CD133 is shown to interact with VEGF. The knockdown model of CD133 caused disruption of capillary formation in vitro and decreased angiogenesis in vivo. Anti-CD133 has been added to stents to enhance endothelialisation (Li et al. 2014).

1.17.3 Nestin and Pax6 expression

Nestin and Pax6 were used as markers to identify the NE4C cell line. Nestin is a type IV intermediate protein that can be found in neuronal (embryonic) progenitor cells and (Lendahl et al. 1990). It is found in early stage embryonic development of the Central Nervous System (CNS), Peripheral Nervous System (PNS) and in myogenic tissue. It is also found to persist throughout development. Nestin expression has also been found in vascular endothelial cells at the proliferative (endothelial progenitors) stage but not the mature vasculature. Nestin is a good marker for undifferentiated cells of the CNS as the loss of the protein indicates commitment of the progeny to another specific lineage (Suzuki et al. 2010). Pax6 is a transcription factors and is associated with a member of a family of transcription factors. Pax6 is required for embryonic development of the central nervous system, the eye and the pancreas (Tyas 2003). There is less information in the literature regarding Pax6 expression and smooth muscle cells in the vasculature, but it has been shown that Pax6 may play a role in the development of smooth muscle in the iris (Jensen 2005).

1.18 The induction of Smooth Muscle Cell differentiation in vitro

1.18.1 TGFβ1

TGFβ1 (Transforming Growth Factor Beta-1) is the founding member of the TGFβ super family. This family is made up of a number of different members including Activins, Bone Morphogenic Proteins (BMPs) and growth and differentiation factors, as well as other TGFβ counter parts (Massagué 1998). Different isoforms of TGFβ have been identified in mammals these are TGFβ1, TGFβ2 and TGFβ3. The isoforms carry out similar functions in a variety of cellular activities including growth, differentiation, proliferation, apoptosis and ECM synthesis in both ECs and

SMCs (Dennler et al. 2002; Schuster & Krieglstein 2002). Vascular smooth muscle cells express distinct TGFβ receptors in culture, two types exist, TGFβ type I receptor and TFGβ type II receptor. TGFβ is found in large amounts in platelet granules and has been found to play a role in wound healing. These two factors demonstrate how TGFβ can contribute to vascular biology (Goodman & Majack 1989).

1.18.2 TGFβ1 and smooth muscle cell differentiation

TGFβ plays an important role in the differentiation of SMCs during embryonic development. (Perrella et al. 1998), discuss how TGFβ plays a role in blood vessel development during embryogenesis but also is vital for a variety of other vascular functions after developmental stages. For example, failure of SMC differentiation has been associated with a loss of TGFβ signalling components. This leads to abnormalities in the maturation of the vascular network (Goumans & Mummery 2000). TGFβ is fundamental for controlling contraction, regulating blood pressure and is responsible for blood flow in postnatal development (Owens 1995). TGFβ can be PDGF (Platelet Derived Growth Factor) dependent and independent in stimulating SMC proliferation (Mallat et al. 2001). TGFβ and Notch activation have been shown to up regulate SMC genes and protein expression in SMC differentiation of Mesenchymal stem cells and human embryonic stem cells (Kurpinski et al. 2010). Moreoever, vascular SMCs have been derived from human pluripotent stem cells via neuroectodermal and mesodermal intermediates by use of TGFβ (Cheung et al. 2012).

1.18.3 The role of TGFβ in SMC related disease

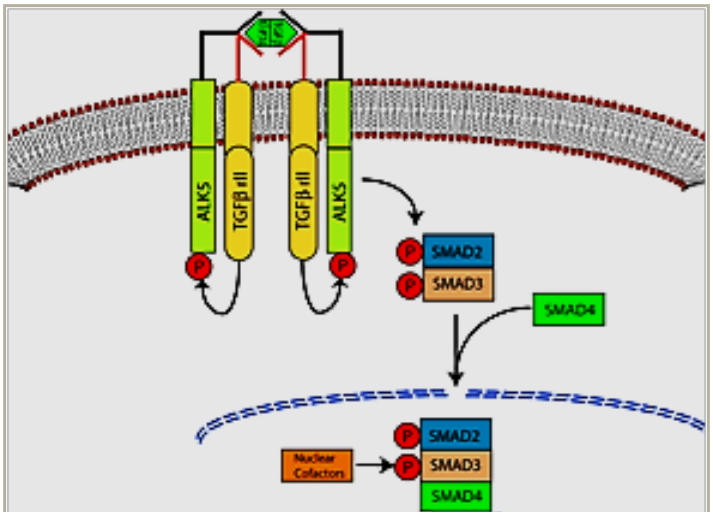
Studies from (Yamamoto et al. 2000; Orlandi et al. 1994) have shown that over expression of TGFβ increases SMC proliferation and differentiation, this has been shown to contribute to neointimal formation in balloon injury models. TGFβ has been directly linked with atherosclerosis, whereby it initiates the response of SMCs within the injured tissue or vessel (Lusis 2000). It has also been shown to affect the properties and the function of other cell types that are found in atherosclerotic lesions including; ECs, monocytes, platelets, T-cells and myofibroblasts (Bobik 2006; Mallat & Tedgui 2002). Studies carried out in humans have shown that genetic

mutations in genes from the TGFβ family (or effector genes working upstream/downstream of TGFβ signalling) can lead to vascular dysfunction and disease, for example, Camurati-Engelmann disease which affects humans. A murine knock out model for the disease shows embryonic lethality with vascular defects or a post natal lethality from auto immune disease (Kulkarni et al. 1993). Research has indicated that TGFβ has a protective role by exertion of anti-atherogenic effects (Grainger 2004). It has been suggested that this is done by regulation of SMC proliferation or by anti-inflammatory functions it carries out (Mallat & Tedgui 2002). More recently, elevated levels of TGFβ/Smad3 have been shown to contribute to Intimal Hyperplasia by inhibiting the apoptosis of SMCs through a mechanism involving VEGF (Shi et al. 2014).

1.18.4 TGFβ and Smad protein signalling

Members of the TGFβ family work by stimulating the formation of specific heteromeric complexes of type I and type II serine/threonine kinase receptors (Moustakas & Heldin 2005). The intracellular effectors of TGFβ signalling are the Smad proteins. These proteins were first identified in Drosophila and C.elegans (Patterson & Padgett 2000; Whitman 1998). They are expressed throughout embryonic development and they persist into adult life (Flanders et al. 2001; Luukko et al. 2001).

Cells can have two types of receptors as previously described; Type I and Type II. Together they form a heterodimer structure. TGFβ/Smad signalling is initiated when TGFβ ligand comes into contact with the receptor. The receptor is phosphorylated and the intracellular part of the TGFβ molecule becomes activated. This causes the phosphorylation of Smad2/3 protein. Smad4 then joins to phosphosmad2/3 forming a Smad complex. This activated complex can then travel to the nucleus where it can act as a transcription factor *(Figure 1.7)*. The heterodimer



receptor can be activated by different ligands including Activin or BMP. The result of the ligand-receptor bond will depend on the initial activator and/or the stage of development (Moustakas & Heldin 2005). TGFβ specifically is associated with SMC signalling and vascular development (Whitman 1998).

Figure 1.7 *TGFβ and Smad2/3 signalling (Wilson Lab).*

Aims of the study:

- To isolate multipotent vacsular stem cells (MVSC) from primary rat aortic medial explants.

- To characterise MVSCs in culture for their multipotent properties via trilineage differentiation assays and by probing the cells for markers associated with neural stem progenitors, such as Sox10, Sox17, S100β and also the cluster of differentiation markers linked to MSC phenotype (CD44, CD29 and CD146).

- To define MVSC transition to SMC lineage following treatment with TGF-β1 and PDGF-BB.

- To determine whether aortic SMCs in culture shared MVSC multipotent properties and/or phenotypic characteristics.

- To determine whether an immortalised murine neuroectodermal progenitor stem cell line, NE-4C mimcked the multipotent properties and phenotypic characteristics of MVSC and further whether they transition and differentiate to SMC following TGF-β1 and PDGF-BB treatment.

Chapter 2:

Materials and Methods

1.0 Materials

All materials used in the following experiments were of the highest purity and grade available.

ATCC

A10 rat aorta, thoracic/medial layer (CRL 1476),

A7r5 rat aorta, thoracic/smooth muscle (CRL 1444),

NE4C mouse neural stem cell/brain/neuroectodermal (CRL 2925),

Embryonic qualified FBS (30-2020),

EMEM (30-2003)

BD Biosciences

BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit # 554714

Fannin

4 X SDS Laemmli Buffer (161-0747)

Gibco

B27 (17504-044)

IDT

Primer Sets

Invitrogen (Groningen, The Netherlands)

Murine Mesenchymal Stem Cells (H+L) (S1502-100)

Alexa Fluor® 546 Goat anti-rabbit (H+L) (A-11035)

Alexa Fluor® 546 Goat anti-mouse (H+L) (A-11030)

Alexa Fluor® 488 Goat anti-rabbit (H+L) (A-11008)

Alexa Fluor® 488 Goat anti-mouse (H+L) (A-11001)

Alexa Fluor® 488 Donkey anti-goat (H+L) (A-11055)

N2 supplement (A13707-01)

StemPro® Adipogenesis Differentiation Kit (A10070-01)

StemPro® Osteogenesis Differentiation Kit (A10072-01)

Cell Start (A10142-01)

2-mercaptoethanol 50mM solution, (31350-010)

DEPC treated water (750024)

HCS Lipotox, (750024)

InvivoGen

Primocin (ant-pm-1)

Millipore

LIF (ESG1107)

MP Biomedical (CA, USA)

Chick embryo extract (2850145)

MSC

Magcore cartridges total RNA Cultured Cells 610 (MRC-01 // MRC-02)

Sensi-fast SYBR No-Rox one step kit (BIO-72005)

PeproTech

TGFβ1, PDGF-BB

Pierce

Dnase I,Rnase-free (89836)

BCA Assay kit (23227)

Page ruler ladder (26620)

Qiagen (West Sussex, UK)

SMA: Mm_Acta2_1_SG (QT00140119)
SM-MHCII isoform 1: Mm_Myh11_1_SG (QT01060843)
SM-MHCII isoform 2: Mm_Myh11_2_SG (QT02327626)
Smoothened: Mm_Smtnl2_1_SG (QT00126455)
Calponin: Mm_Cnn1_1_SG (QT00105420)
Sox10: Mm_Sox10_1_SG (QT00295204)
Sox17: Mm_Sox17_1_SG (QT00160720)
S100β: Mm_S100b_1_SG (QT00151536)
GAPDH: Mm_Gapdh_3_SG (QT01658692)

R&D Systems (Minneapolis, USA)

rmFGF (3139-FB-025)

TGFβ1 (7666-MB-005/CF)

Sigma-Aldrich (Dorset, UK)

2-mercaptoethanol (M-7154)

30% bis/acrylamide solution (A3699)

Alizarin Red (A5533)

Anti-goat 2° antibody, HRP conjugated (A5420)

Anti-mouse 2° antibody, HRP conjugated (A5278)

Anti-rabbit 2° antibody, HRP conjugated (A0545)

APS (A3678)

BSA (A4503)

Collagenase (C6885)

DMEM (D5796)

DMSO (C6164)

Elastase type III (E0127-5MG)

Fatty acid free BSA (A7030)

FBS (F9665)

Gel Blotting Paper (Z698172)

Glycine (G8898)

L-Glutamine (G7513)

Oil Red O (O0625)

Pen-Strep (P4333)

Poly-L-Lysine (P9155)

Ponceau S stain (P7170)

Protease inhibitor (P8340)

Protran membrane (Z670979)

Retinoic acid (R2656)

RIPA (R0278)

Soybean Trypsin inhibitor (T6414)

TEMED (T7024)

TMB (T0565)

Triton X-100 (T8787)

Trypan Blue (T8154)

Trypsin (T4174)

Tween (P1379)

α-MEM (M0644)

2.1 Methods

2.2 Cell culture protocols

All cell culturing techniques were carried out in aseptically in a Biosciences Air 2000 Mac laminar flow cabinet. Cells were maintained in a Hera water jacketed cell culture incubator at 37°C and 5% CO_2. Cells were visualised using a Nikon Eclipse TS100 phase-contrast microscope.

2.2.1 Embryonic Vascular Smooth Muscle cell culture

Two embryonic smooth muscle cell lines were studied; A10 and A7r5. The cells were derived from rat embryos and were isolated from the thoracic aorta/medial layer and thoracic aorta/smooth muscle, respectively. A10 cells were cultured in Embryonic Stem Cell approved medium and 10% (v/v) Fetal Bovine Serum (FBS) supplemented with 1% Penicillin-Streptomycin (P/S), 0.001% of Leukemia Inhibitory Factor (LIF) and 0.01% of β mercaptoethanol (50mM). A7r5 cells were cultured in DMEM medium supplemented with 10% FBS and 1% P/S. For experimental purposes cells were cultured in T25cm^2, T75cm^2 and 6 well plates.

2.2.2 Rat Mesenchymal Stem Cell Culture

Rat Mesenchymal Stem Cells were used at low passages between 4 and 11. Low passages were used to ensure the cells still had high mulipotent potential. The cells were maintained in growth media made up of 50:50 minimal essential medium (α MEM) an Ham's F12 supplemented with 10% MSC defined FBS, 150unit/ml penicillin and 150µg/ml streptomycin.

2.2.3 NE4C Cell Culture

NE4C cells were maintained in media as recommended. The media was composed of 10% FBS embryonic qualified in EMEM. The media contained an initial 2mM L-Glutamine and was supplemented with an extra 2mM of L-Glutamine to give a final concentration of 4mM in the maintenance media. Before NE4C cells were seeded onto plates/dishes the surface of the container was pre-coated with 15µg/ml Poly-L-

Lysine. The plates were pre-coated and left to dry for 5 minutes. After this they were washed briefly with water and left to dry for at least 2 hours before the cells were seeded.

2.2.4 Adult Smooth Muscle Cell line Culture

Rat, mouse and bovine adult smooth muscle cell lines were cultured in 10% FBS and DMEM supplemented with 1% P/S. The cells were used up to passage 20. Murine aortic SMCs were obtained from ATCC Rockville, MD [MOVAS (ATCC CRL-2797)]. Rat aortic SMCs [rSMCs, R354-05a) were obtained from Cell Applications, CA. Bovine aortic SMCs were obtained from Coriell Cell Repositories [Coriell AG08504, NJ, USA].

2.2.5 Isolation of Rat MVSC

Male Sprague Dawley rats were anesthetized with pentobarbital sodium (0.1mg/g) and then perfused with 10ml of PBS. The ribcage was cut open at the front of the animal to expose internal organs. These were removed so the back of the ribcage was in view. The aorta was carefully excised using a tweezers and a scissors. The tweezers was carefully run along the side of the aorta to loosen it from the back bone. The aorta tissue was harvested as fast as possible from the lower thoracic aorta to the upper abdominal aorta. Following this, the tissue was briefly washed in cold PBS to remove any blood an excess fatty tissue. It was then placed in pre-warmed DMEM supplemented with 1% FBS and 1% P/S. The endothelium was removed by gently scraping off the cell layer on the luminal surface using a sterile scalpel blade. The aorta was placed in an enzyme solution containing 2.5mg/ml of collagenase for 20 minutes at 37°C. After, the adventitia was carefully removed using a forceps under a dissection microscope. The layer of the artery that remains is the tunica media only. This section was cut up into pieces measuring 1mm in diameter. The tissue sections were placed on 6-well plates which were pre-coated with 1% CellStart (Invitrogen). Approximately 5-6 pieces of tissue were added per well. The cell start was diluted 1:50 in in Dulbecco's Phosphate Buffered Saline with calcium and magnesium.

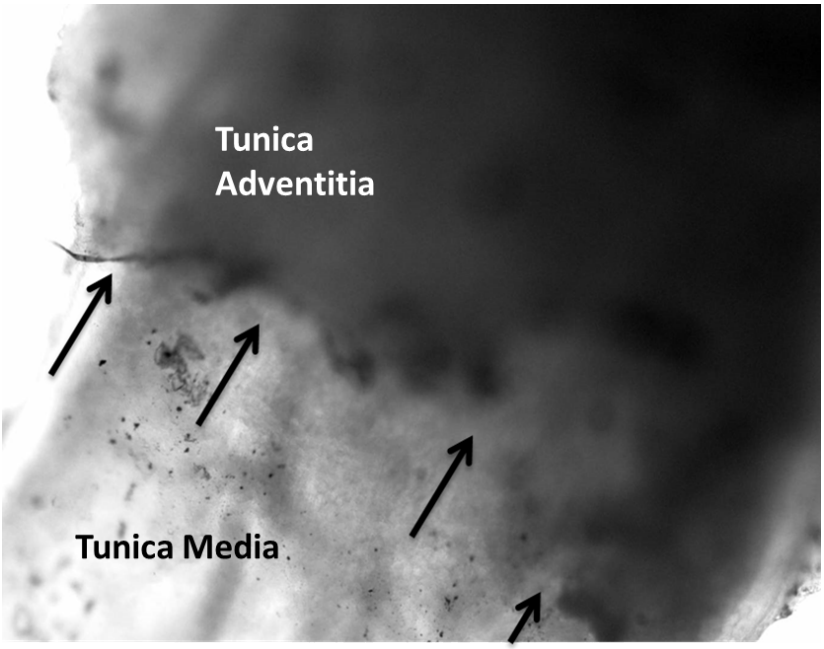


Figure 2.0 The adventitia was carefully removed and peeled back to isolate the medial layer only.

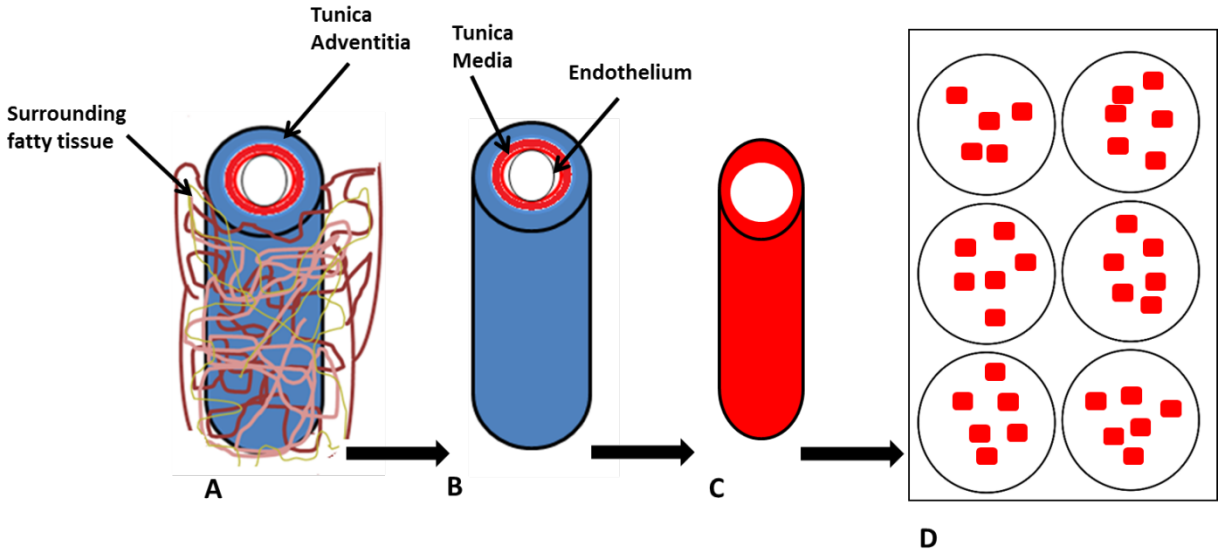


Figure 2.1 *MVSC isolation protocol. A) The surrounding fatty tissue is removed, B) the endothelial layer is gently removed, after incubation with an enzyme cocktail the adventitial layer is removed, C) the medial layer is the only remaining layer, D) the tunica media is cut up into small pieces and 6-7 pieces of tissue are placed in a pre-coated 6 well plate.*

2.2.6 Explant cell culture

The tissue pieces were initially grown in 10% FBS in DMEM supplemented with 1% P/S and primocin at 100ug/ml. After three days cells began to explant out of the tissue. Once this was observed the tissue pieces were removed. Each well was washed briefly and the media was replaced with a maintenance media specifically designed to keep the MVSCs in a multipotent state. The media used to maintain MVSC multipotency was made up in DMEM and supplemented with 2% chick embryo extract (MP Biomedical), 1% FBS, 1% N2 (Invitrogen), 2% B27 (Invitrogen), 100 nM retinoic acid (Sigma-Aldrich), 50 nM 2-mercaptoethanol (Sigma-Aldrich), 1% P/S and 20 ngml−1 bFGF (R&D Systems). Cells were re-fed every 2-3 days and passaged every 3days depending on confluency (usually around 70%). The cells had a very low adherence rate after trypsonization.

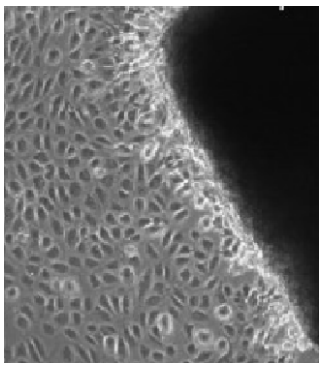


Figure 2.2 An example of progenitor cells in culture explanting from isolated tissue (Smart and Riley, 2006).

2.2.7 Isolation of Enzymatically digested SMCs

The same procedure was used as described previously in order to isolate the aorta. This time the cells of interest are the majority of cells in tunica media rather than the minority of mulipotent cells which explant out first from the vessel. As previously described, connective and outer fatty tissue was removed from the vessel. The aorta was then left in a collagenase solution at 2.5mg/ml for 20 minutes at 37°C. After this treatment the adventitia was removed carefully using a fine forceps. The tunica media was then incubated in a solution containing 0.25mg/ml elastase, 0.7mg/ml collagenase, 0.4mg/ml Soybean trypsin inhibitor and 1mg/ml essentially fatty acid free BSA for 2 hours at 37°C. At 15 minute intervals the tissue was removed from

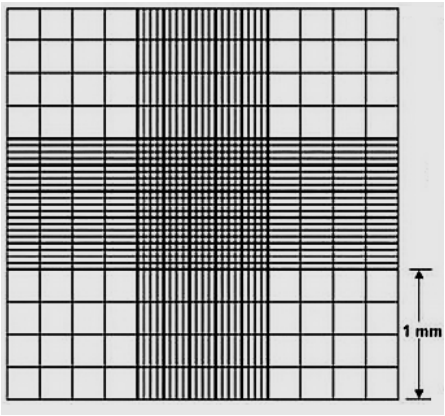
the incubator and vortexed for 20 seconds to promote the dissociation of cells from the tissue. After two hours media containing 10% FBS in DMEM was added to the tissue and enzyme solution to neutralize the actions of the enzymes. The subsequent solution was spun down at 200 *xg* for 5 minutes and re-suspended in media before being plated onto 6 well plates. The media used for these cells was DMEM supplemented with 10% FBS and 1% P/S. The cells were allowed to settle down for 48 hours before the media was changed and before analysis.

2.2.8 Trypsinisation of cells

All cell lines used were adherent therefore trypsinisation was required to sub culture the cells. Trypsin is a proteolytic enzyme that detaches cells from the surface they are cultured onto. Briefly, growth medium was removed from flasks/culture dishes and the cells were washed twice in sterile PBS to facilitate the removal of α-2-macroglobulin, a component of FBS which inhibits the action of trypsin. 1X trypsin was added to the flask which was then incubated at 37°C for 2-5 minutes (depending on cell type). Regular cell growth medium containing FBS was added to neutralise the cleaving action of the trypsin. Cells were harvested by centrifugation at 115 *xg* for 5 minutes at room temperature. Cells were re-suspended in the appropriate volume of growth medium and counted using a haemocytometer. Cells were split at different ratios according to the specifications of individual cell lines.

2.2.9 Cell Counting

After trypsinisation cells were re-suspended in media and counted using a haemocytometer to obtain the number of viable cells. A 20µl cell suspension was mixed gently at 1:1 with 20µl of Trypan Blue. 20µl of this suspension was added to the haemocytometer *(Figure 2.3)*. Trypan blue will stain cells which are not viable due to their leaky membrane. Viable healthy cells do not take up the dye and will appear bright under the microscope in comparison to the dead cells. Four of the chambers were counted excluding any cells which touched the line indicating the left side of the square and the top side of the square.



The cell number was calculated using the formula:

(Cell Number)/4 * (Dilution Factor) * 1x10^4 (area under cover slip mm) = Number of viable cells/ml

Figure 2.3 *Haemocytometer cell counting*

2.2.10 Cryogenic preservation and recovery of cells

Cells were stored in cryovials suspended in Liquid Nitrogen at -196°C for long term storage. Cells were trypsinised and pelleted as per usual and re-suspended in freeze down media which was composed of 20% (v/v) FBS and 5% (v/v) DMSO in regular growth medium supplemented with 1% (v/v) P/S. Cells were divided into 1 ml aliquots in sterile cryovials. They were then placed in the Liquid Nitrogen cell and were frozen at the rate of –1°C/minute using a Mr Freeze® cryofreezing container. The cells were left in the -80°C freezer overnight and then transferred to a cryo-freezer unit.

2.3 Immunocytochemistry

Cells were seeded at low density (5-10,000 cells/well) for immunocytochemistry to allow for clear visualisation of the cells. They were seeded onto 6 well plates containing sterile coverslips. Coverslips were sterilized in IMS for 2 hours and washed up to 5times in sterile PBS before the addition of cells.

For preservation and fixation of samples cells were treated with formaldehyde at 3.7% for 15 minutes at room temperature. For intracellular staining

cells were permeabilized for 10-15 minutes using 0.025% Triton X-100. The samples were blocked for non-specific binding sites using 1ml of blocking buffer which was added to each well. The blocking solution was composed of 5% BSA and 0.3M glycine in 1% Tween PBS solution. Samples were incubated for 1 hour at RT. The primary antibody was diluted in blocking buffer at the appropriate concentration and added to the coverslips. It was allowed to bind overnight at 4°C protected from the light. The secondary antibody was diluted in the blocking buffer at the appropriate concentration (Alexa Fluor 1:1000), added to the wells and incubated for 1hr at room temperature. Cell nuclei were stained using DAPI (1:1000) and incubated for 15 minutes at room temperature. For each secondary antibody used a no primary control was prepared to ensure that there was no non-specific binding of the secondary antibody to the cells *(Figure 2.4)*. All of the secondary alone controls can be found in the Appendices. Cells were washed in between each step using 1% Tween in PBS 2-3 times for five minutes, with gentle agitation. Exposure to light was minimal throughout the process. 8-10 images were taken per sample at magnifications of 10X, 20X and 60X or 100X power. Results are representative of experiments that were repeated multiple times (n=3 or more).

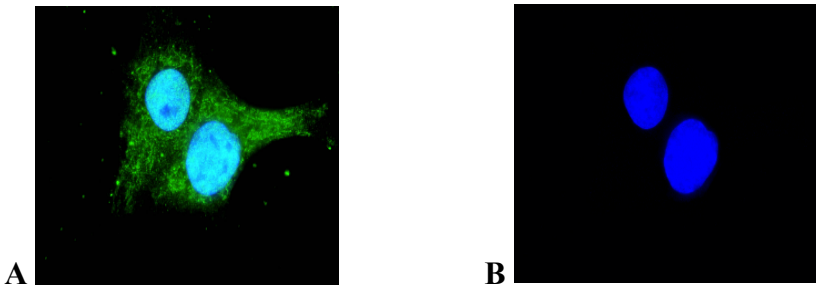


Figure 2.4 *Sample immunocytochemistry images of cells probed with primary and secondary antibody and DAPI (A) and cells probed with secondary antibody and DAPI (B) - primary antibody control. Cells shown here are MVSCs expressing Sox10. DAPI stain used for neucli observation.*

2.4 Confocal Imaging

Cells were plated onto glass coverslips and placed in six well culture plates. The cells were incubated for at least 48 hours, allowing the cell attachment and spreading

for optimal protein visualisation. Before probing the cells with antibody they were fixed and permeabilized using ice-cold acetone for 1 min. The cells were then washed three times with PBS containing 0.5% TWEEN-20. Non-specific binding of the antibody was prevented by blocking the cells in a solution of 5% BSA made up in PBS containing 0.5% Tween-20 for ten minutes at room temperature. Specific primary antibodies were added to the cells and they were incubated overnight at 4°C. The following day the appropriate secondary Alexa 488-labelled antibody was added to the samples and the cells were incubated at room temperature for 60 minutes. The coverslips containing the cells were mounted onto slides with antifade medium (Dako). Slide preparations were observed using a Zeiss Axio Observer. Z1 equipped with a Zeiss 710 and ConfoCor3 laser scanning confocal head (Carl Zeiss, Inc.). Images were analyzed using Zen 2008 software *(Figure 2.5)*. 8-10 images were taken per sample at magnifications of 10X, 20X and 60X or 100X power. All results shown are representative of experiments that were repeated multiple times (n=3 or more). Secondary controls were used for all samples.

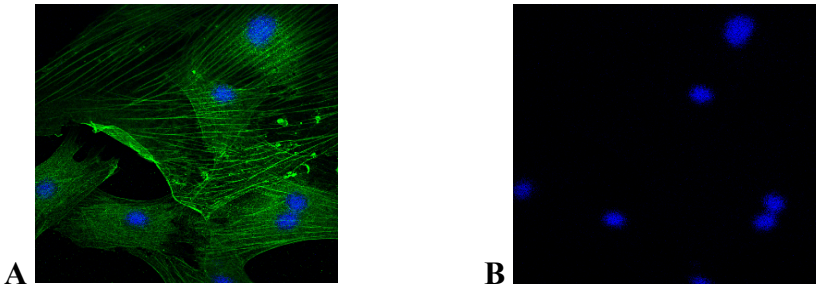


Figure 2.5 *Sample confocal images of cells probed with primary and secondary antibody (A) and cells probed with secondary antibody only (B) - primary antibody control. Cells shown here are MVSCs expressing SMA.*

2.5 SDS-PAGE and Western Blot Analysis

2.5.1 Preparation of Whole Cell Lysates – Protein Harvest

Protein samples were harvested using radioimmunoprecipitation assay (RIPA) lysis buffer (composed of; 20mM Tris, 150mM NaCl; 1mM Na2EDTA; 1mM EGTA; 1% Triton X-100 (v/v); 2.5mM sodium pyrophosphate; 1mM-glycerophosphate; 1mM sodium orthovanadate; 1µg/ml leupeptin) supplemented with protease - phosphate inhibitor cocktails (1/100 dilution of each, Sigma Aldrich). Cells were washed 2-3

times in ice cold PBS. A small volume of ice-cold PBS was added to each well and a cell scraper was used to detach the cells from the plate surface. The cells were collected and centrifuged for ten minutes at 1000rpm to pellet the cells. The PBS was removed and an appropriate volume of RIPA buffer (depending on pellet/samples size) was added to the pellet to lyse the cells. The amount added was minimal to ensure a high concentration (mg/ml) of protein was achieved. The cell lysate suspension was re-suspended in the RIPA buffer using a pipette to ensure equal mixing and yielding the maximum amount of protein from the cells. The tubes were sonicated for 10 seconds at 40% using a Branson Digital Sonifier (AGB Scientific). The samples were either stored at -80°C (long term), -20°C (short term) or used immediately in a BCA assay to determine the overall protein concentration or for western blot analysis.

2.5.2 Preparation of fractionated Lysates

Protein was isolated in the same manner as whole cell lysates. After suspension in RIPA buffer the cells were kept on ice for an hour. Every ten minutes the samples were vortexted for 10 seconds. The samples were then spun down at 13,000rpm for 10 minutes at 4°C. The supernatant was removed, representing the cytosolic fraction and the pellet (nuclear fraction) was re-suspended in a defined quantity of RIPA buffer and sonicated to release any membrane bound proteins. The fractions were either used immediately for Western Blot analysis or stored at -80°C.

2.5.3 Protein Sonication

Protein sonication agitates the samples using sound energy. The purpose of this is to release any membrane bound proteins from the nuclear fraction or total protein lysate. This was carried out for 10-15 seconds depending on the viscosity/size of the cell lysate/pellet at 40%.

2.5.4 Bicinchoninic acid (BCA) Assay

The Bicinchoninic acid (BCA) protein microassay is a method used for protein quantification. Cell lysates were analysed using a commercially available “Pierce

BCA Protein Assay Kit" to determine the total protein concentration per sample. The assay uses the Biuret reaction and protein concentration is determined calorimetrically using a colour change approach. The assay depends on selective calorimetric detection of Cu^{+} ions by the bicinchoninic acid (BCA). Cu^{2+} reacts with protein under alkaline conditions and is reduced, producing the cuprous cation (Cu^{+}), with selective colorimetric detection of Cu^{+} using a reagent containing BCA. Bicinchoninic acid, sodium salt, is a stable water-soluble compound that forms a purple complex (originally yellow/green) with cuprous ions in an alkaline environment. The complex formed exhibits strong absorbance at 562nm and is measured using a spectrophotometer (Thermo Scientific). The absorbance is linear with increasing protein concentrations from standards ranging between 0.2-2mg/ml. Protein lysates and the bovine serum albumin (BSA) protein standards were added to a 96 well plate in triplicate (10µl). The kit supplies two different solutions A and B. A is an alkaline bicarbonate solution and B is a copper sulphate solution. A dilution of 1:50 is made by mixing B:A. 200µl of this solution was added to the protein cell lysate or BSA protein standard. The plate was protected from light and incubated at 37°C in a CO_2 incubator for 30 minutes. A standard curve was prepared using BSA (0-2mg/ml) for each experiment and from this the protein concentrations were determined quantitatively *(Figure 2.6)*.

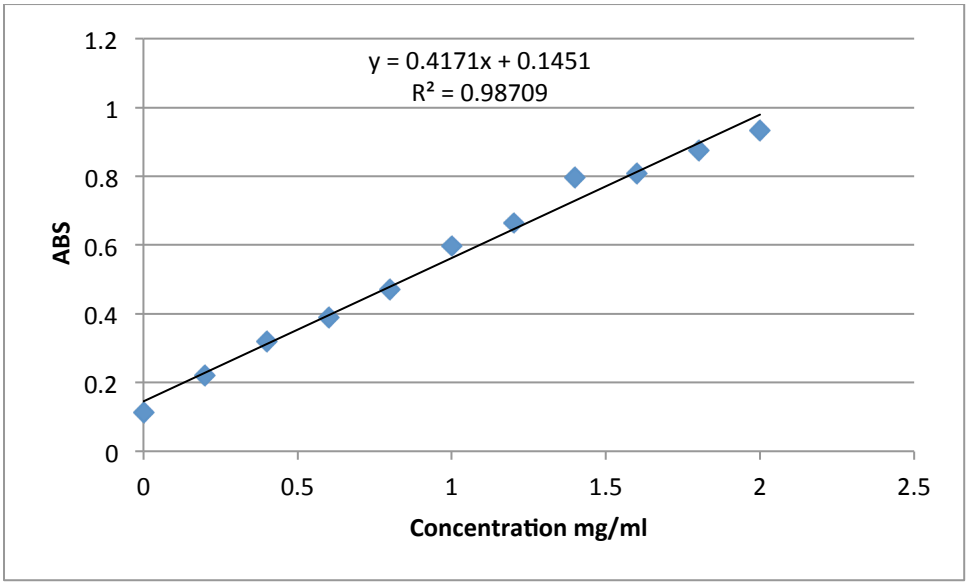


Figure 2.6 *Example of BCA assay standard curve to determine protein concentration.*

2.6 Western Blotting

SDS-PAGE was performed as described by Laemmli using 12.5%, 7.5% and 5% polyacrylamide gels (Laemmli et al., 1970). The semi-dry Western blot method was used. The gels (stacking and resolving) were prepared as follows:

Resolving Gel:

Component	7.5%	12.5%
30% bis/acrylamide solution (ml)	1.875	3.12
1.5M Tris-HCl pH8.8 (ml)	1.875	1.875
10% SDS (µl)	75	75
dH_2O (ml)	3.63	2.38
10% APS (µl)	37.5	37.5
TEMED (µl)	7.5	7.5

Table 2.1 *SDS PAGE Resolving Gel recipe*

Stacking Gel (5%):

30% bis/acrylamide (µl)	**325**
1M Tris-HCl (µl)	625
10% SDS (µl)	25
dH_2O (ml)	1.54
10% APS (µl)	12.5
TEMED (µl)	5

Table 2.2 *SDS PAGE Stacking Gel recipe*

For analysis of cell lysate protein concentration was determined using the BCA assay as described previously. From this equal amounts of protein were added to each well in the gel. Before addition to the gel the samples were mixed with a 4X laemmli loading buffer (8% SDS, 20% β-mercaptoethanol, 40% glycerol, Brilliant Blue R in 0.32M Tris pH6.8). The samples were boiled for 5 minutes at 95 °C and immediately placed on ice. The gel electrophoresed in reservoir buffer (0.025M Tris pH8.3; 0.192M Glycine; 0.1% (w/v) SDS) at 30mA using a power pack (BioRad). Pierce page ruler ladder was used as a marker for protein size, 10µl of the ladder was added to the appropriate well. The gel was pre run for 30 minutes before the samples were added, upon addition of the samples the gel was run until the dye front reached the bottom of the gel.

Following gel electrophoresis the gel was soaked in transfer buffer (0.025M Tris pH8.3; 0.192M Glycine; 15% v/v methanol) for 10 minutes at 4°C. For each gel one sheet of Whatman (Life Sciences) nitrocellulose membrane was cut and 6 sheets of Whatman gel blotting paper (Sigma) were cut to fit the size of the gel and pre-soaked in transfer buffer. Proteins were transferred to the membrane using an Atto gel box (MSC) semi-dry automated system for 30 minutes at 17V. Ponceau S staining was used to confirm the transfer of the proteins from the gel to the membrane. The Ponceau S stain was left on the blots for 2-3 minutes and washed briefly using dH_2O revealing defined pink bands demonstrating the transfer of the proteins *(Figure 2.7)*. Once protein transfer was confirmed the blots were blocking in

a blocking buffer (0.5% BSA and 0.1% Tween in PBS) for one hour at room temperature on agitation. Following the blocking step, blots were probed with the appropriate antibody at the appropriate concentration overnight at 4°C. The next day the blot were washed three times for five minutes each with 0.1% Tween. The appropriate HRP-conjugated secondary antibody was added to the blots for 2 hours at room temperature, away from light with gentle agitation. Both primary and secondary antibodies were diluted in blocking buffer solution. After the incubation the washing step was repeated on the blot. The blot was washed as before and 5ml of 3, 3',5,5'-Tetramethylbenzidine (TMB) was added to each blot to determine if the desired protein was present on the blot. The protein bands will appear within minutes displaying a dark blue colour on the blot. dH_2O used to wash off the TMB and stop the further development of non-specific bands/background staining. TMB is a chromogenic substrate that allows for the visualisation of the protein bands when used for the western blot method *(Figure 2.8).* Adobe software and densitometry was used to analyse the blots. GAPDH or β-Actin (depending on the experiment) primary antibodies were used for each blot as a loading control as well as the Ponceau S stain.

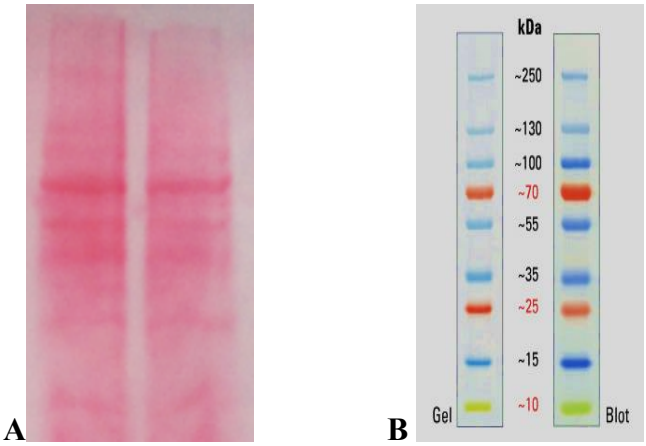


Figure 2.7A *Example of Ponceau S staining protein on blot, Figure 2.7B protein ladder used for molecular weight marker.*

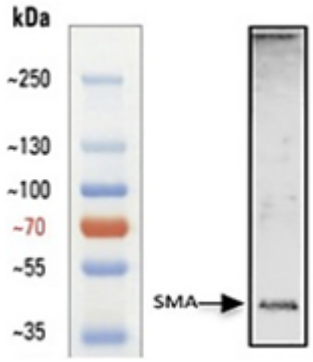


Figure 2.8 *Representative western blot of smooth muscle actin in MVSCs. Visualisation was carried out using TMB after secondary antibody incubation.*

2.6.1 Antibody Dilutions

Primary Antibody	Dilution	Secondary Antibody	Dilution
CNN1 (Sigma)	1:5000	HRP-Conjugated Anti-mouse IgG	1:5000
αActin (Sigma)	1:5000	HRP-Conjugated Anti-mouse IgG	1:5000
SM-MHCII *(1)* **(Abcam)**	1:1000	HRP-Conjugated Anti-rabbit IgG	1:5000
SM-MHCII *(2)* **(Sigma)**	1:1000	HRP-Conjugated Anti-mouse IgG	1:5000
SM-MHCII *(3)* **(Santa Cruz)**	1:1000	HRP-Conjugated Anti-goat IgG	1:5000
Sox17 (Millipore)	1:2000	HRP-Conjugated Anti-rabbit IgG	1:5000
Sox10 (Abcam)	1:2000	HRP-Conjugated Anti-rabbit IgG	1:5000
S100β (Millipore)	1:2000	HRP-Conjugated Anti-rabbit IgG	1:5000
SMAD3	1:5000	HRP-Conjugated Anti-rabbit IgG	1:5000

(Abcam)			
GAPDH (Sigma)	1:5000	HRP-Conjugated Anti-rabbit IgG	1:5000
B-Actin (Sigma)	1:5000	HRP-Conjugated Anti-mouse IgG	1:5000

Table 2.3 *Outline of antibody dilutions used in experiments*

2.7 Flow Cytometry

Flow Cytometry is a method used in biotechnology and other industries to count and sort cells. FACS™ (Fluorescent Activated Cell Sorting) is a type of flow cytometry whereby cell samples flow through the cytometer in a single cell fluid suspension. Cell samples are treated with flouresecent particles in preparation for this method of analysis. Particles which can be used include Annexin V and Alexa Fluor 488/546 for example. The cytometer contains a laser which analyses the particles as they pass by. This laser measures the forward scatter and side scatter of the cells analysing the size of the cell and granularity of the cell respectively. The laser measures these parameters based on fluorescence of each individual cell as it passes a certain point. The fluorescent particles are excited at a certain wavelength and then emit at another wavelength depending on each individual particle. For example Alexa Fluor 488 excites at 499nm and emits and 519nm. There are filters in front of the detectors in the cytometer that restrict the light that reaches the detector to a specific range of wavelengths.

2.7.1 Protein Marker Analysis

FACS was used to screen a series of cell lines for different markers representing smooth muscle and stem cell phenotypes. The kit used was BD Bioscience Cytofix/Cytoperm and the protocol provided was used. Briefly, 500,000 cells were used per sample (equal cell number). After the cells were pelleted they were fixed (if appropriate for the antigen) using BD cytoperm solution for 20 minutes at 4°C. Following two brief wash steps the appropriate primary antibody was added at a

final concentration of 1µg per sample. The cells were incubated at 4°C for 30 minutes. The cells were washed again and incubated with appropriate secondary antibody (Alexa Fluor 488 or Alexa Fluor 546) for 30 minutes at 4°X. Cells were analysed using BD FACS Calibur using the corresponding detector according to the secondary fluorescent probe used *(Figure 2.9)*.

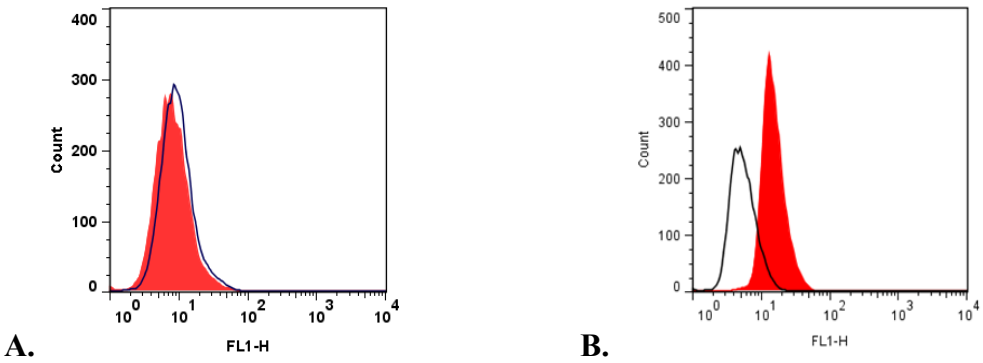


Figure 2.9 *Example of FACS data analysis. A) Secondary antibody control, B) MVSC expression of CNN1.*

2.7.2 FACS Data Analysis

Data analysis was performed using FloJo and De Novo software FCS Express 4 Flow Cytometry.

2.8 Polymerase Chain Reaction (PCR)

2.8.1 Preparation of total RNA

RNA was isolated from cultured cells using the MagCore® HF16 System of RBC Bioscience Corp. *(Figure 2).* This instrument purifies Nucleic acids (RNA and DNA) from many different tissue types, but for the purpose of these experiments kit no. 610 was used for RNA extraction. The kit is a cartridge that contains different solutions/buffers pre-prepared that are required for the isolation of RNA *[Figure 2.3]*. These cartridges are slotted into racks within the machine. Two sets of racks are within the machine, the smaller T-rack holds the samples and buffers prepared by

the user and the second larger rack hold the pre-prepared cartridges. Pipette tips are added to well 2 and the machine picks up the raw sample and extracts the RNA allowing everything to be carried out in a controlled, sterile environment. The instrument provides a protocol for tissue processing and this was followed. Briefly, Cells were trypsinised from 6-well plates and washed once using PBS. A Reaction Buffer supplemented with 1% β-mercaptoethanol was prepared and 200µl of this solution was added to the cell pellet. The cell and buffer solution and mixed using a vortex until the cell pellet was re-suspended. This suspension was added to well 4 on the rack. A DNase solution was prepared using DNase I and a DNase buffer solution provided and this was added to well 3 *(Figure 2.10)*. The 610 option program was run and the total duration for pure RNA extraction was 73 minutes. Samples were eluted in 60µl of DEPC treated water into an elution tube inserted to Well 1.

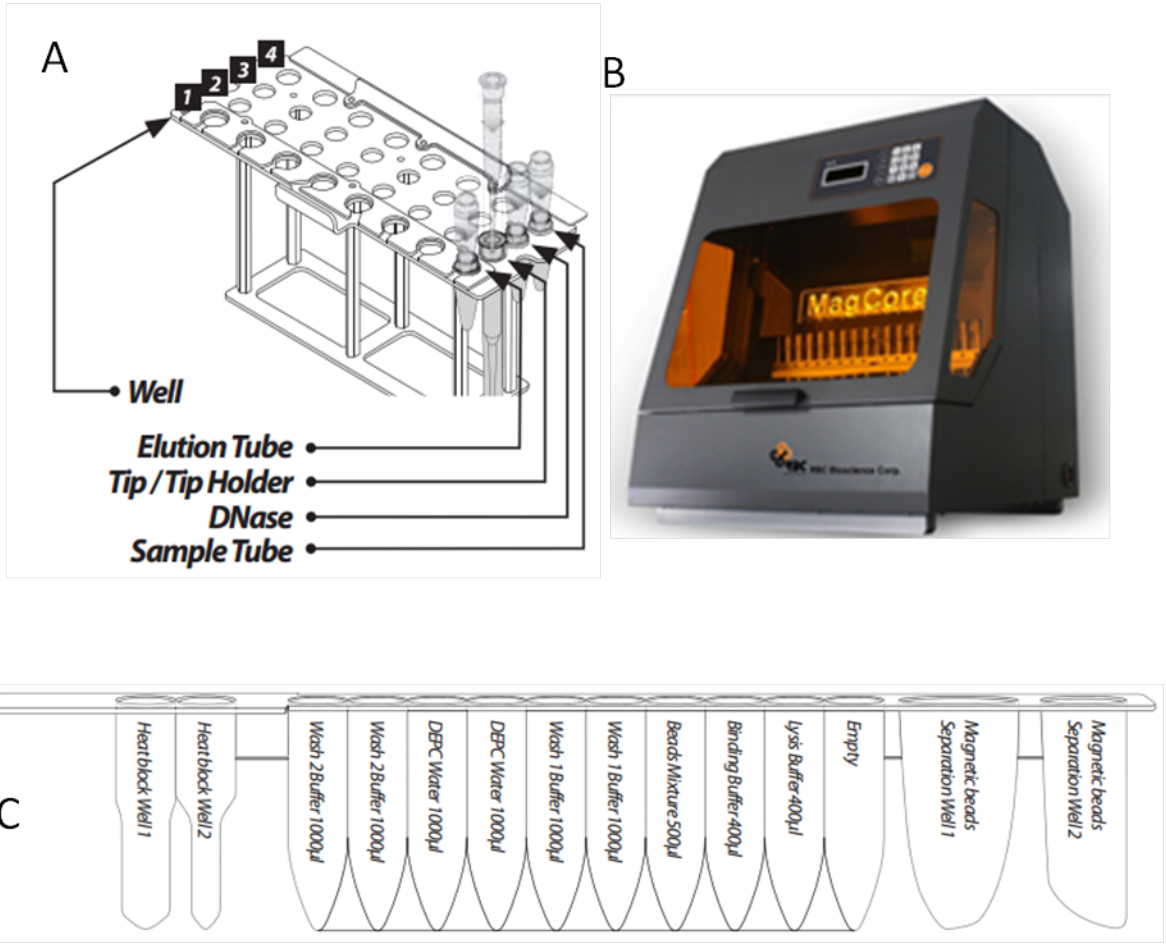


Figure 2.10 *Magcore components A) T-rack for holding samples and DNase solution. B) Magcore instrument. C) Pre-prepared cartridge of solutions.*

2.8.2 RNA Measurement

2.8.3 The NanoDrop® ND-1000 Spectrophotometer

The NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific) was used to determine the concentration of mRNA in the samples obtained from the Magcore. 2µl of DEPC treated water was used as a blank before measuring protein samples. 2µl of sample was pipetted onto the end of a fibre optic wire. The second fibre optic wire was brought forward so it was in contact with the sample as well. The liquid in between the two fibres bridges the gap closing up the system. A pulsed xenon flash lamp provides the light source and a spectrophotometer using a linear charged coupled device (CCD) array analyses the light after passing through the sample. The

NanoDrop® automatically calculates the purity of the mRNA sample added by taking two absorbance readings at 260nm and 280nm and determining the ratio between them. Pure RNA should have a ratio of 2.0. A lower ratio may indicate phenol or another contamination that is absorbed at 280nm. The NanoDrop® also takes readings at 230nm and calculates a 230nm:260nm ratio which is a second ratio indicator of RNA purity. These values can be higher than the 260/280 ratios but are generally between 2.0-2.2. If the ratio is lower this may indicate the presence of a contaminant that absorbs at 230nm. The instrument is connected to a PC and controlled by specialised software. Samples are analysed in triplicate and kept on ice at all times.

2.8.4 Primer Sets

Primer sets were purchased from Qiagen and IDT, as outlined in materials and methods. A SYBR no-ROX master mix was used with the primers.

2.8.5 Real time qRT-PCR

Quantitative PCR was carried out using a Real Time Rotor-GeneRG-3000TM lightcycler (Corbett Research). This type of PCR is a more accurate method of quantification in comparison with older gel based methods. All components needed for this reaction are contained within the one tube. The principle behind this method is that the amount of fluorescence measured is directly related to the product amplification and this is proportional to the amount of target gene present in the sample *(Figure 2.11)*.

This method is used to quantify specific RNA target amplifications using the designated primer sets. This procedure permits for streamlined samples and allows for higher throughput analysis, whereby reverse transcription and PCR take place sequentially in the same reaction tube. The kit used was QuantiTect SYBR Green PCR kit. The kit contains a master mix which contains; SYBR Green dye (the fluorescent component for the detection of the amplicon), a SYBR Green buffer containing a balanced combination of K^+ and NH_4^+ ions (encouraging specific primer annealing) and Taq polymerase. The kit also supplies reverse transcriptase

which is added to the sample reaction tubes only. Samples were prepared in triplicate with reverse transcriptase and a control sample for each sample was set up excluding the reverse transcriptase. GAPDH was used as the reference "house keeping" gene for comparison for all reactions.

Component	Sample + Reverse Transcriptase	Sample – Reverse Transcriptase
RNA (10ng)	5µl	5µl
Primer	2.5µl	2.5µl
Rnase free H_2O	4.75µl	5µl
SYBR Green	12.5µl	12.5µl
RT	0.25µl	-

The program used on the instrument for the different primer sets were as follows:

Denaturing Phase	95°C - 20s
Annealing Phase	55-60°C - 30s (55 cycles)
Elongation Phase	72°C - 30s

The results obtained were analysed using the Comparative CT method (ΔΔCT), described by Livak and Schmittgen, 2001.

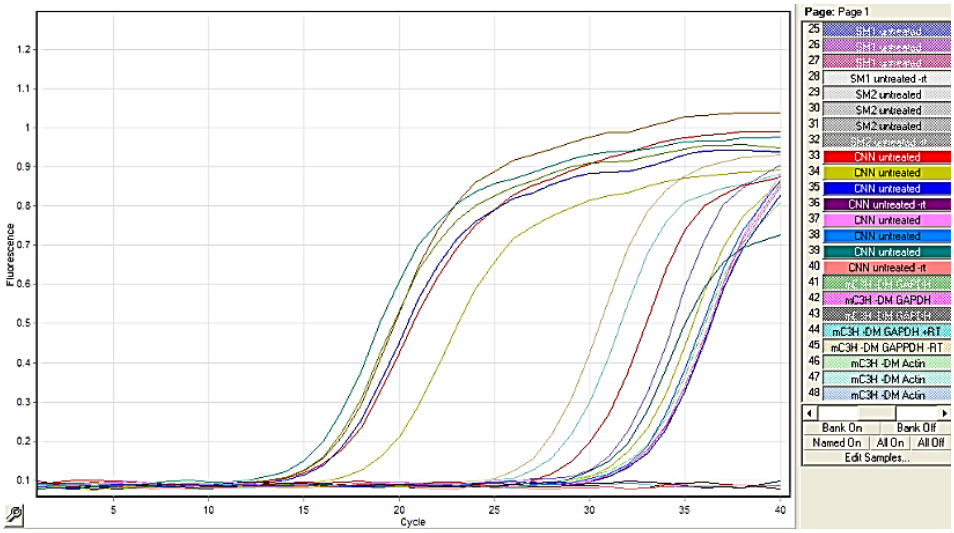


Figure 2.11 *Example of qRT-PCR output data. Curved lines indicate +RT samples and straight lines indicate –RT controls.*

2.9 Cell manipulations

2.9.1 Serum Deprivation

Where appropriate for marker/phenotype analysis cells were serum deprived. This was done by using 0.5% FBS in DMEM supplemented with 1% P/S.

2.9.2 Adipogenesis Differentiation

StemPro® Adipogenesis Differentiation Kit (Invitrogen) was used to differentiate multiple different cell lines to test their stem like properties, including A10, A7r5, rat derived multipotent vascular stem cells and rat mesenchymal stem cells. Arterial derived rat vascular smooth muscle cells were used as a control. Briefly, cells were cultured in 12 well plates to 60-80% confluency. Media provided with the kit was added to the cells once they were ready. The transformation took between 7-14 days. Cells were fixed and stained using a 0.5% Oil Red O solution (Sigma) to determine if the differentiation to an adipocyte cell took place. A fluorescent stain HCS lipidTOX was used to confirm the adipocyte differentiation results

2.9.3 Oil Red O Stain Preparation

300mg of oil red O powder was added to 100ml of 99% isopropanol. In the fume hood 30ml of the oil red o stock was added to 20ml of deionized water. The solution was sterile filtered using a 0.2µM filter. After fixation of the cells were washed three times in deionized water. 2ml of 60% isopropanol was then added to cells for 2 minutes. The cells were washed briefly again in PBS and the working concentration solution of oil red o was added, ensuring the cells were fully covered with the dye. The samples were left at room temperature for 15 minutes. After the incubation the cells five times in deionized water ensuring that most of the stain had been removed. The cells were then analysed using an Olympus CK30 microscope under phase contrast *(Figure 2.12)*.

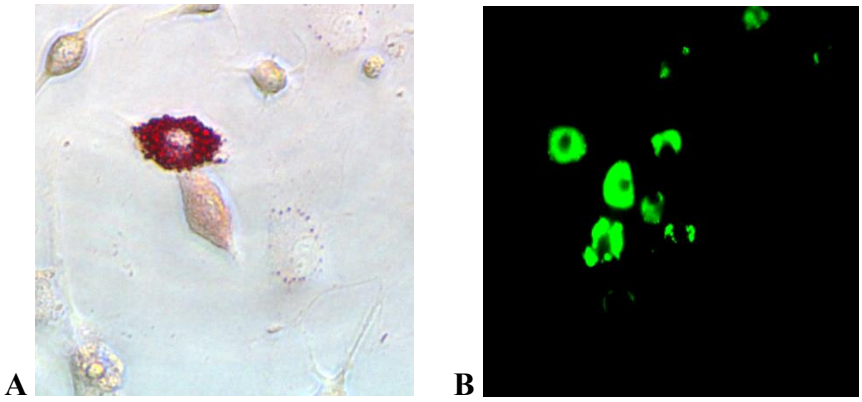


Figure 2.12 *Example of rMSC transition to adipocyte, visualisation under phase contrast using A) Oil Red O staining and B) LipidTox fluorescent staining*

2.9.4 HCS LipidTox

HCS LipidTox was applied to the cells after fixation at a concentration of 200X. The cells were incubated for at least 30 minutes before visualisation. The fluorescent dye was visible under the 488 (green) filter on the microscope.

2.9.5 Osteogenesis Differentiation

StemPro® Osteogenesis Differentiation Kit (Invitrogen) was used to differentiate A10, A7r5, MVSC, MSC, rSMC cell lines as described in the manual. Mesenchymal stem cells served as a positive control for the differentiation process. The cells were

plated at a density of 10 x10^3 cells/cm^2 and were left to grow for 2 days before the addition of the osteogenic supplement. Cells were left to grow for 21 days, the media was replenished every 3 days. After 21 days the cells were stained for Calcium deposits using Alizarin Red Dye. Large red globular structures were observed where differentiation had occurred.

2.9.6 Alizarin Red Dye

Alizarin red dye was prepared to a concentration of 2% in dH_2O. The pH of the dye was adjusted to 4.1-4.3 if required using NH_4OH. Afterward, the dye was filtered using a 0.45µ pore filter. After cell fixation the cells were stained with alizarin red dye and incubated for 10-15 minutes at room temperature. The cells were washed 5 times and left in PBS for analysis. The samples were visualised under Nikon microscope using phase contrast. Mineralized tissue nodules form where the differentiation has taken place. These calcium deposits will stain bright red/orange *(Figure 2.13)*.

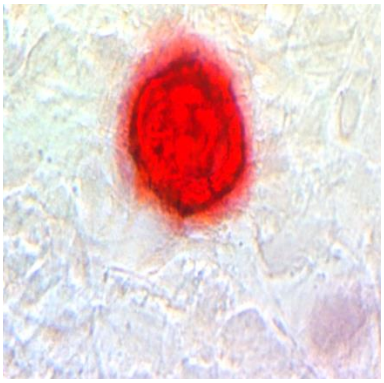


Figure 2.13 *Example of rMSC transition to osteocyte, visualisation under phase contrast using Alizarin Red staining method.*

2.10 Data Anlaysis

Results are expressed as mean ± SEM. Experimental points were performed in triplicate, with a minimum of three independent runs. A t-test was used for comparison of two groups. A value of $p \leq 0.05$ was considered significant. For immunocytochemistry and confocal imaging the stain intensity and location was analysed.

Results

Chapter 3:

Multipotent Vascular Stem Cell Isolation and Characterisation

3.0 Introduction

As previously described the topic of SMC infiltration in vascular remodelling is highly controversial at the moment (Tang et al 2012, Nguyen et al 2013). The latest population of cells proposed to be involved with intimal lesions and subsequent disease progression are resident multipotent vascular stem cells (MVSCs). They were originally isolated from the medial explants of rat tissue but other studies have encountered cells with related properties within the adventitia of both arterial and venous vessels from both animals and humans (Gallagher et al. 2000; Owens et al. 2004; Tang et al. 2012; Gomez & Owens 2012). MVSCs are derived from the neural crest and therefore co express markers associated with this phenotype; S100β, Sox10 and Sox17. In culture these cells have been found to poorly express SMC differentiation markers CNN1 and especially SM-MHCII. When these cells are treated with TGFβ1 they transition to SMCs and begin to express SMC differentiation markers (Wang et al 2012). It is hypothesized that under injury *in vitro* these cells transition to MSC-like cells and subsequently to SMCs, contributing to vascular remodelling (Wang et al 2012, 2013).

For the purpose of this study a population of MVSC cells were isolated from rat medial aortic tissue using the explant method. The cells were screened for panels of markers associated with SMCs [SMA, CNN1, SM-MHCII], MVSCs [Sox10, Sox17, S100β] and MSCs [CD29, CD44, CD146]. The cells were also treated with induction media for osteocyte and adipocyte lineage differentiation. Adult rat SMCs and Rat MSCs were used as appropriate controls for all experiments investigating the characteristics and phenotype of the MVSC cell line.

3.1 Materials and methods

All materials used were of the highest purity available commercially. Primary cell culture was carried out as previously described in Chapter 2 using the explant method. Protein analysis was carried out using FACS, western blotting, immunocytochemistry and confocal imaging. The cells were treated with induction media for adipogenic and osteogenic differentiation as previously described. MVSCs were also treated with TGFβ1 and PDGFBB for smooth muscle cell differentiation.

3.2 Cell lines and Reagents

A primary cell line was generated by explant method using rat aortic tissue. The cells were taken from the medial layer of the aorta. The cells were grown in a maintenance media as previously described. The first set of cells that explanted from the tissue were the multipotent Vascular Stem Cell (MVSC) population. These are the resident stem-like cells present in the medial tissue possessing characteristics associated with multi-potent stem cells. These cells were immediately grown in a specific maintenance media (previously described). Once isolated these cells were cultured and used for experiments up to Passage 8. In a parallel experiment the medial layer of the aorta was digested in enzymes collagenase and elastase. Both cell lines were analysed for MVSC and SMC marker expression. Enzymatically dissolved cells were only screened briefly. This was to confirm the hypothesis that the cells explanting first off the tissue are those with multipotent properties and the majority of cells remaining in the tissue do not possess stem cell characteristics. Therefore for the purpose of this work the cell line that was of main focus was the MVSC cell line.

3.3 MVSC appearance phase contrast

MVSCs were observed under the light microscope. Their appearance was slightly different to that of Bovine aortic Smooth Muscle Cells (BaSMCs). Although both cell lines were elongated, MVSCs displayed narrower spindles. When confluent they grew at a fast rate in comparison with SMCs and their growth pattern was one presenting star-like arrays as opposed to the hill and valley growth pattern of SMCs *(Figure 3.0)*.

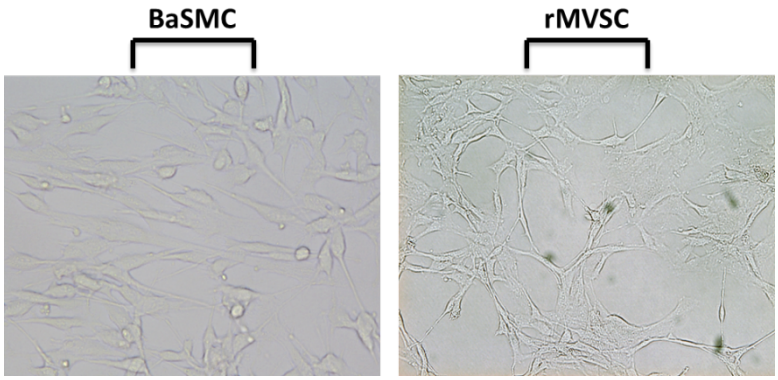


Figure 3.0 Phase contrast images of BaSMCs and rMVSCs. Cells were fixed in 3.7% formaldehyde and observed under phase contrast on the microscope for visualisation.

3.4 Expression of Sox10 and SM-MHCII proteins in situ

Collaborators that also worked on this project freshly isolated aortic tissue from murine source. Protein isolates were made and analysed for the presence of Sox10 and SM-MHCII. For comparison, protein isolates from cultured human SMC were ran in parallel. Sox10 expression was found to be very low in the aortic tissue, in comparison with the smooth muscle cells that showed high expression. SM-MHCII was very highly expressed in the fresh vessel with a lower amount found in the cultured smooth muscle cells *(Figure 3.1)*. Tang et al 2012 completed a similar study whereby they found very little Sox10 expression via in situ analysis yet the cells they explanted from the same tissue were all Sox10 positive. These results led to the isolation of an MVSC population and further analysis on their Sox10 and SM-MHCII expression was investigated (Tang et al 2012).

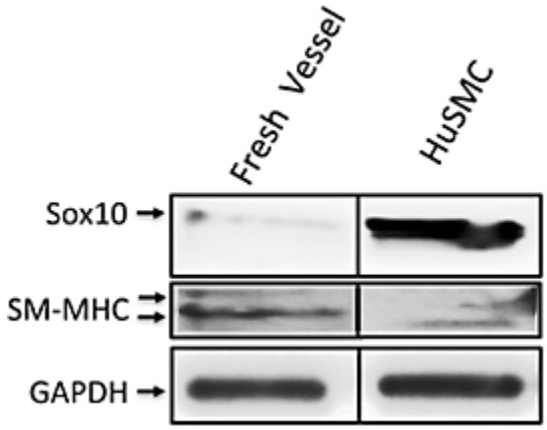


Figure 3.1 *The expression of Sox10 and SM-MHCII in fresh vessel lysates and a human smooth muscle cell line were evaluated using a western blot. Lysates and analysis were carried out by collaborators on this project (Kennedy et al 2014).*

3.5 Sox10 and SM-MHCII expression in MVSCs

Following the isolation of the MVSC population, their characterisation was very important before these cells could be used in any differentiation experiments. The isolated population needed to be deemed multipotent not only for differentiation experiments but also to confirm the hypothesis that the correct population of cells had been isolated from the tissue. The explanted MVSC cells were characterised extensively using immunocytocehemistry, FACS and western blot for protein analysis. The multipotent markers used included S100β (astrocyte marker), Sox10 (neural stem cell marker) and Sox17 (endoderm marker) (Tang et al 2012). These markers are known as stem/pluripotent markers therefore the presence of these markers indicated that the population of cells isolated were of stem cell origin. Sox10 and SM-MHCII expression were the two first important proteins to be analysed. The cells expressed the nuclear Sox10 protein and a small amount of cytoplasmic expression was found *(Figure 3.2)*. The cells were negative for SM-MHCII with no filaments visible and very little fluorescent staining found *(Figure 3.3)*. A double stain was carried out to show the two markers together in one cell that represents the MVSC population $Sox10^{+}$/SM-$MHCII^{-}$ *(Figure 3.4)*. The cells expressed SM-MHCII via FACS and this raised confusion as the immune data was negative. Western blot data confirmed that the antibody was non specific and also picked up on SM-MHCI *(Figure 3.5)*. The absence of the late SMC marker SM-MHCII shows that these cells do not represent a differentiated contractile phenotype.

The presence of sox10^{+}/SMA^{+}/SM-MHCII^{-} cell showed that this cell population stemmed from smooth muscle origin, and was not defined as an adult contractile cell line due to the absence of smooth muscle myosin heavy chain II.

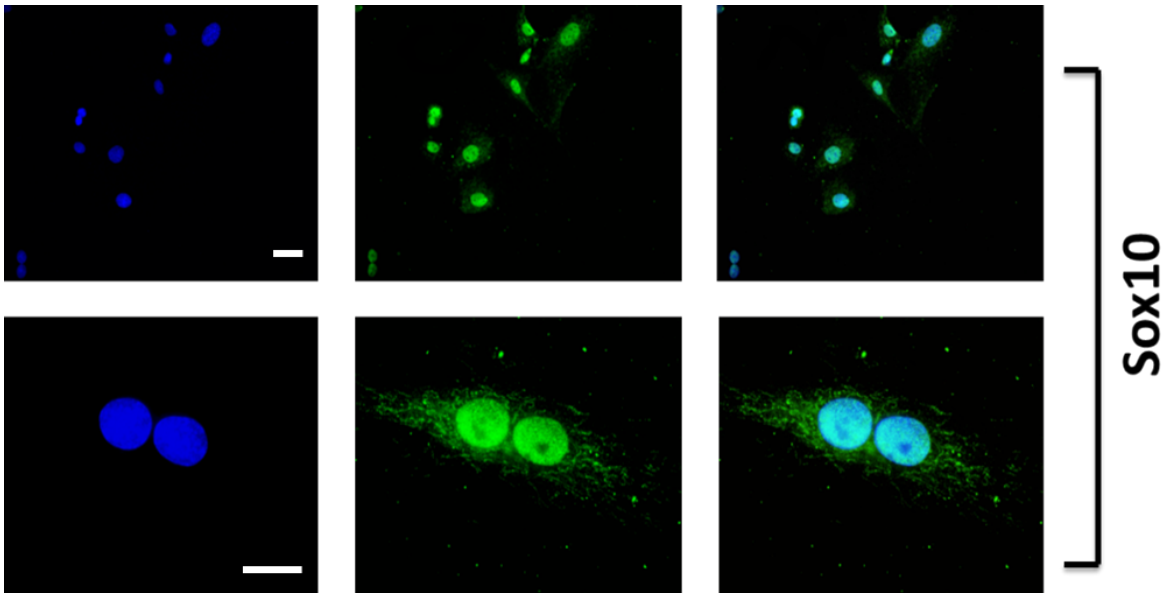


Figure 3.2 *MVSCs in maintenance media stained immunocytochemically for Sox10. Cells were fixed using formaldehyde and stained for the respective stem cell marker using primary antibody. Cells were probed with an appropriate secondary antibody and stained with DAPI for nuclei visualisation. Scale Bar indicates 50nm.*

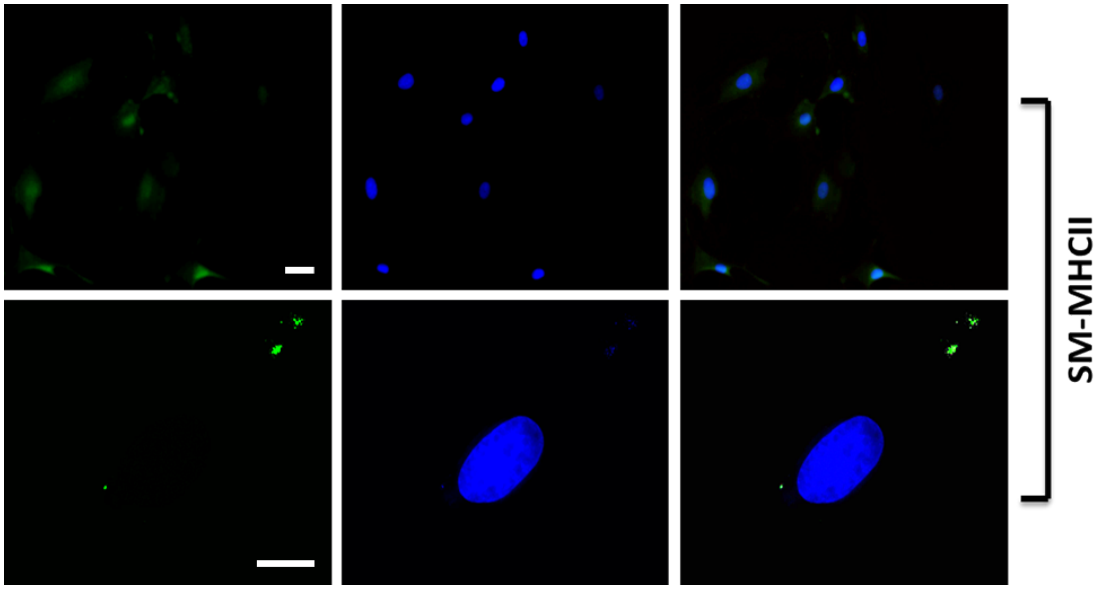


Figure 3.3 *MVSCs stained immunocytochemically for SM-MHCII. Cells were fixed using formaldehyde and stained for the respective stem cell marker using primary antibody. Cells were probed with an appropriate secondary antibody and stained with DAPI for nuclei visualisation. Scale Bar indicates 50nm.*

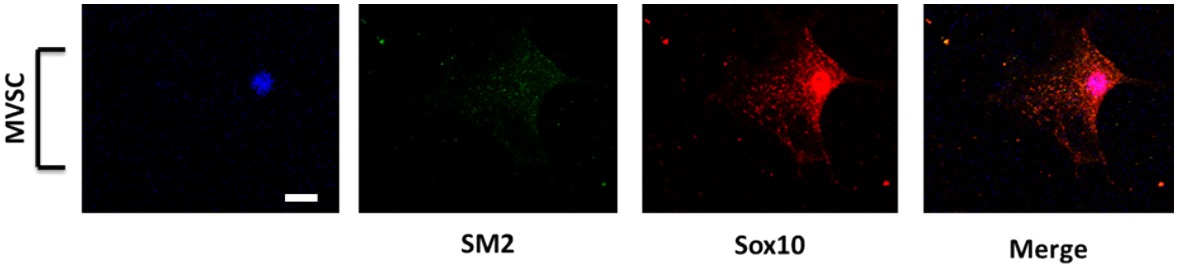


Figure 3.4 *Sox10/SM-MHCII double stain on MVSCs. Cells were seeded at a low density (5,000 cells/well) and incubated for 48 hours before probing with the appropriate primary and secondary antibody. Scale Bar indicates 50nm.*

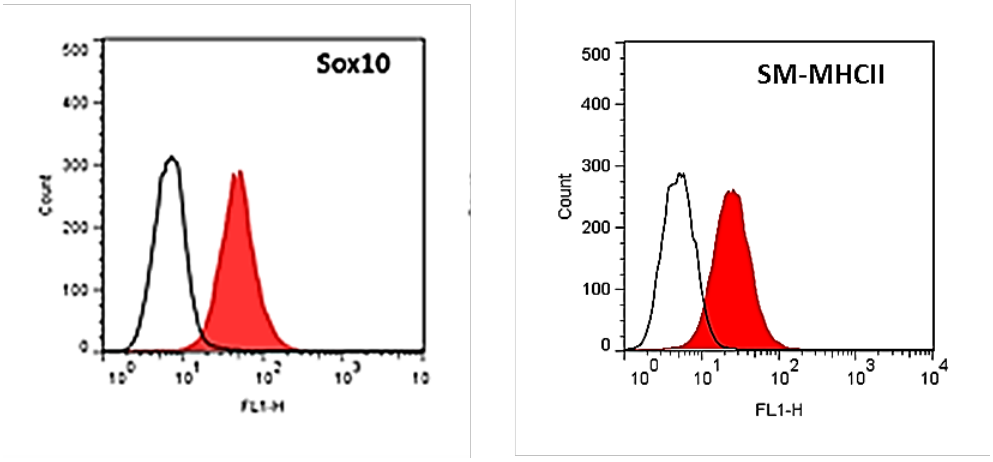


Figure 3.5 *FACS analysis of Sox10 and SM-MHCII on MVSCs. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

3.6 Cellular localisation of Stem Cell Markers

MVSCs protein analysis was carried out using immunocytochemistry, FACS and western blotting for neural stem cell marker Sox17 and glial cell marker S100β after Sox10 expression was confirmed. The cells expressed both proteins. Images for immunocytochemistry were taken and both high and low power lens *(Figure 3.6)*. FACS confirmed the expression of these markers *(Figure 3.7)*. Western Blots were performed on lysates for MVSCs and the membranes were probed for Sox17 (44kDa), Sox10 (49Dka) ans S100β (11kDa) *(Figure 3.8)*.

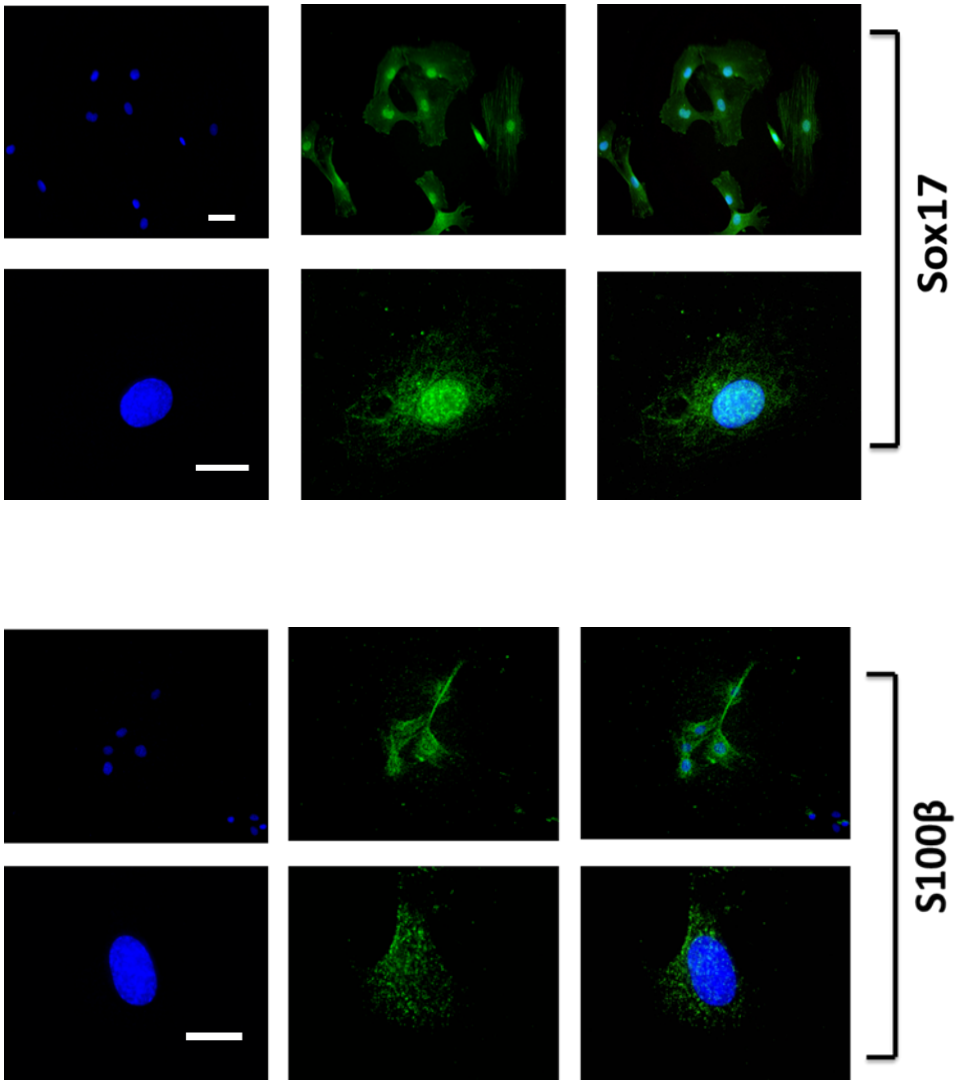


Figure 3.6 *MVSCs stained immunocytochemically for Sox17 and S100β. Cells were fixed using formaldehyde and stained for the respective stem cell marker using primary antibody. Cells were probed with an appropriate secondary antibody and stained with DAPI for nuclei visualisation. Scale bar indicates 50nm for all images.*

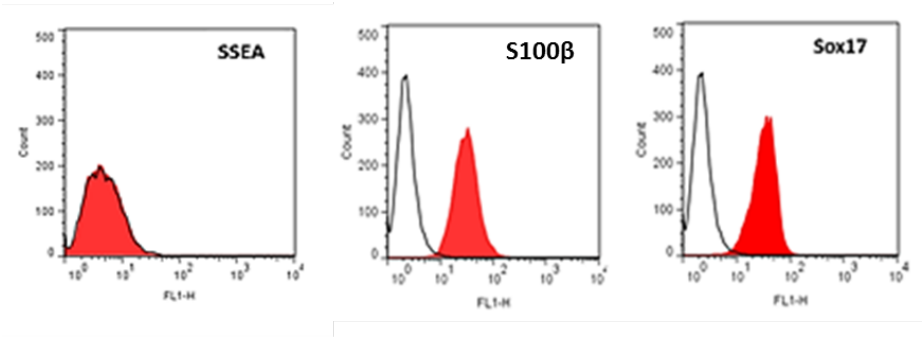


Figure 3.7 *FACS analysis of Sox17, S100β and SSEA-1 (Stage Specific Embryonic Antigen1) on MVSCs. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

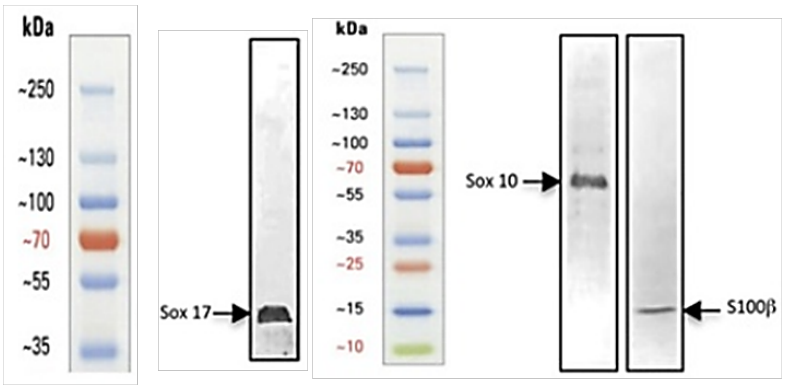


Figure 3.8 *Western Blot analysis of multipotent markers Sox10, Sox17 and S100β. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis.*

3.7 Cellular localiasation of smooth muscle cell markers in MVSCs

Although the MVSCs posess stem cell markers, it was important to analyse the cell line for smooth muscle cell markers since they were derived from smooth muscle tissue. These cells were generated to be used as a differentiation model of stem cells to smooth muscle cells, therefore before any treatment of the cells it was of great importance to establish the basal line expression of SMC markers in the population.

Apart from SM-MHCII expression as already discussed the two other markers used were SMA and CNN. These three markers as a team cover late (SM-MHCII), middle (CNN1) and early (SMA) stage differention of smooth muscle cells (Campbell et. al 1979). Using this panel allowed the assessment of how far along the differentiation pathway the MVSCs were. Immunocytochemically, the generated cell line was positive for SMA and CNN1 *(Figure 3.9)*, which has been previously described (Wang et. al 2012). The population showed that some cells had not yet begun to express Calponin 1. This phenotype is associated with a more juvenile SMC population. Cultured smooth muscle cells derived from rat and murine sources were used as positive controls for the smooth muscle cell markers. Confocal (murine) and immunofluorescence (rat) techniques were used *(Figure 3.10, 3.11)*. FACS analysis was used to compliment the immunocytochemistry data to show SMA and CNN1 positivity *(Figure 3.12)*. This method gives insight into a larger population of cells as it analysis 10,000 cells/count in comparison with ICC which captured tens of cells per frame. Lysates isolated from MVSCs were probed against SMC markers SMA, CNN1, SM-MHCII *(Figure 3.13)*. Protein analysis showed that MVSCs expressed SMA and CNN1 but did not show any SM-MHCII expression, the bands that appeared were specific to SM-MHCI indicating that MVSCs express some amount of SM-MHCI.

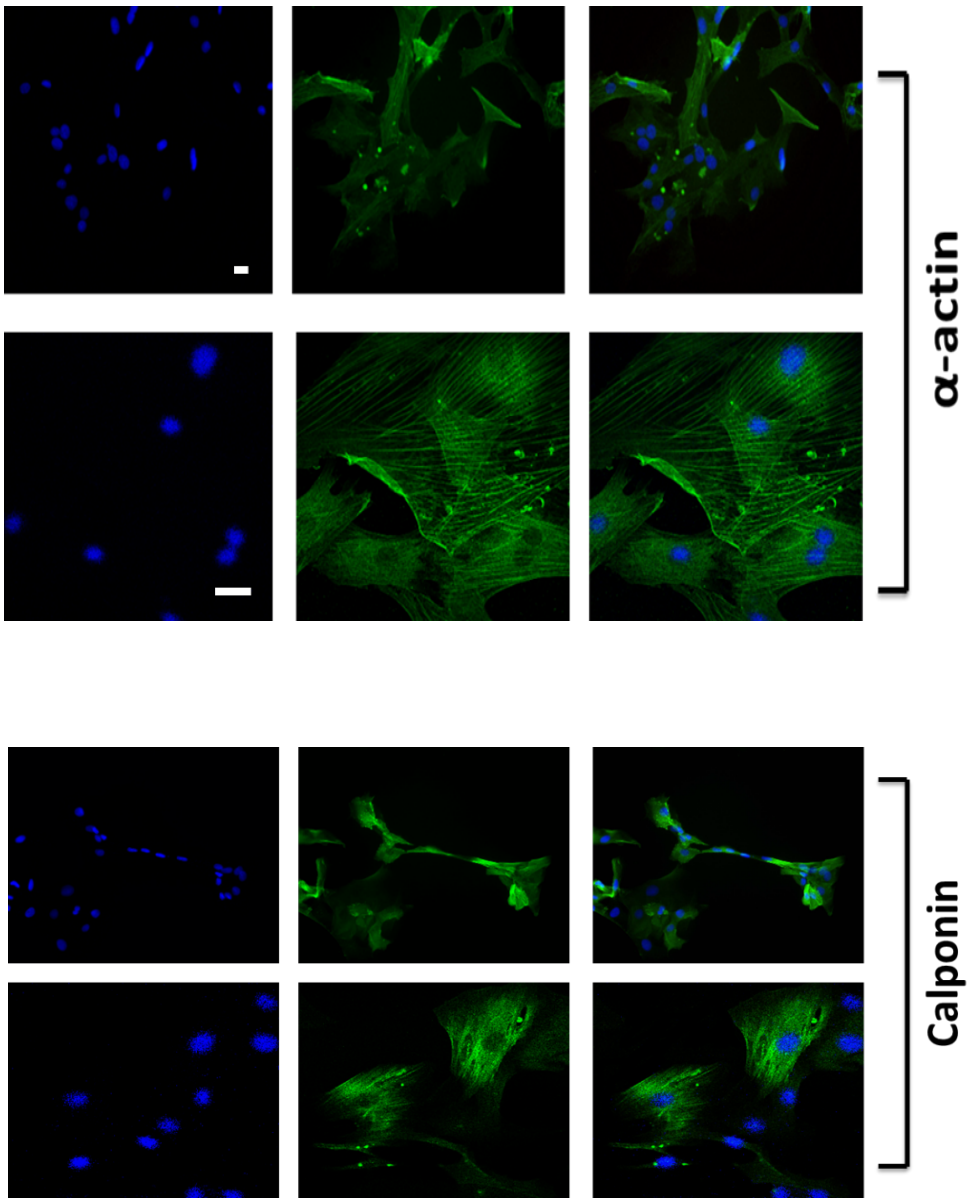


Figure 3.9 *SMA and CNN1 protein expression in MVSCs grown in maintenance media. Cells were seeded at a low density (5,000 cells/well) and incubated for 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

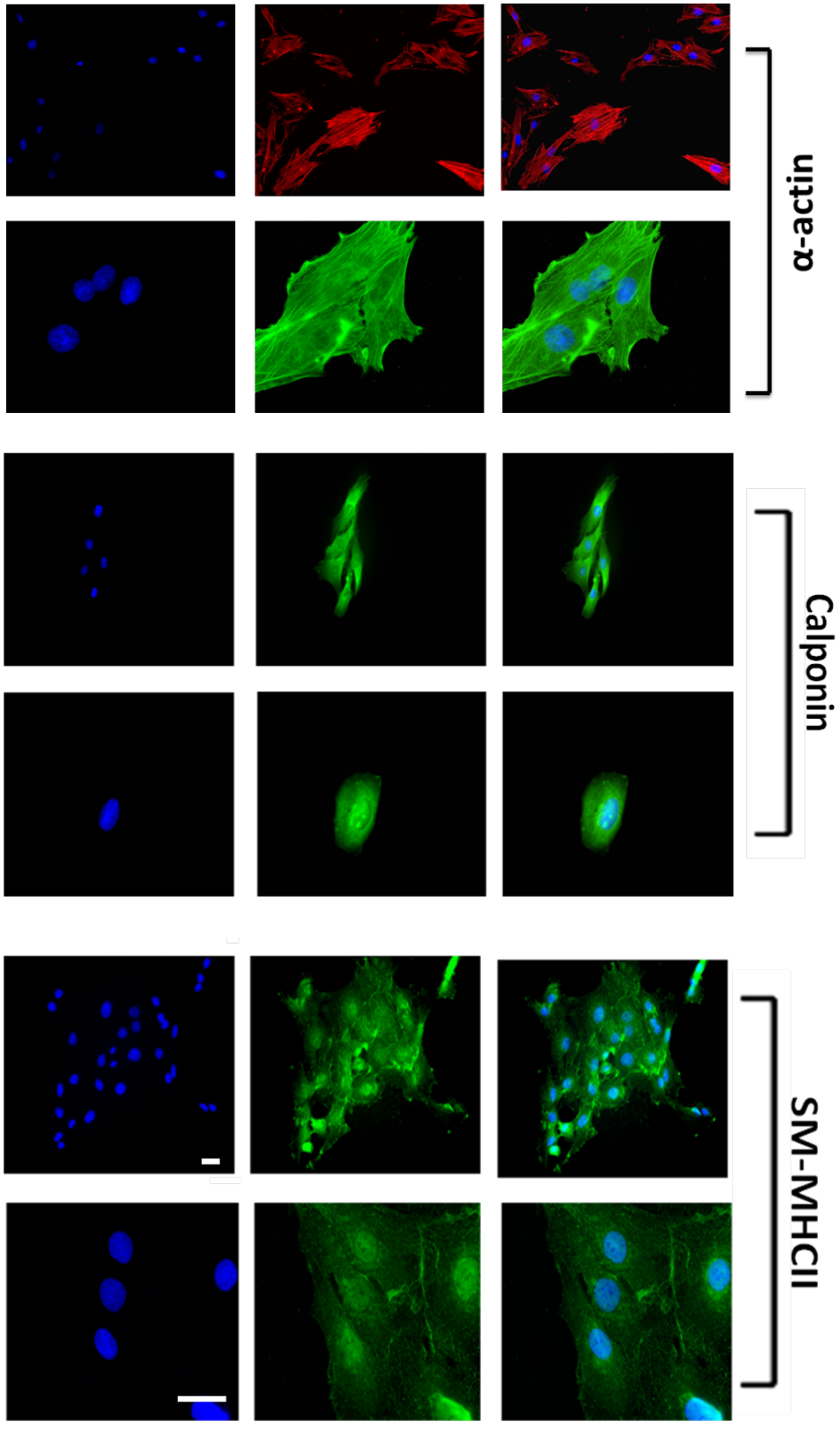


Figure 3.10 *Immunocytochemistry of rat SMC expressing SMC markers in differentiation media [SMA, CNN1, SM-MHCII]. Cells were seeded at a low density (5,000 cells/well) and incubated for 48 hours before probing with the appropriate primary and secondary antibody. Scale bar indicates 50nm for all image*

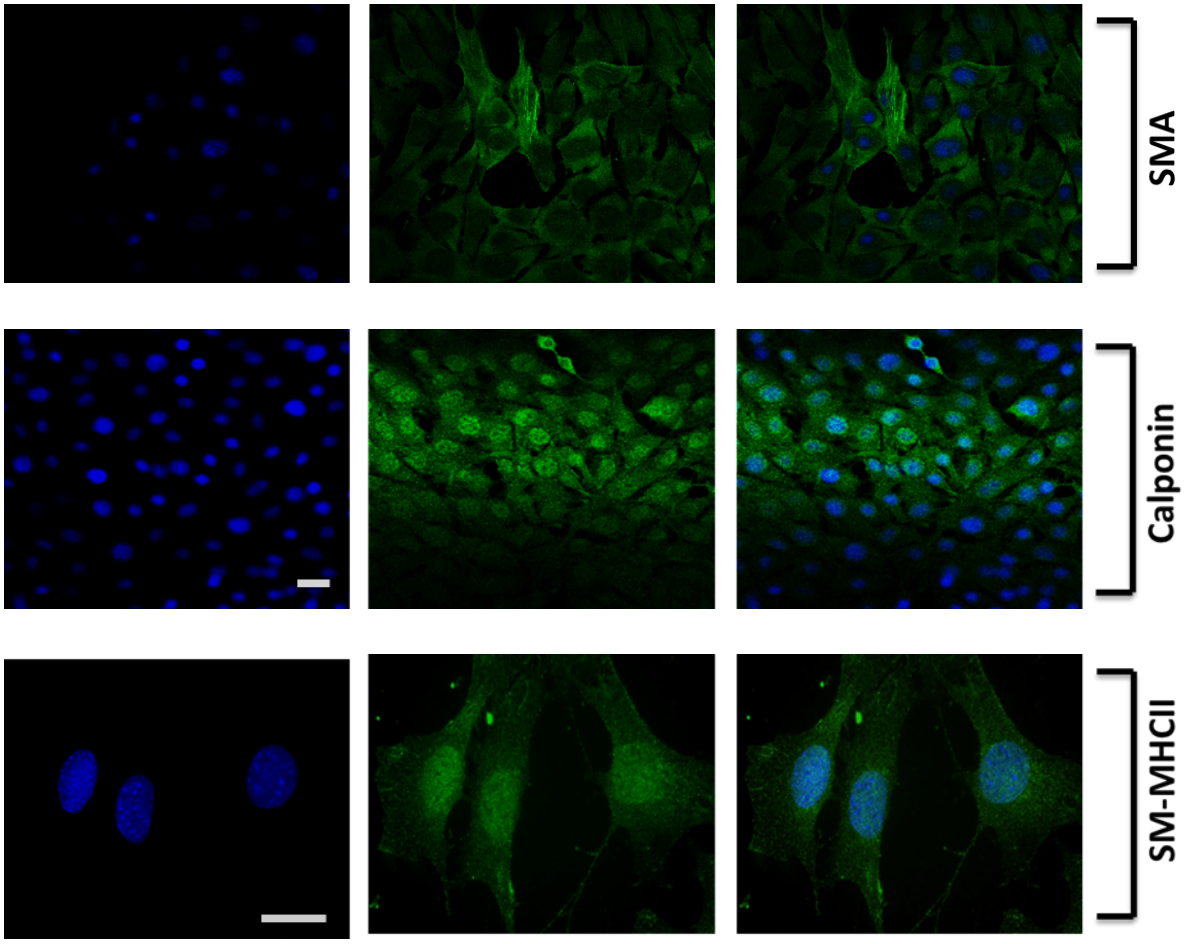


Figure 3.11 *Murine SMC grown in differentiation media expression of smooth muscle cell markers SMA, CNN1, SM-MHCII. Cells were seeded at a low density (5,000 cells/well) and incubated for 48 hours before probing with the appropriate primary and secondary antibody.*

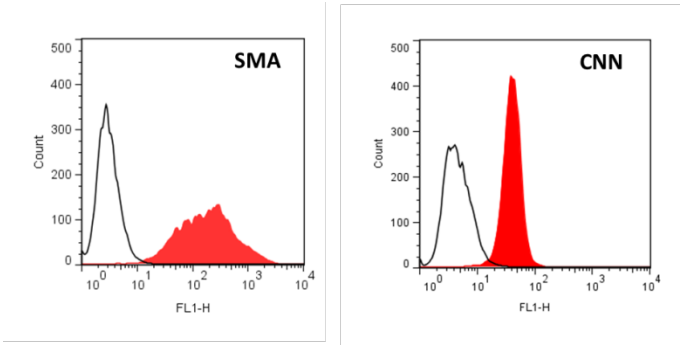


Figure 3.12 *FACS analysis of SMA and CNN1 for MVSCs. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

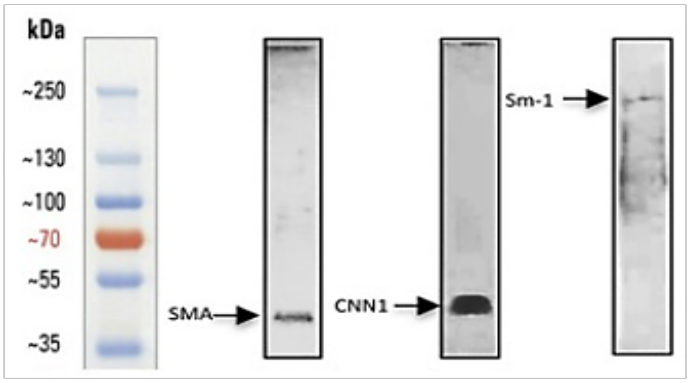


Figure 3.13 *Representative immunoblot of SMC differentiation markers [SMA, CNN1, SM-MHC] on MVSCs. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis*

3.8 Expression pattern of enzymatically digested SMCs from rat aortic medial layer

As described previously, two different populations were isolated. Firstly, those cells which migrated off the explant tissue in early days and secondly the media vessel as a whole after enzymatic digestion. Both cell populations were characterised at P1 using immunocytochemistry for SM-MHII and Sox10. The cells were analysed using an Olypmus BX51 microscope at low power X 20 and X 100 magnifications. The purpose of this experiment was to show that not all cells in the medial layer present the $Sox10^{+}$/$SM\text{-}MHCII^{-}$ phenotype and that these characteristics only apply to a small percentage of the cells in the tissue ie the resident stem cell population.

The enzymatically digested SMCs were highly positive for SM-MHCII in comparison with the MVSCs *(Figure 3.14, 3.15)*. Myosin filaments were clearly visible at low and high power magnifications in enzymatic SMCs. There was a small amount of staining in the MVSCs at high and low power magnification but this appears to be non-specific binding of the primary antibody as the small amount of staining was not uniform between different samples/images.

Sox10 protein is a nuclear protein. MVSCs stained strongly positive for Sox10 in the nucleus as predicted. In comparison, the enzymatically digested SMCs

had very low Sox10 staining in the nucleus with a small amount of staining seen in the cytoplasm *(Figure 3.14, 3.15)*.

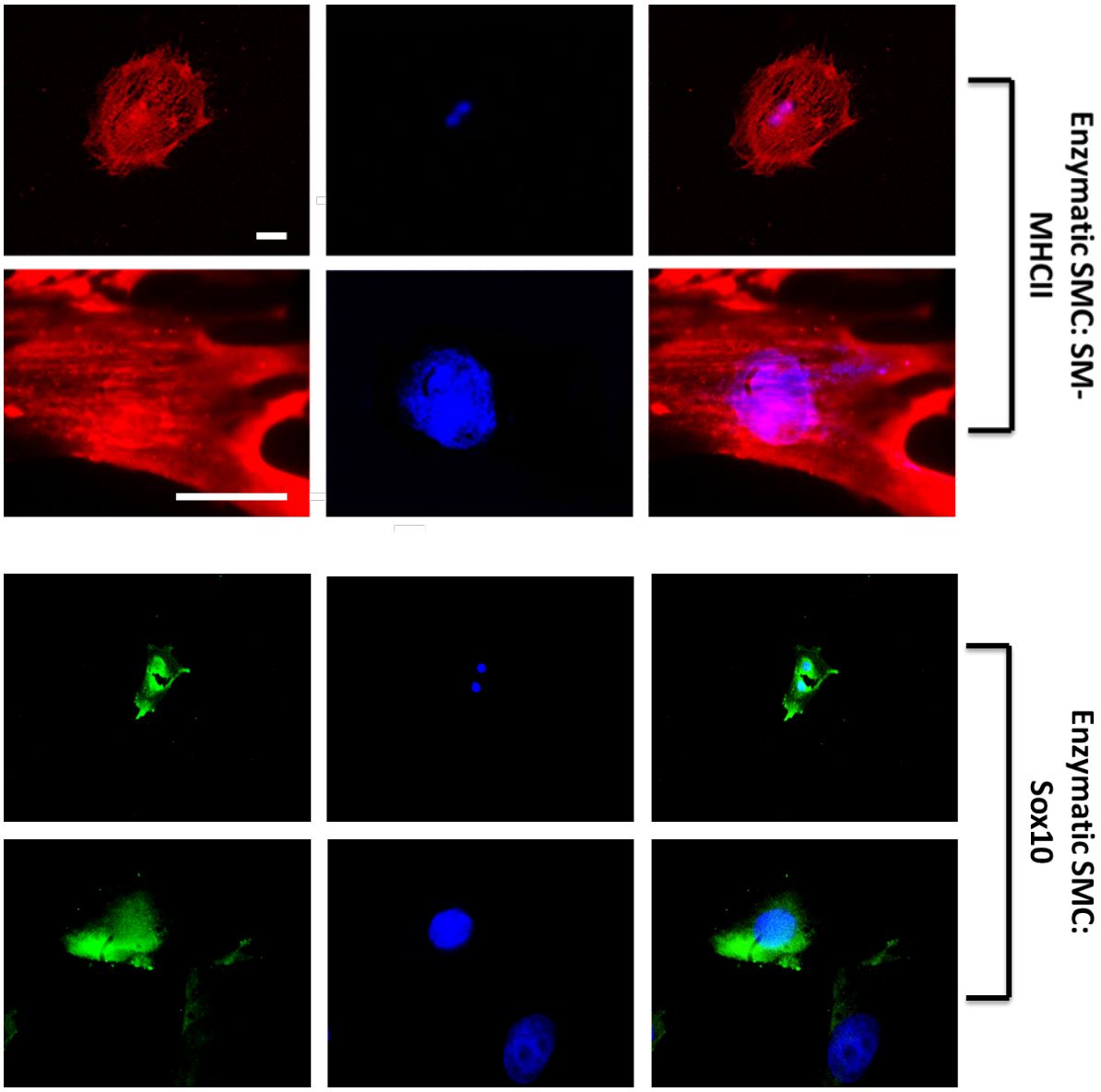


Figure 3.14 *Freshly isolated enzymatically digested SMC in differentiation media expression of SM marker SM-MHCII and Neural stem cell marker Sox10. Cells were at a low density (5,000 cells/well) and incubated for 48 hours after all tissue was removed. Samples were then probed with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images*

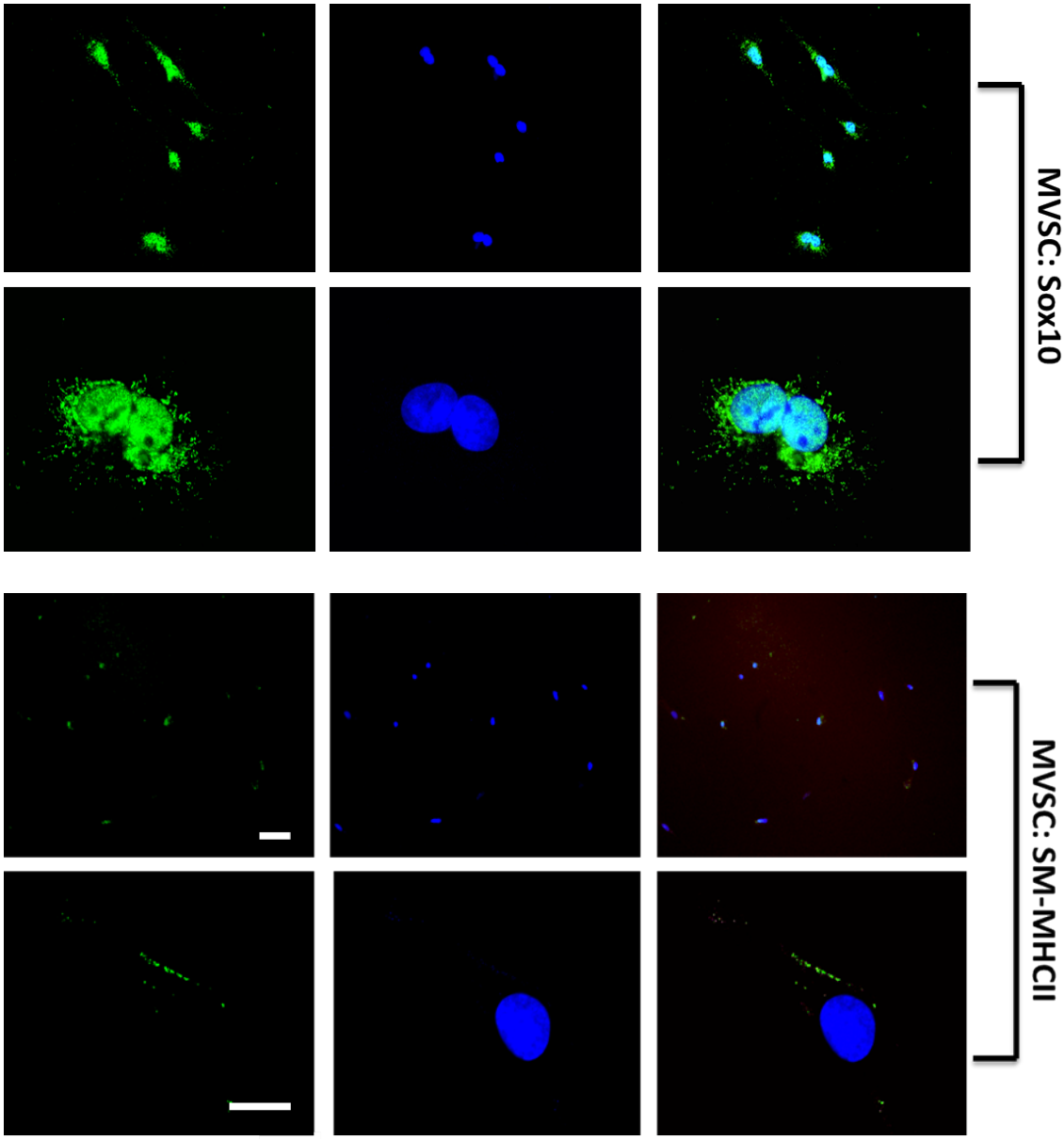


Figure 3.15 *Freshly isolated MVSC in maintenance media expression of SM marker SM-MHCII and Neural stem cell marker Sox10. Cells were at a low density (5,000 cells/well) and incubated for 48 hours after all tissue was removed. Samples were then probed with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images*

3.9 Mesenchymal Stem Cell Marker expression in MVSCs

MVSCs expression of mesenchymal stem cell markers CD29, CD44 and CD146 was determined using immunocytochemistry *(Figure 3.16)*. Rat mesenchymal stem cells were used as a positive control for this assay. FACS analysis was used to confirm the presence of CD29 and CD44 in MVSCs *(Figure 3.17)*.

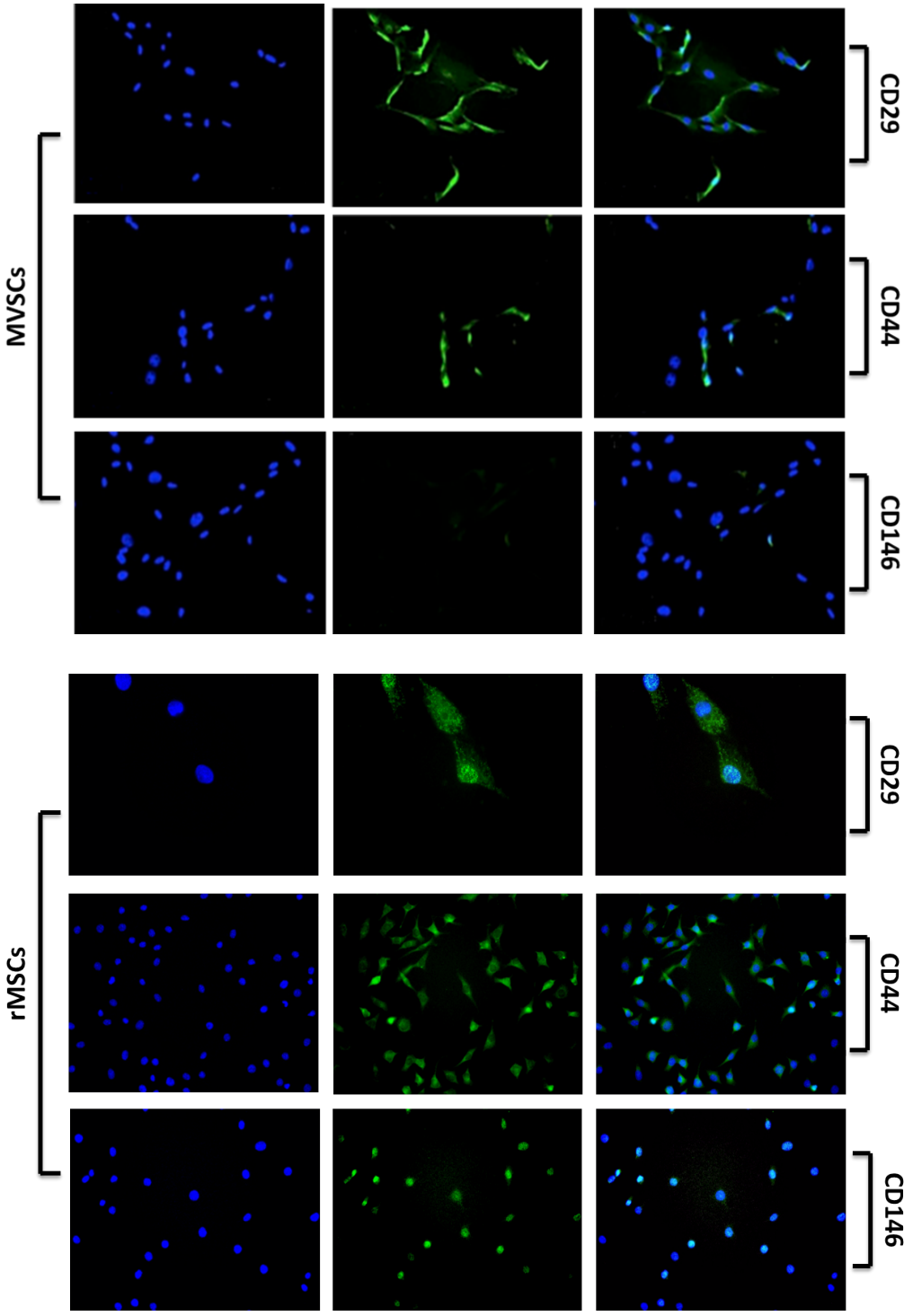


Figure 3.16 *Immunocytochemical data for MVSC expression of MSC markers [CD29, CD44, CD146]. Rat MSC used as positive control. Cells were seeded at a low density (5,000 cells/well) and incubated for 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

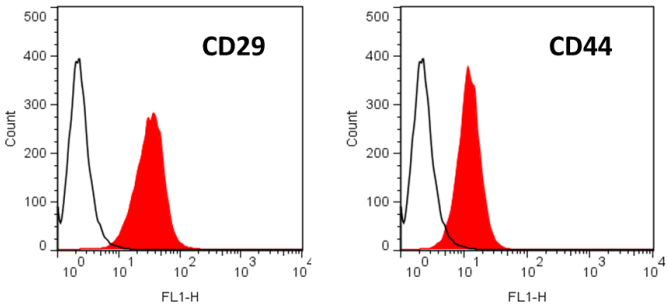
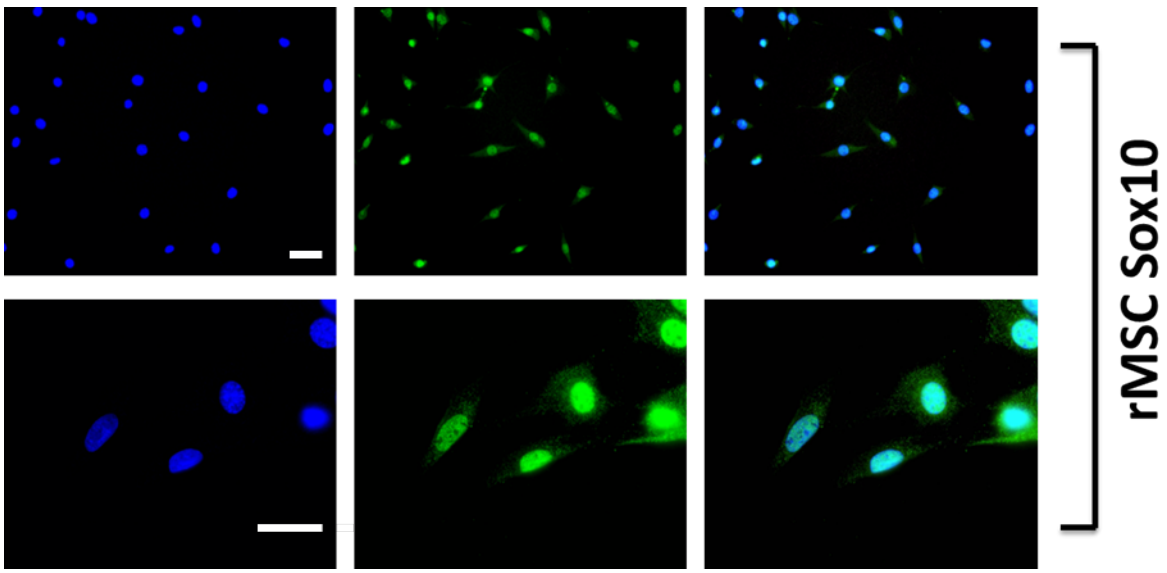


Figure 3.17 *FACS analysis for CD29 and CD44 in MVSCs. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

3.10 Expression of neural and glial stem cell markers in rat Mesenchymal Stem cells.

Rat MSCs were analysed for the presence of the three stem cell markers associated with MVSCs (Sox10, Sox17, S100β) *(Figure 3.18).* This was carried out to monitor the transition of MVSC differentiation to MSCs. It was hypothesized that MSCs would still retain some of these stem cell markers in culture, to fit in with the profile of MVSC differentiation to MSC and finally to SMC. MSCs are the intermediate cell type and could possibly still retain some of the MVSC properties.



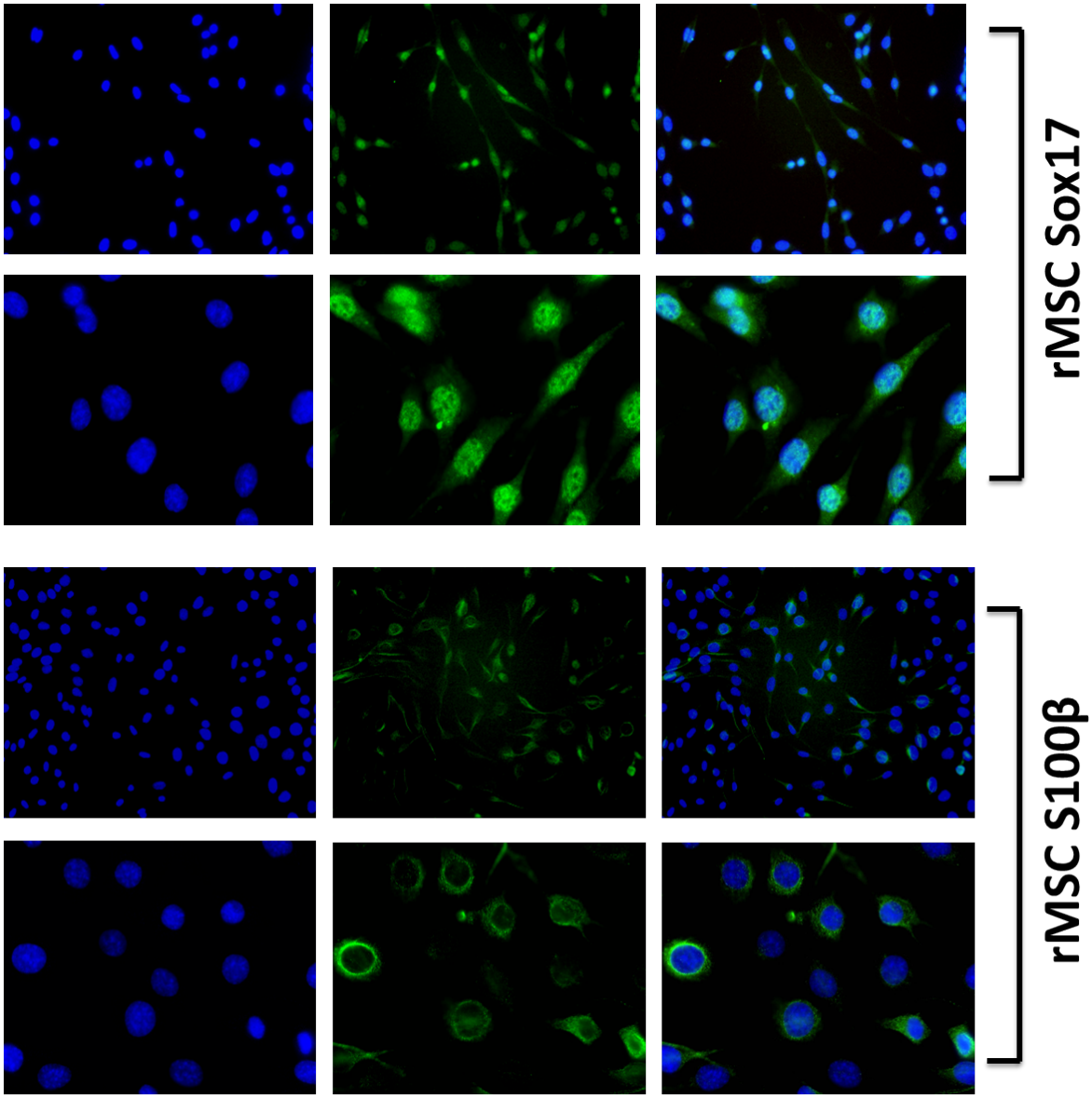


Figure 3.18 *Rat MSC expression of markers associated with MVSC phenotype [Sox10, Sox17, S100β]. Cells were seeded at a low density (5,000 cells/well) and incubated for 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

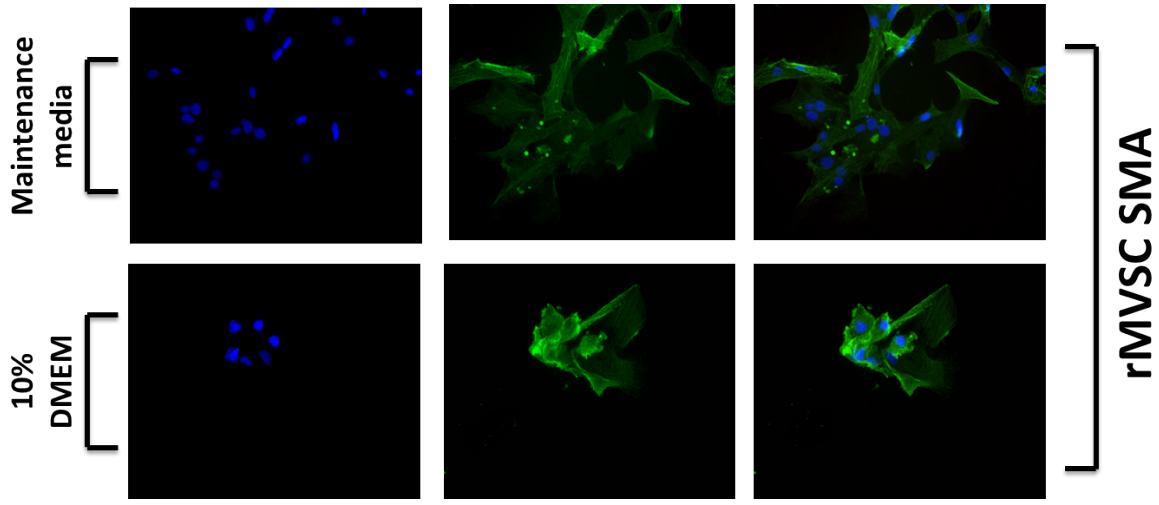
3.11 Differentiation potential of MVSCs

In order for this isolated cell population to be considered stem cell like, the cells must retain some (as much as possible) of the multipotent traits associated with stem cells. Multipotent stem cells should be capable to differentiate into bone, muscle, fat, cartilage and other tissues. Smooth muscle cell potential of the MVSC population was shown firstly using high serum concentration (10%) to examine spontaneous

differentiation and then the use of TGFβ1 and PDGFBB for induced SMC differentiation.

3.12 Smooth Muscle Cell differentiation of MVSCs

Two different methods were used to investigate the differentiation of MVSCs to SMCs. The first was using media containing 10% Fetal Calf Serum (FCS) in regular DMEM which was also carried out by Tang et. al 2012 The second was using TGFβ1 and PDGFBB in media containing 5% serum. In a parallel experiment MVSCs were cultured continuously for 3 weeks in 10% FCS DMEM media. Each experiment involved the assessment of smooth muscle cell markers and/or stem cell markers. Expression of SMA was already apparent in MVSCs. The treatment with differentiation media slightly increased fluorescent intensity but overall there was no huge change in expression. CNN1 expression increased over the ten day stimulation. To begin with there was a small population of $CNN1^-$ cells, after treatment the cells all expressed some levels of CNN1. SM-MHCII expression was slightly increased but there were no myosin filaments observed *(Figure 3.19)*.



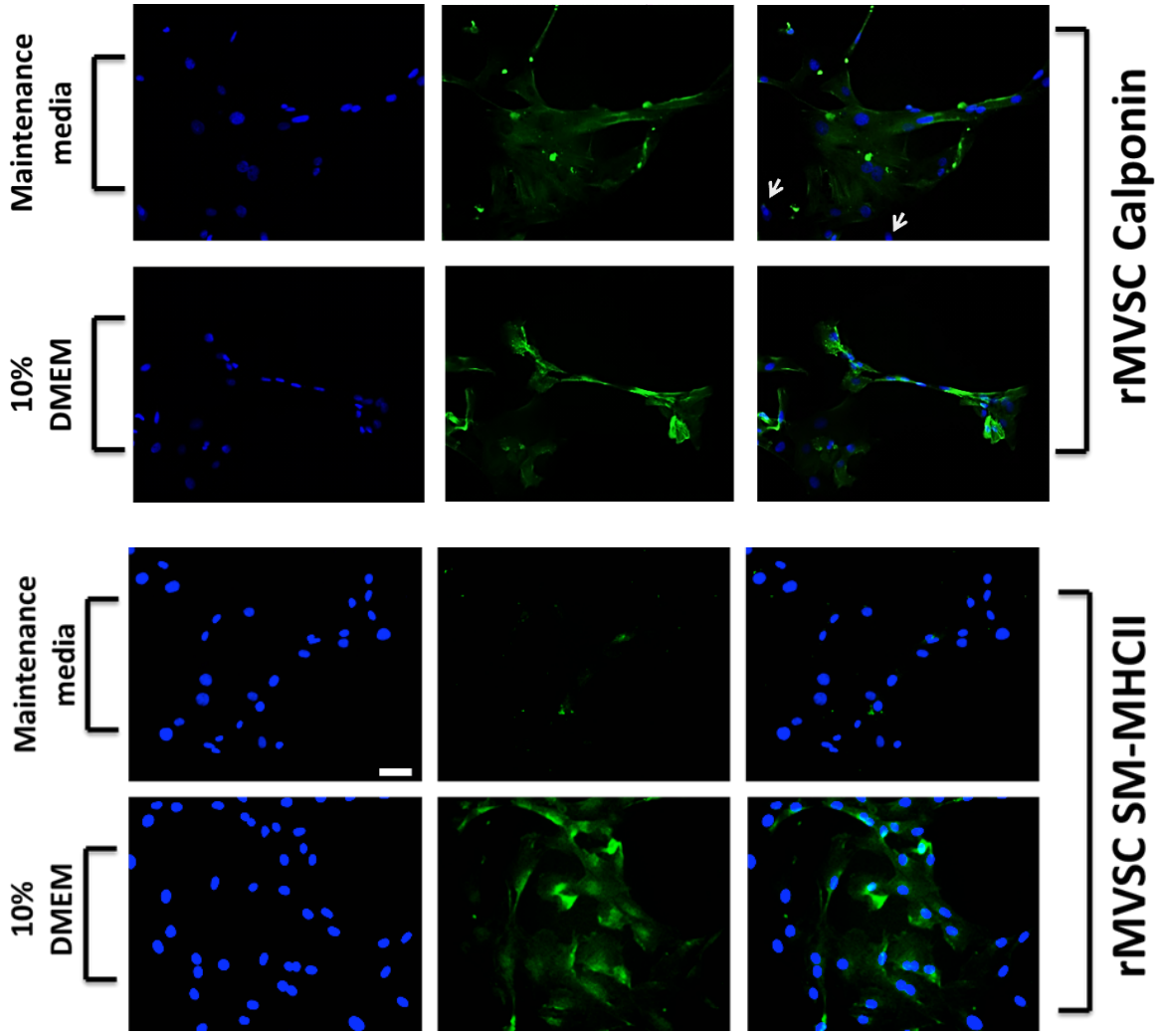


Figure 3.19 *SMC marker expression after 10 days in 10% FCS DMEM differentiation media. Cells were seeded at a low density (5,000 cells/well) and incubated for 10 days in media containing 10% FCS. The media was replenished every 2-3 days. The cells were fixed in formaldehyde and treated with the appropriate primary and secondary antibodies. Scale bar represents 50nm for all images.*

3.12.1 Protein expression analysis after 21 day serum induction

MVSCs were screened for the late SMC marker, SM-MHCII expression after culture in differentiation media for 21 days *(Figure 3.20)*. The presence of this marker would indicate SMC differentiation. MVSCs reached Passage 14. Stem cell markers were maintained after 10 days in differentiation media so these markers (Sox10, Sox17, S100β) were further analysed the MVSCs cultured for 21 days. MVSCs

started off with a $Sox10^{+}/Sox17^{+}/S100\beta^{+}/SMA^{+}/CNN1^{+}/SM\text{-}MHCII^{-}$ phenotype. Following 21 days in culture it was found that the cells maintained their a $Sox10^{+}/Sox17^{+}/S100\beta^{+}/SMA^{+}/CNN1^{+}$ expression and also became slightly SM-MHCII positive in comparison with MVSCs in maintenance media as previously described.

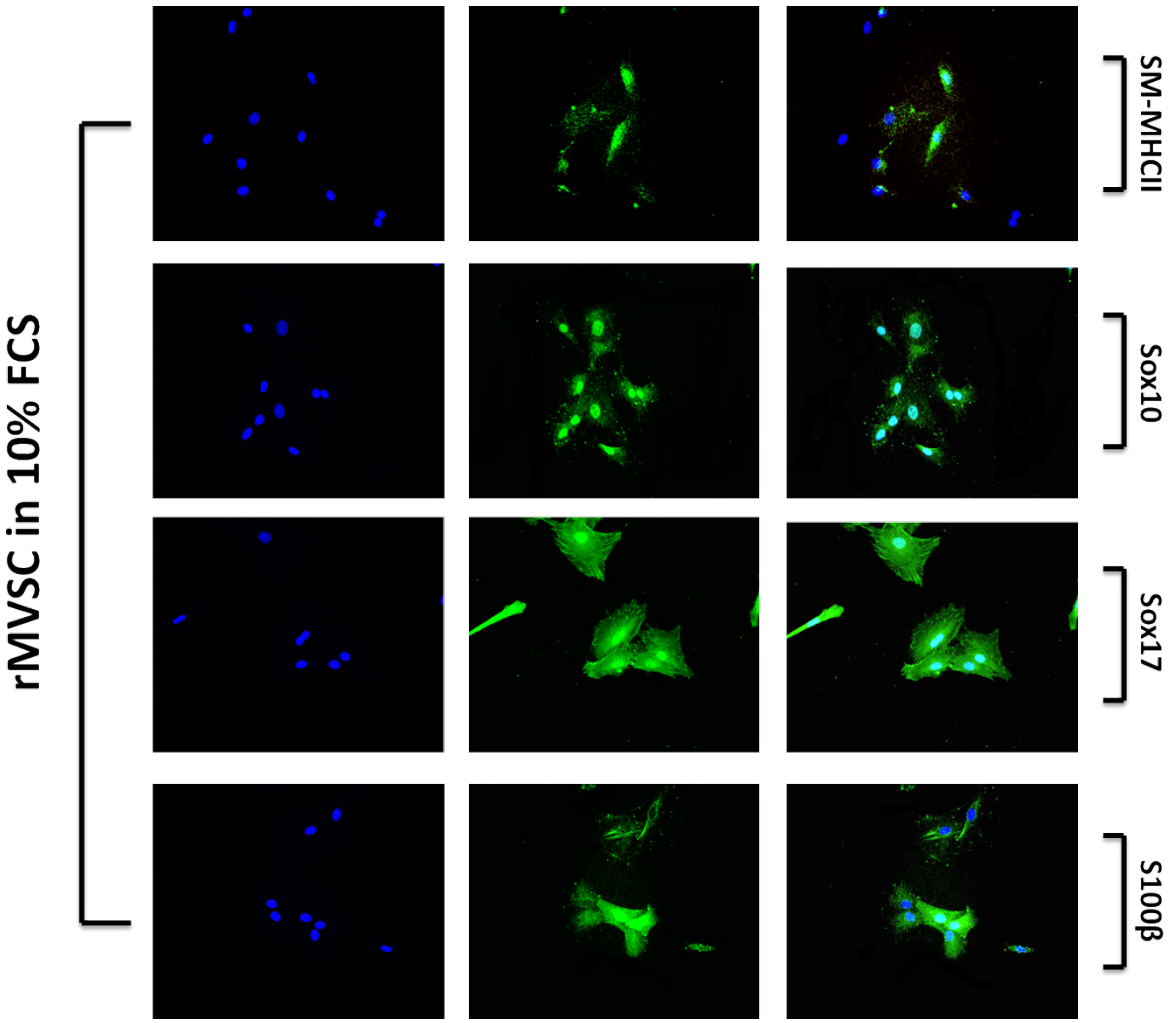


Figure 3.20 *SM-MHCII, Sox10, Sox 17 and S100β expression in MVSCs after 21 days culture in 10% FCS DMEM. Cells were seeded at a low density (5,000 cells/well) and incubated for 21 days in media containing 10% FCS. The media was replenished every 2-3 days. The cells were fixed in formaldehyde and treated with the appropriate primary and secondary antibodies. Scale bar represents 50nm for all images.*

3.12.2 Smooth Muscle Cell induction of MVSCs using TGFβ1and PDGFBB

The activation of the TGFβ1 pathway is an established method of driving smooth muscle cell differentiation. Serum naturally contains some components that will initiate this therefore naturally MVSCs in serum will slowly become more smooth muscle cell like as they are passaged, as observed previously when cells were left for 10 and 21 days in FCS. To achieve full differentiation of SMCs TGFβ1 was used. Two different concentrations were used to compare what dose of TGFβ1 was most effective in smooth muscle cell differentiation. Both 2ng/ml and 5ng/ml were used with the addition of 10ng/ml PDGFBB in each case. 5ng/ml gave greater differentiation and this was the concentration chosen for future experiments.

The concentration of FCS is important for TGFβ1 activation. Too high a serum concentration can inhibit the action of TGFβ1, and too low of a concentration will mean that the cells cannot grow properly and the TGFβ1 may induce apoptosis. Optimal serum concentration was determined using immunocytochemistry.

3.12.3 TGFβ1 optimisation in FCS

In order to maximise cell growth in TGFβ1 the media in which the cells were grown in, the FCS concentration was optimised *(Figure 3.21)*. Three different concentrations of FCS were used in parallel experiments; 2% FCS, 5% FCS and 10% FCS (all in DMEM). The SMC differentiation marker examined was SM-MHCII as it was the best indicator of smooth muscle cell differentiation.

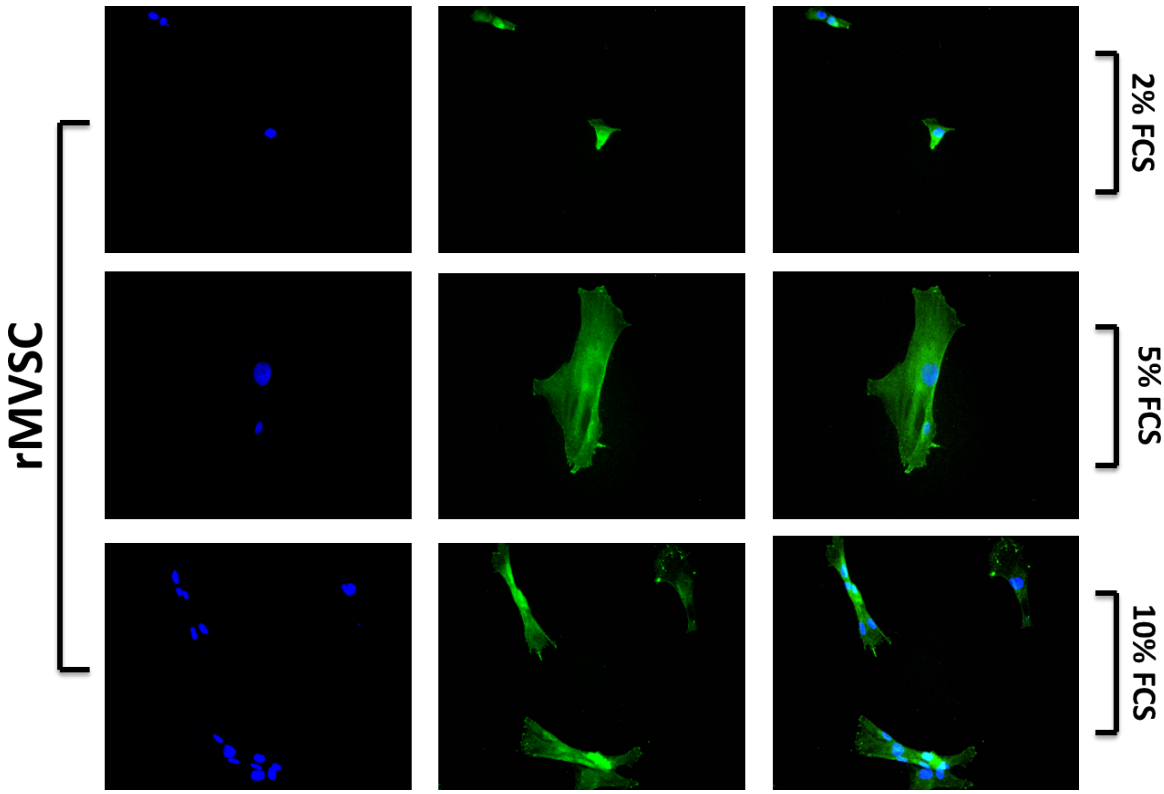
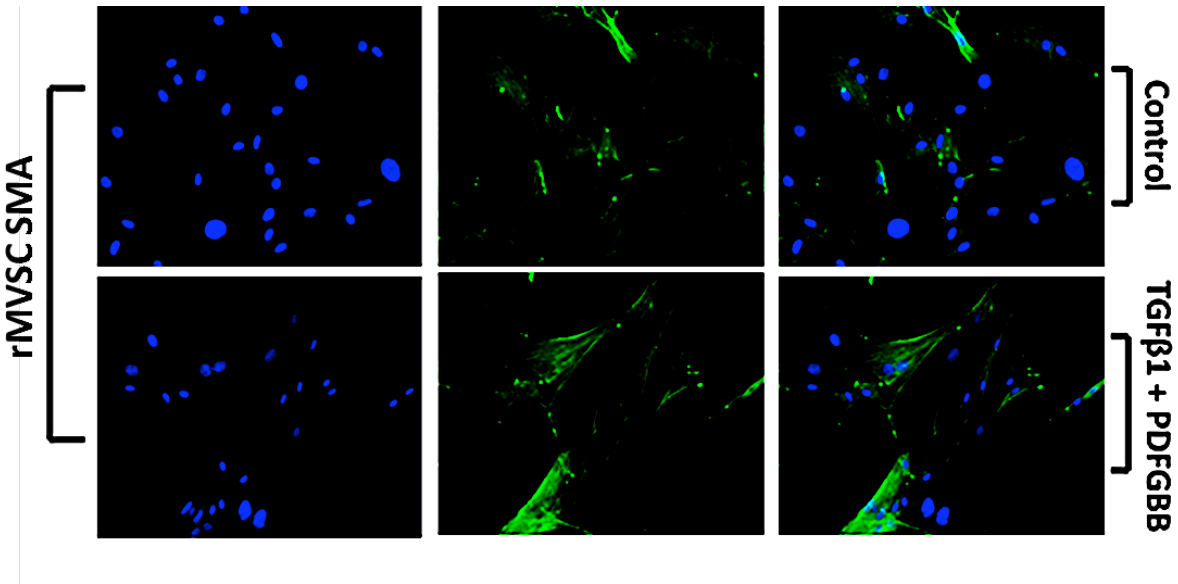


Figure 3.21 *Optimisation of FCS concentration for TGFβ1 differentiation of MVSC to SMC at concentrations of 2, 5, 10%. Cells were seeded at a low density (5,000 cells/well) and incubated for 5 days in media containing either 2, 5 or 10% FCS. The media was replenished every 2-3 days. The cells were fixed in formaldehyde and treated with antibody against SM-MHCII antigen and the appropriate secondary antibody. Scale bar represents 50nm for all images.*

MVSCs were treated for 5 days with 5ng/ml TGFβ1 and 10ng/ml of PDGFBB in the different serum conditions. All three serum conditions stimulated MVSC differentiation. Cells at 2% FCS were shrunken, after fixation of the cells in formaldehyde the majority of the cells had lifted off. The low serum conditions had compromised their state. Cells treated in the differentiation media with 5% FBS were healthy and flattened. 10% serum treated cells were healthier than the cells in 2% serum but still appeared to be shrunken and elongated. Therefore, 5% serum was chosen as the optimal concentration for further analysis. Control samples containing just 2%, 5% and 10% FCS were run simultaneously (data not shown).

3.12.4 Expression of smooth muscle, neural and glial cell markers in MVSCs after TGFβ1 and PDGFBB treatment

After optimisation of the TGFβ1, PDGFBB and FCS cocktail, further analysis was undertaken to investigate the expression of SMA and CNN1 SMC markers (other markers including Sox10, Sox17 and S100β) *(Figure 3.22)*. As before rat smooth muscle cells were used as positive controls for immunocytochemistry analysis. Because MVSCs expressed SMA before any treatment it was not predicted that the SMA fluorescent intensity would increase. There was a slight increase in frequency of filaments but overall there was no huge change in SMA expression after TGF β1 stimulation. After treatment with TGFβ1 and PDGFBB, MVSCs appeared to express increased levels of CNN. The staining was more intense in the treated samples and the filaments became more obvious indicating a mature smooth muscle cell phenotype. The induction of SM-MHCII was the most important result for this experiment. The MVSC cell population went from SM-MHCII^{-} to SM-MHCII^{+}. Analysis was under taken in high and low power magnifications. The staining was intense and myosin filaments were visible indicating that the cells had transitioned to mature SMCs.



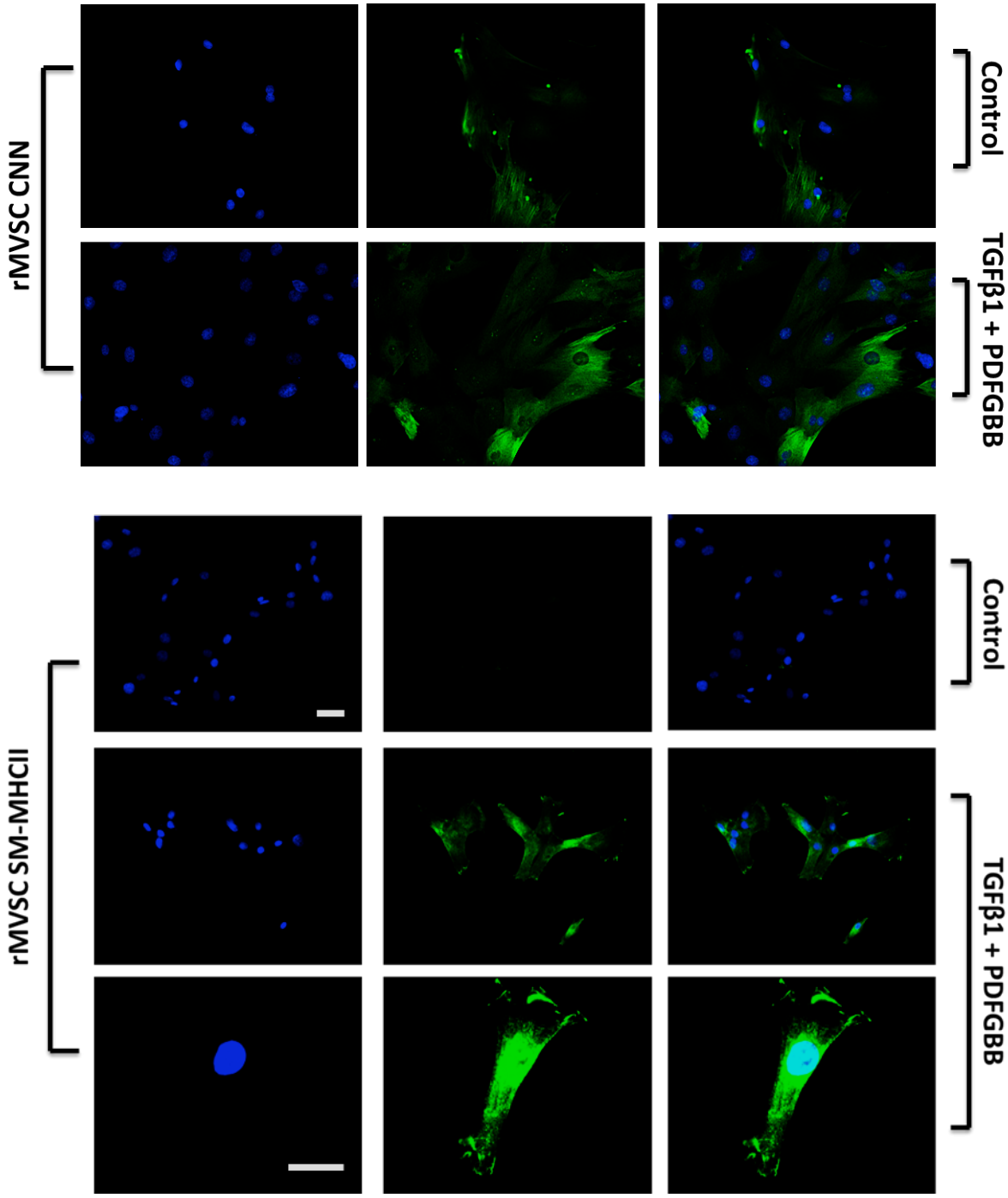


Figure 3.22 *SMA, CNN1 and SM-MHCII expression after TGFβ1 stimulation in MVSCs. Cells were seeded at a low density (5,000 cells/well) and incubated for 5 days in media containing 10% FCS with differentiation components 5ng/ml TGFβ1 and 10ng/ml PDGFBB. The media was replenished every 2-3 days. The cells were fixed in formaldehyde and treated with the appropriate primary and secondary antibodies. Scale bar represents 50nm for all images.*

3.13 Osteocyte and Adipocyte differentiation

The assays used here to determine the multipotency of MVSCs were an osteogenesis assay (bone cells) and an adipogenesis assay (fat cells). MVSC were prepared in parallel expriments and treated according to each protocol *(Figure 3.23, 3.24)*. Rat mesechymal stem cells were used for both experiments as a positive control. Rat smooth muscle cells were used as a negative control. Untreated samples for each cell line were used as another control. Alizarin red (osteocyte) and Oil Red O (Adipocyte and LipidTox) stains were used for visualisation. Both treated and untreated samples were stained. The use of an untreated sample acted as an internal control and eliminated non-specific binding of the stains. MVSCs demonstrated SMC differentiation, Osteogenic differentiation and Adipogenic differentiation. There was a clear induction of osteocyte differentiation in both MVSCs and MSCs. Bright red calcium-rich deposits were observed in treated samples showing that differentiation has occurred. In adipogenic cultures red globular structured were found in samples treated with the induction media. The results from these differentiation assays indicated the multipotency of the cell line and the capability of this cell line to be used as an *in vitro* model for MVSC to smooth muscle cell differentiation.

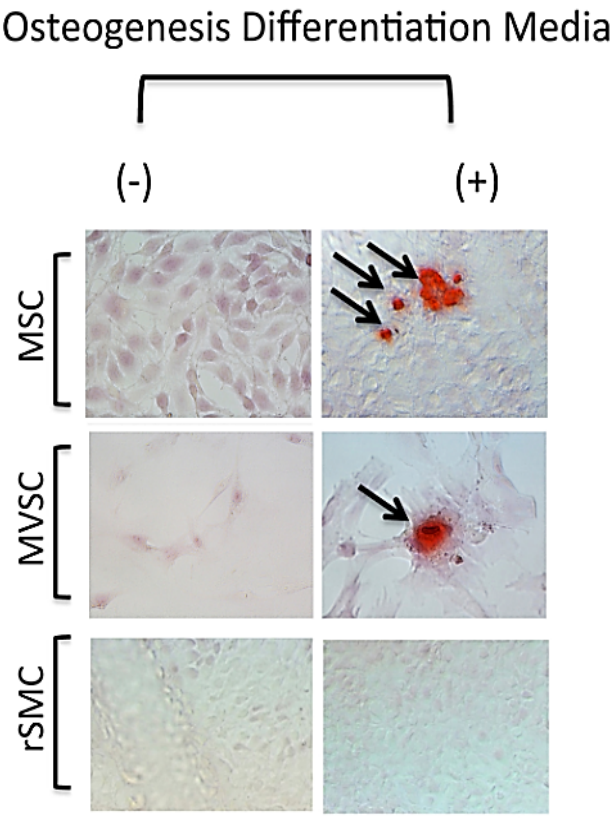


Figure 3.23 *Generation of osteocytes from MVSCs, rMSCs (positive control) and rSMCs (negative control). Cells were seeded at low density (5,000 cells/well) and treated for 21 days with osteogenic induction media. The samples were then stained using Alizarin Red dye to visualise differentiated osteocytes. Scale bar represents 50nm for all images.*

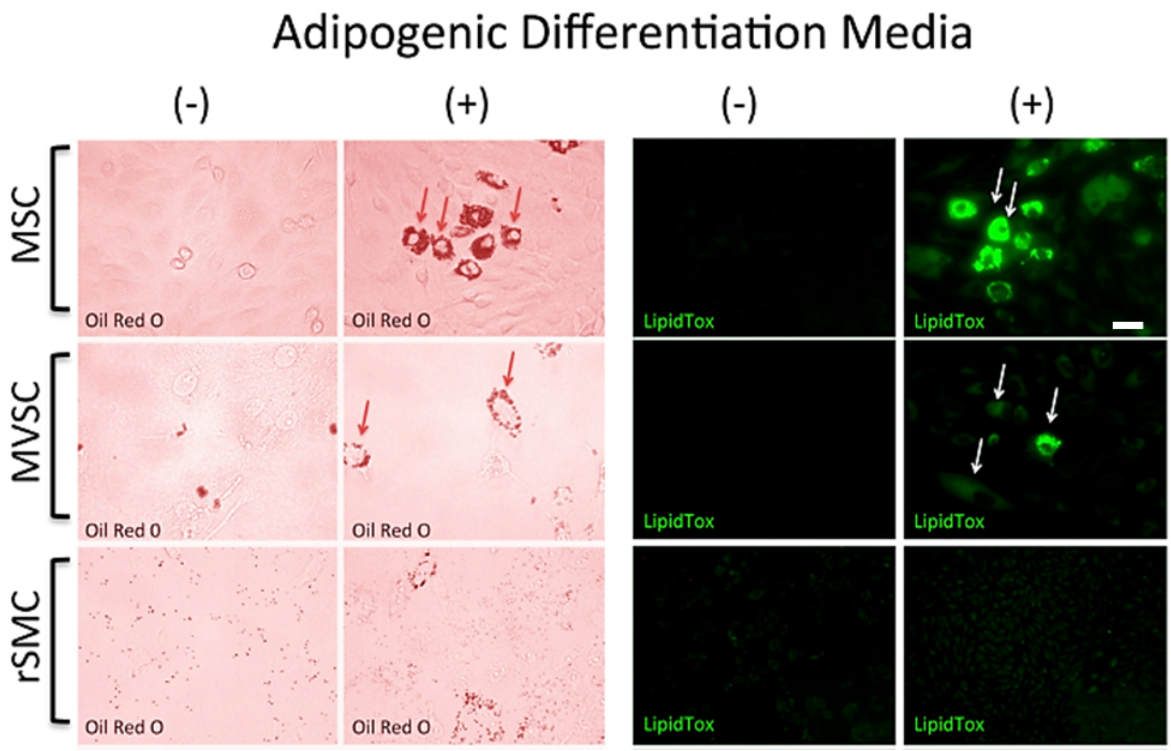


Figure 3.24 *Differentiation of MVSCs, MSCs and rSMCs to adipocytes. Analysis carried out using both Oil Red O and a fluorescent LipidTox stain. Cells were seeded at low density (5,000 cells/well) and treated for 7-14 days with adipogenic induction media. The samples were then stained using Oil Red O dye/LipidTox fluorescent probe to visualise differentiated osteocytes. Scale bar represents 50nm for all images.*

3.14 Summary

- A Multipotent Vascular Stem Cell population was generated by explant method (representing the stem-like population) from the tunica media of aortas isolated from Sprague Dawley rats.
- MVSC appearance was more spindle-like and star shaped in comparison to adult bovine aortic SMCs that were used as a control.
- MVSCs were characterised for stem cell like markers, of particular interest was Sox10, Sox17 and S100β. MVSCs were positive for these markers by immunocytochemistry, western blot and FACS analysis.

- MVSCs were also characterised for markers associated with smooth muscle cells [SMA, CNN1, SM-MHCII]. They were found to be $SMA^{+}/CNN1^{+}/SM\text{-}MHCII^{-}$. Adult SMCs were used as positive controls.
- Cells enzymatically digested from the tunica media (representing the majority of cells in the vessel) were characterised for Sox10 and SM-MHCII, the cells displayed a $Sox10^{+}/SM\text{-}MHCII^{-}$ phenotype.
- The MVSCs were analysed for mesenchymal stem cell markers CD29 and CD44 to determine how much in common they have with their MSC intermediates. MVSCs were positive for both markers.
- MVSCs were deemed multipotent by use of induction media to differentiate them to adipocytes and osteocytes in parallel experiments
- After three weeks in a differentiation media (10% FBS in DMEM), MVSCs showed some evidence of SM-MHCII expression. This was noted by the increase in fluorescent stain.
- MVSCs were treated with 5ng/ml TGFβ1and10ng/ml PDGFBB for 5days and were analysed for SM-MHCII using immunocytochemistry. The cells expressed high levels of SM-MHCII, indicating that smooth muscle differentiation had occurred.

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Chapter 4:

Evaluation of Stem Cell markers in Embryonic and Adult SMC lines

4.0 Introduction

A10 and A7r5 cell lines are embryonic cells lines that have been derived from the thoracic aorta of 14-17 day old embryonic BD1X rats. These cells are commonly used as representative models for vSMCs in cultures (Rao et al. 1997). The cell lines have been used in different studies relating to smooth muscle cell research (Zhou et al. 2010; D C Graves & Yablonka-Reuveni 2000; Capey et al. 2007); (Watanabe 2002). Both cell lines are considered models of non-differentiated, neonatal and neotintimal vascular smooth muscle cells in culture. Initial studies carried out by Roa et al 1997 described the cells as non-differentiated SMCs, the research suggests that the cells differ from the neonatal cells but have a significant resemblance to neoinitmal cells. The functionality and relevance of A10 and A7r5 cells as representative model smooth muscle cell lines in research is questionable, specifically speaking, when used to identify mechanisms underlying the contractile properties of highly differentiated vascular smooth muscle cells. However, these cell lines still present an adult smooth muscle cell phenotype, showing protein expression and evidence of several highly restricted smooth muscle cell markers including SM CNN1, SM22 and to a lesser extent SM-MHCII (Firulli et al. 1998). Graves et al 2000, carried out a detailed analysis of a gene expression program which reported a phenotypic transition of the cell lines from vascular smooth muscle to skeletal muscle, this research suggests that these cells may still retain multipotent and some stem cell like properties. The cells have been shown to contract by both calcium dependent and calcium independent mechanisms, a characteristic associated with a highly differentiated smooth muscle cell (Nakajima et al. 1993).

However, the actin cytomatrix of these cells shares structural similarities to fibroblasts, similar to other smooth muscle cell types that can revert back to presenting a less differentiated SMC phenotype when grown in culture. Despite this the cells are still used in research due to their apparent similarities to neointimal cells and they offer an excellent model for investigating transcriptional regulation of vSMC markers and signalling cascades involved in neointima formation (American et al. 1979; Rao et al. 1997; Firulli et al. 1998).

More recently, multipotent vascular stem cells have been identified and characterised within adventitial and medial layers of the vessel wall. The transition of these cells and their response after injury is highly topical (Tang et al 2012, 2013).

It has been suggested that A10 and A7r5 cell lines that are defined as proliferative or synthetic SMCs due to their phenotype are in fact SMCs which have been derived from these resident stem cells in culture, as opposed to being de-differentiated immature/mature SMCs (Tang et al. 2012; Hu et al. 2004). This discovery of these resident progenitors prompted a re-evaluation of the origin and identity associated with these embryonic smooth muscle cell lines and to investigate whether or not these cells share properties associated with resident vascular stem cells in culture. The cell lines were analysed for stem cell markers associated with MVSCs [Sox10. Sox17, S100β], adventitial cell marker [Sca-1^{+}], established smooth muscle cell markers [SMA, CNN1, SM-MHCII] and also analysed for their multipotent potential.

It was of interest to investigate commercially available adult smooth muscle cell lines in culture and establish whether these SMC lines exhibit neural stem cell markers that are typical of MVSCs. Cell lines were murine, rat and bovine, interestingly isolated by different culture methods, enzymatic dispersal (murine and rat) and explant (bovine). SMC differentiation expression markers were analysed as well as neural stem cell marker expression and the multipotent capabilities of the adult SMC lines following lineage induction *in vitro.*

4.1 Materials and Methods

All materials used were of the highest purity available commercially. Cell culture was carried out as previously described in Chapter 2. Embryonic smooth muscle cell lines A10 and A7r5 were used in this chapter as well as commercially available adult smooth muscle cell lines from three different sources; Rat, Murine and Bovine. Serum deprivation for cellular manipulations was carried out using 0.5% serum containing media. Protein analysis was carried out using FACS, western blotting, immunocytochemistry and confocal imaging. Analysis of mRNA was carried out using qRT-PCR. The cells were treated with induction media for adipogenic differentiation as previously described.

4.2 Results

MVSCs remained sox10, sox17 positive and concomitantly expressed SM-MHC after treatment in 10% DMEM as a differentiation media. For this reason A10 and A7r5 cell lines were screened with sox10 and SM-MHC antibodies to identify whether any 'MVSC-like' positive cells were present in the two populations.

4.3 Cell lines and reagents

The A10 cell line is a rat embryonic cell line derived from the aorta, thoracic/medial layer, similarly, A7r5 cell lines is a rat embryonic derived cell line from the aorta, thoracic/smooth muscle. Both cell lines were cultured in regular DMEM media supplemented with 10% FBS before phenotypic analysis. A10 cell line was supplemented with Leukemia Inhibitory Factor (LIF) and 2-Mercaptoethanol. The A10 cell line possess the typical myoblast morphology *(Figure 4.0)* and when grown in culture all cells screened positive for early, middle and late stage smooth muscle cell markers; smooth muscle α-actin, Calponin1 and SM-MHCII *(Figure 4.1)*. This expression is indicative of their SMC traits. A7r5 cell line had a different morphology with a flat ribbon like structure growing in parallel arrays of spindle shaped cells when confluent *(Figure 4.0)*. These cells were also positive for the three smooth muscle cell markers, yet in contrast to the A10 cell line there was a proportion of A7r5 cells that appeared to be weakly positive for SM-MHCII and CNN1 *(Figure 4.2)*. Rat mesenchymal stem cells were used as positive controls for multipotency assays (adipogenesis and osteogenesis differentiation). These cells were maintained in growth media made up of 50:50 minimal essential medium (α-MEM) and supplemented with 10% FBS. These cells were characterised for the expression of MSC markers using antibodies against CD29, CD44, CD90 and CD146.

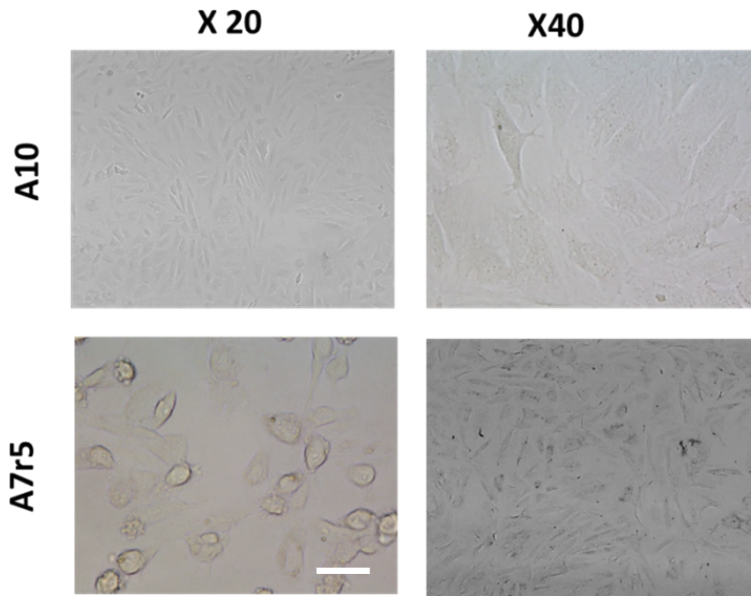


Figure 4.0 *Phase contrast images A10 and A7r5 Cell lines. Cells were seeded at a low density (5,000 cells/well) and fixed in 3.7% formaldehyde before visualisation under phase contrast. Scale bar represents 50nm for each image.*

4.4 Local expression of SMC markers in embryonic stem cell lines

SMC protein expression [SMA, CNN1, SM-MHCII] was assessed using immunocytochemistry. This was carried out initially before any further experiments to characterise the expression of these proteins in the cell lines. Cells were seeded at low density (5,000 cells/well) for visualisation and left for 3 days before being probed with antibodies. Nuclei were stained with DAPI. Both A10 and A7r5 cell lines expressed all three markers. Some cells were found to be slightly more CNN1 positive. This phenotype is representative of a SMC that is not fully differentiated. Flow Cytometry was used as a second method of protein expression to reaffirm results that were achieved with immunocytochemistry *(Figure 4.3, 4.4)*.

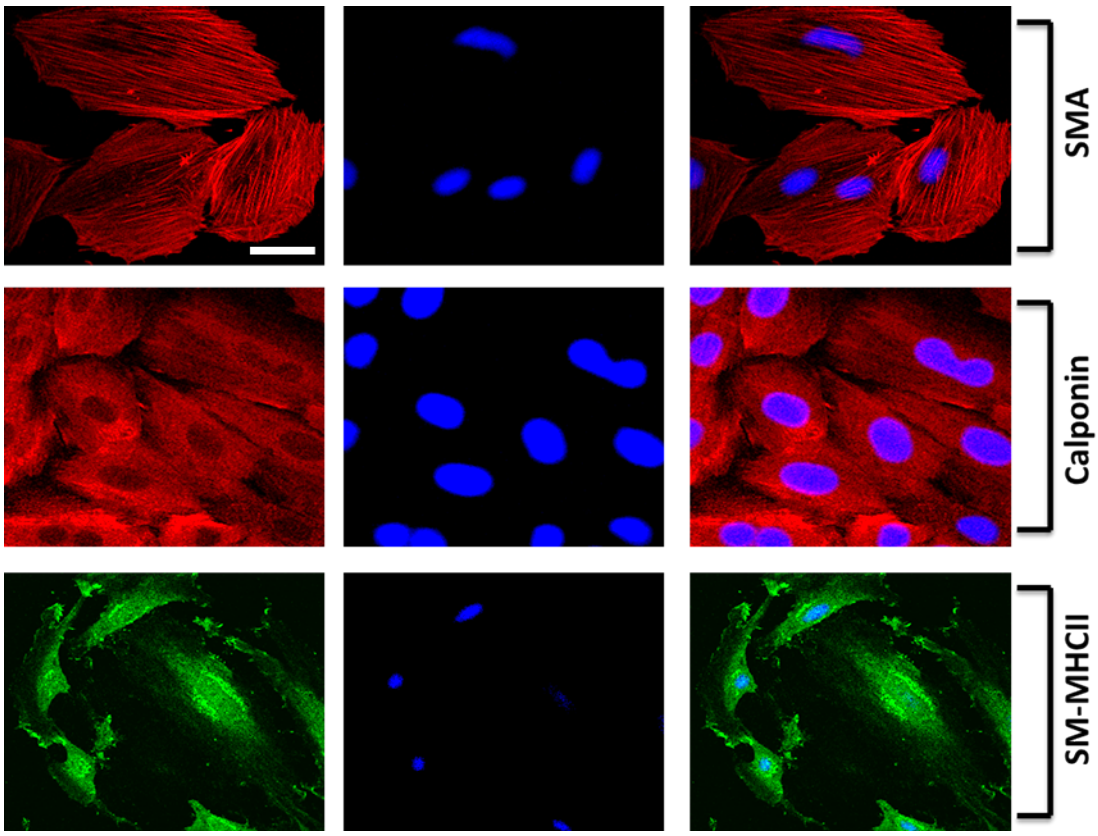


Figure 4.1 *A10 expression of SMC markers [SMA, CNN1, SM-MHCII], confocal and flourescent microscpe images. Cells were seeded at a low density (5,000 cells/well) and incubated for 72 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

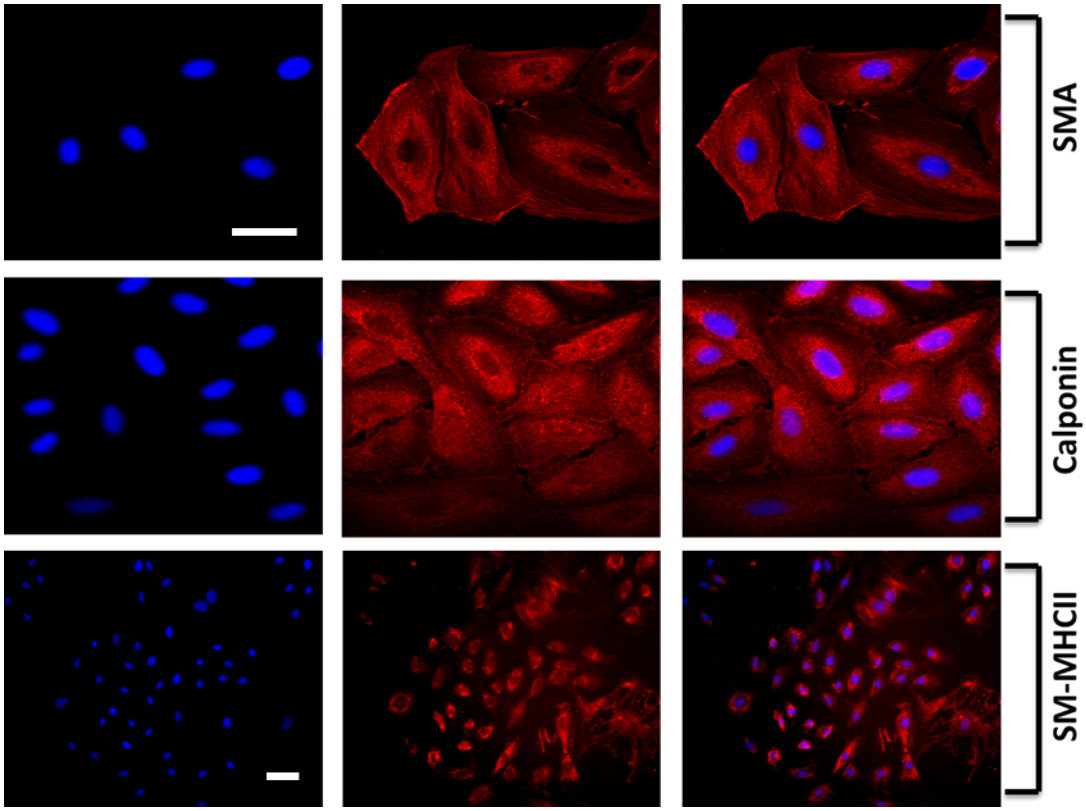


Figure 4.2 *A7R5 expression of SMC markers [SMA, CNN1, SM-MHCII], confocal and flourescent microscpe images. Cells were seeded at a low density (5,000 cells/well) and incubated for 72 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

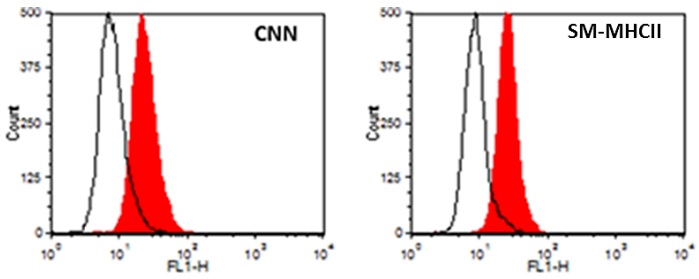


Figure 4.3 *SMC protein expression in A10 via FACS [CNN1, SM-MHCII]. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

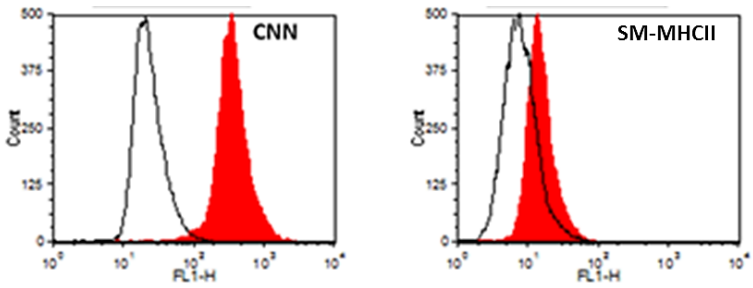


Figure 4.4 *SMC protein expression in A7r5 via FACS [CNN1, SM-MHCII]. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

4.5 Evaluation of the MVSC Sox10^{+}/SM-MHCII^{-} population in embryonic cell lines

The desired cell population according to Tang et. al 2012 would be a smooth muscle precursor cell that expresses neural stem cell marker Sox10 and lack the expression of differentiated SMC marker SM-MHCII. MVSCs were characterised using a double stain for these two proteins, for this reason the same experiment was carried out on A10 and A7r5 cell lines *(Figure 4.5)*.

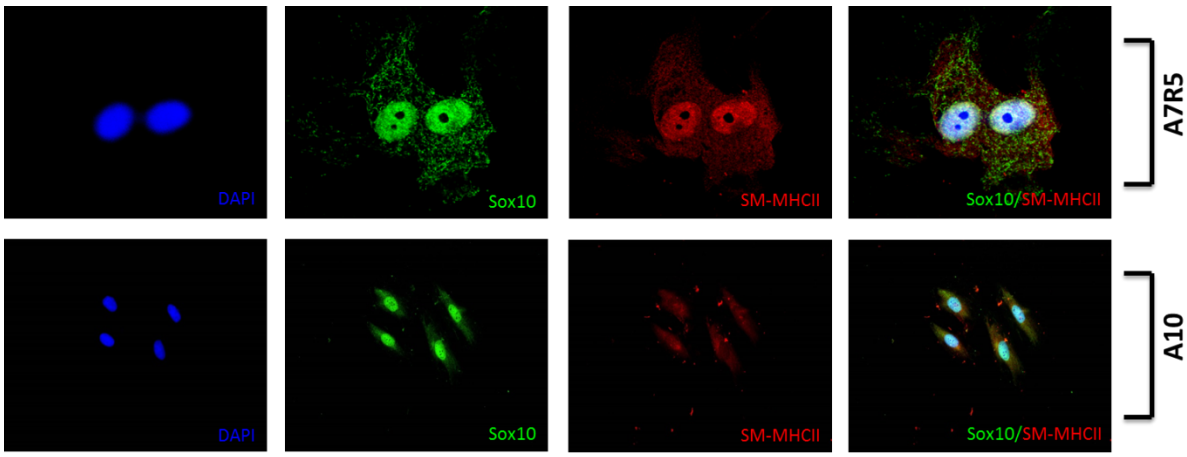


Figure 4.5 *Double Stain on A10 and A7r5 for Sox10 and SM-MHCII. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

4.6 Expression profile of neural and glial cell markers in A10 and A7r5 cell lines

A10 and A7r5 both represent embryonic cell lines. It was hypothesized that these cell lines may retain some of the stem cell like properties, including expression of stem cell markers. MVSCs which had been analysed as previously described were used as positive controls. The cell lines were analysed using immunocytochemistry and FACS analysis *(Figure 4.6 & 4.7)*. Both cell lines were also screen for Stem Cell Antigen 1 *(Figure 4.8)*. This marker is associated with stem cells in the adventitial layer of the smooth muscle vessel. The cell lines A10 and A7r5 were more than likely generated by enzymatic dispersal of the full vessel. It was highly likely that the cells would retain this stem cell associated marker as well as continual expression of the markers associated with the medial layer. In contrast to A10, A7r5 cells stained poorly for SM-MHC by ICC yet the shift in FACS data showed that the cells display a SM-MHC phenotype. Like the A10 cells the A7r5 cells stained strongly for the neural stem makers Sox10, Sox17 and S100β. A7r5 cells stained positive for $Sca1^{+}$ by both FACS and by immunocytochemistry.

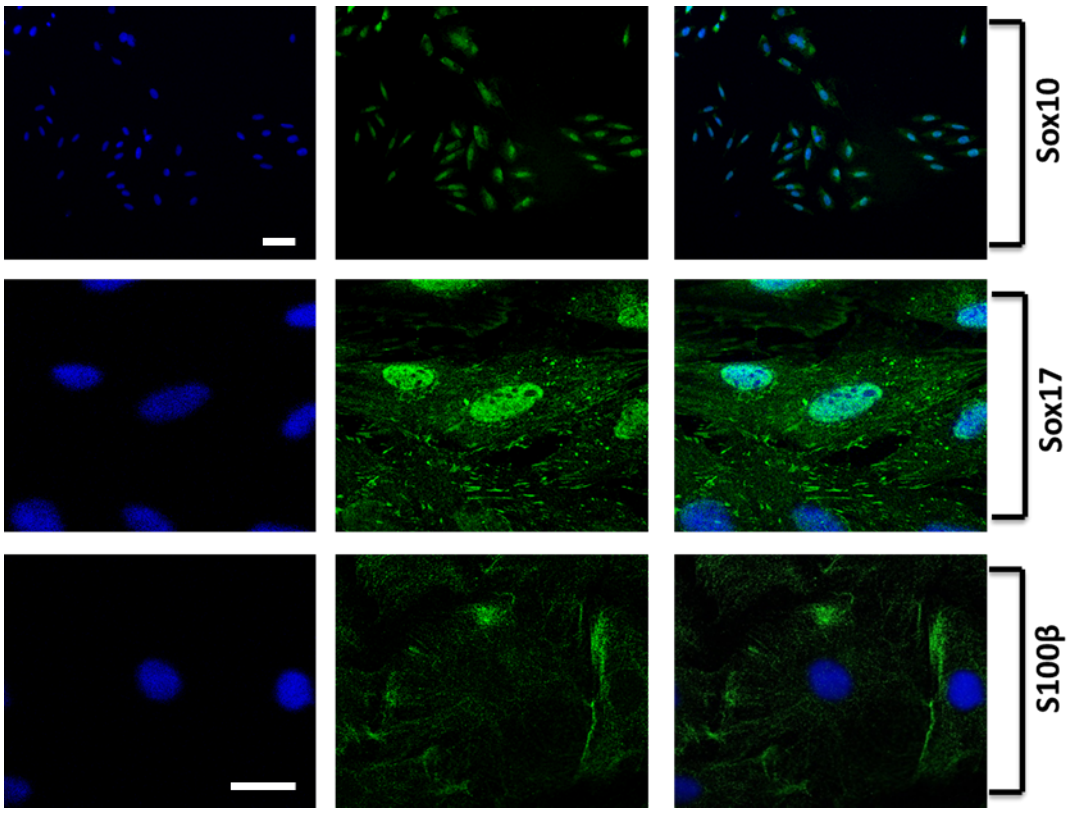


Figure 4.6 *A10 immunocytochemical expression of MVSC markers [Sox10, Sox17 and S100β]. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

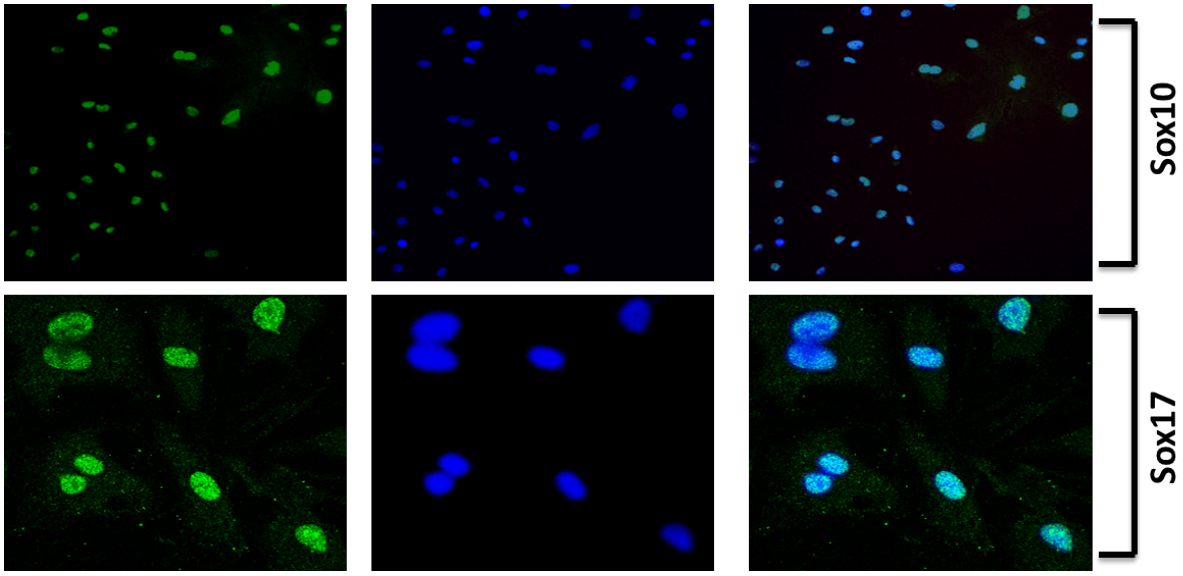


Figure 4.7 *A7r5 immunocytochemical expression of associated stem cell markers [Sox10 and Sox17]. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

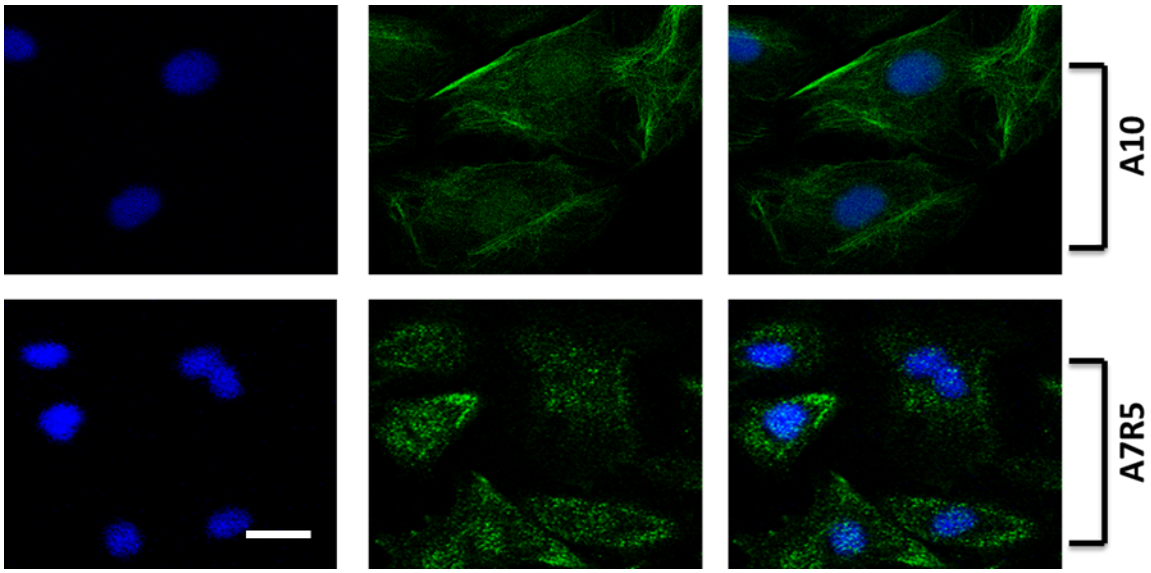


Figure 4.8 *A10 and A7r5 Sca1^{+} expression using immunocytochemistry. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody.*

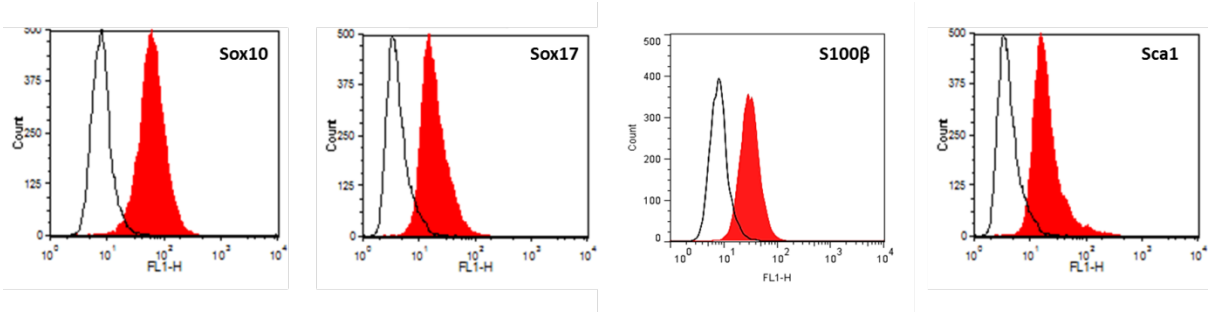


Figure 4.9 *Stem cell marker (neural and glial) protein expression on A10 cells [Sox10, Sox17, S100β and Sca1^{+}]. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

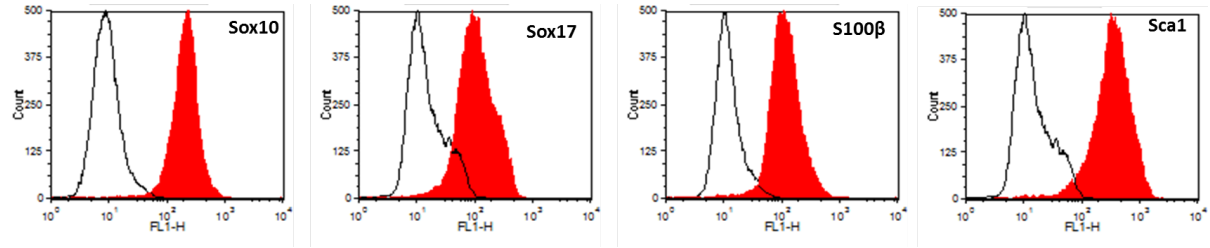


Figure 4.10 *Stem cell marker (neural and glial) protein expression on A7r5 cells [Sox10, Sox17, S100β and Sca1^{+}]. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

4.7 Relative protein expression levels of Sox10, Sox17, S100β, CNN, SMA and SM-MHCII.

Further protein analysis on A10 and A7r5 was carried out using immublotting. MVSC cells were used as a positive control for the stem cell markers [Sox10, Sox17 and S100β] and SMC markers [SMA and CNN1] *(Figure 4.11)*. Maximum amount of protein was added to each well. β-Actin was used as a loading control as well as Ponceau S. stain.

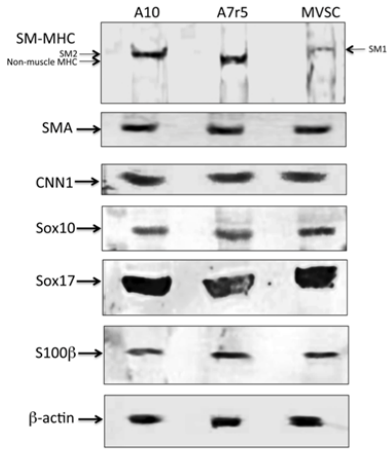


Figure 4.11 *Western Blot analysis of Sox10, Sox17, S100β, SMA, CNN1, SM-MHCII on A10 and A7r5 cell lines, MVSCs acting as positive control. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis*.

4.8 Cell Manipulations

4.8.1 Expression analysis after quiescence of A10 and A7r5 Cell lines

When SMC in culture are quiesced via serum deprivation, SMC differentiation is increased and can be measured by increase in SMC marker expression; SMA, CNN1, SM-MHC. A10 and A7r5 cells were quiesced for 72 hours and compared to untreated serum containing samples. Following the quiescence A10 and A7r5 cells phenotypic changes in the expression of SMC markers SMA and CNN1 was analysed using confocal microscopy. With regard to the A7r5 cell line there was a clear redistribution of SMA and CNN1 filaments after the 72h serum deprivation in comparison with regular serum conditions *(Figure 4.12).* A clear reorganization of actin filaments can be seen under serum conditions in comparison with serum deprived cells. The serum deprived cells overall have a lower intensity of staining. In comparison with serum deprived cells the filaments are less visible with a higher staining intensity being visible at the periphery. With regard to CNN1, the intensity of the stain is increased in serum deprived conditions and there is a presence of filaments which cannot be found in cells cultured in full serum.

A10 cell line showed a similar pattern whereby the expression of CNN1 and SMA increased when the cells were serum deprived. There was an overall increase in fluorescent intensity in samples which were quiesced *(Figure 4.13)*. The actin filaments were much more pronounced in these samples in comparison with the serum containing samples.

The effect of serum deprivation on rat MSC was investigated in order to see how MSCs respond in comparison with the embryonic A10 and A7r5 cell lines. MSCs were seeded at low density (5,000 cells/well) and quiesced for 72 hours. Smooth muscle actin was chosen as the SMC differentiation marker of choice, an increase in SMC markers such as SMA is associated with MSC to SMC differentiation *(Figure 4.14)*.

There was a clear rearrangement of actin filaments after serum deprivation. rMSCs in 5% media displayed a lower range of fluorescence and there were fewer filaments observed with most of the staining on the periphery of the cells. In comparison serum deprived cells displayed much greater staining and an increase in the presence of filaments. The full circumference of the cell displayed the spindle like filaments. SMCs were cultured in 5% FBS and used as an indicator for the MSC differentiation. From the images it is clear that the SMA phenotype of the serum deprived MSCs is very similar to that of the adult smooth muscle cells.

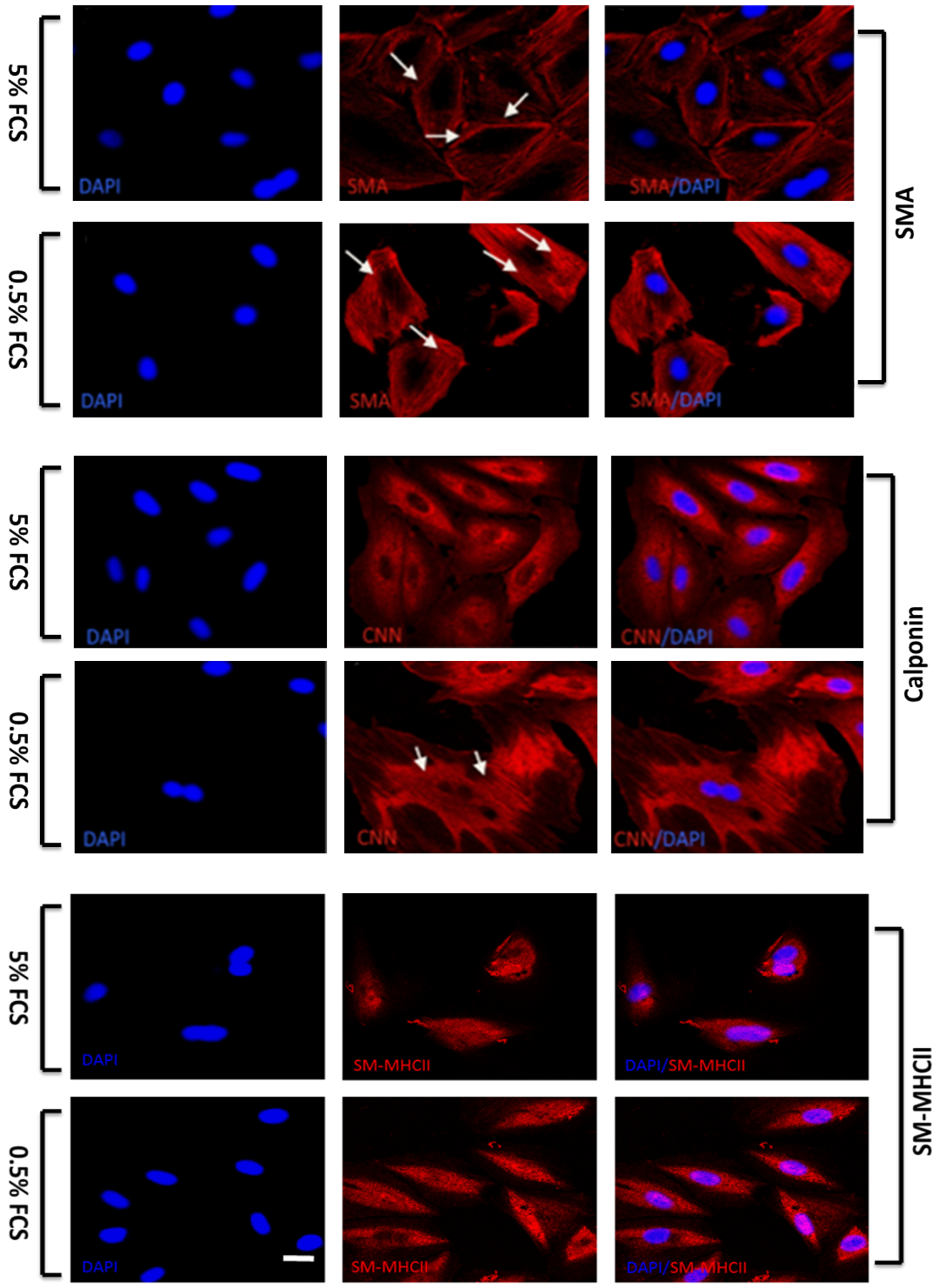
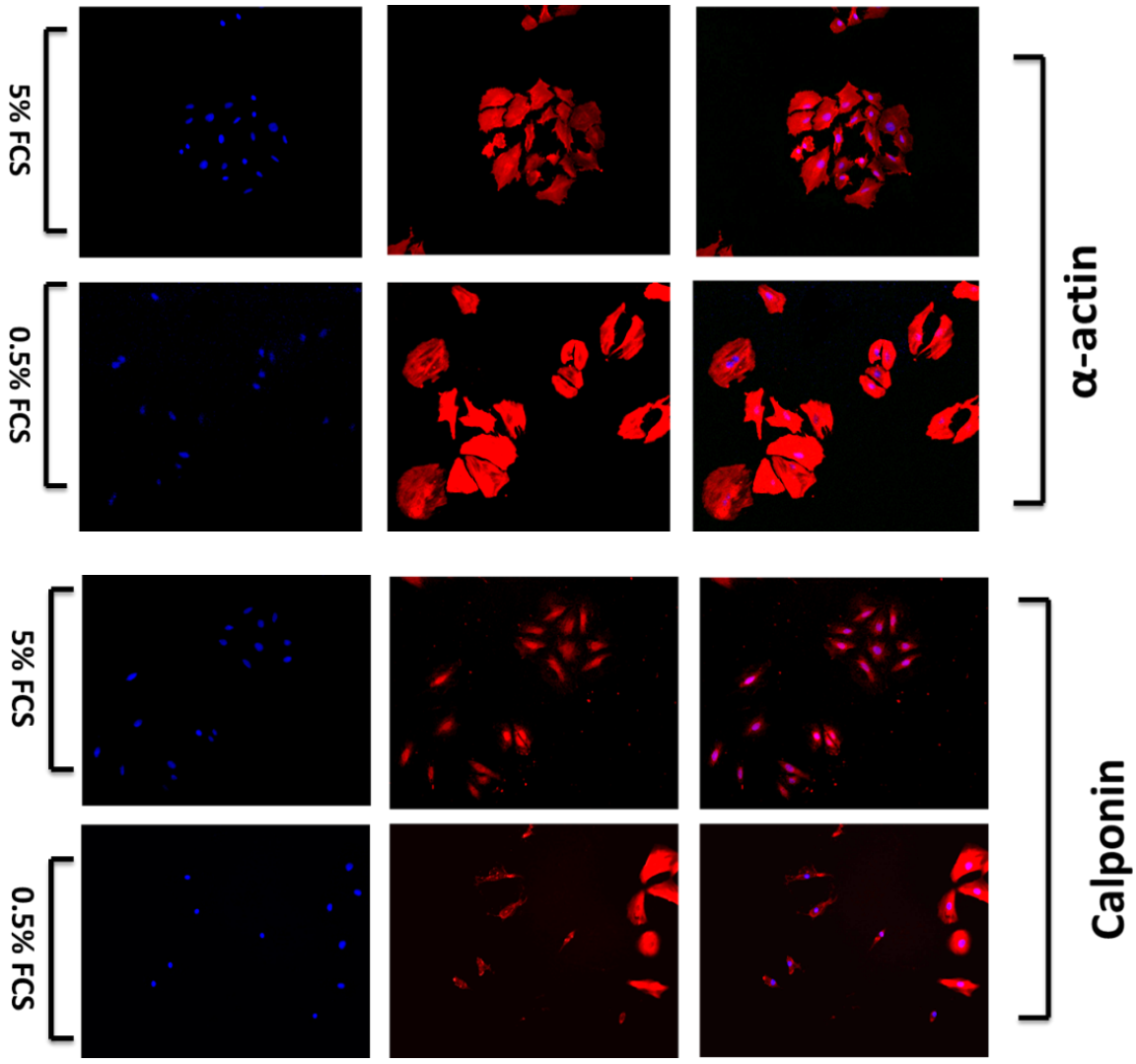


Figure 4.12 *Confocal immunocytochemical of SMC differentiation markers in A7r5 cells. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before switched to media containing either 5% or 0.5% FCS for a further 72 hours. The samples were then probed with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*


5% FCS
0.5% FCS
α-actin
5% FCS
0.5% FCS
Calponin

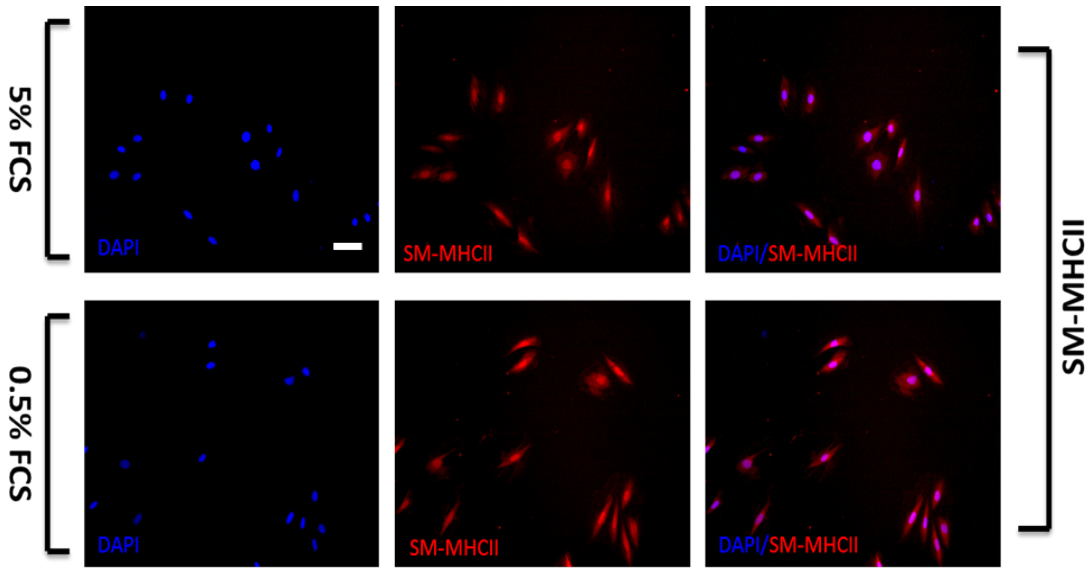


Figure 4.13 *Fluorescent immunocytochemical of SMC differentiation markers in A10 cells, SMA, CNN1 and SM-MHCII. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before switched to media containing either 5% or 0.5% FCS for a further 72 hours. The samples were then probed with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

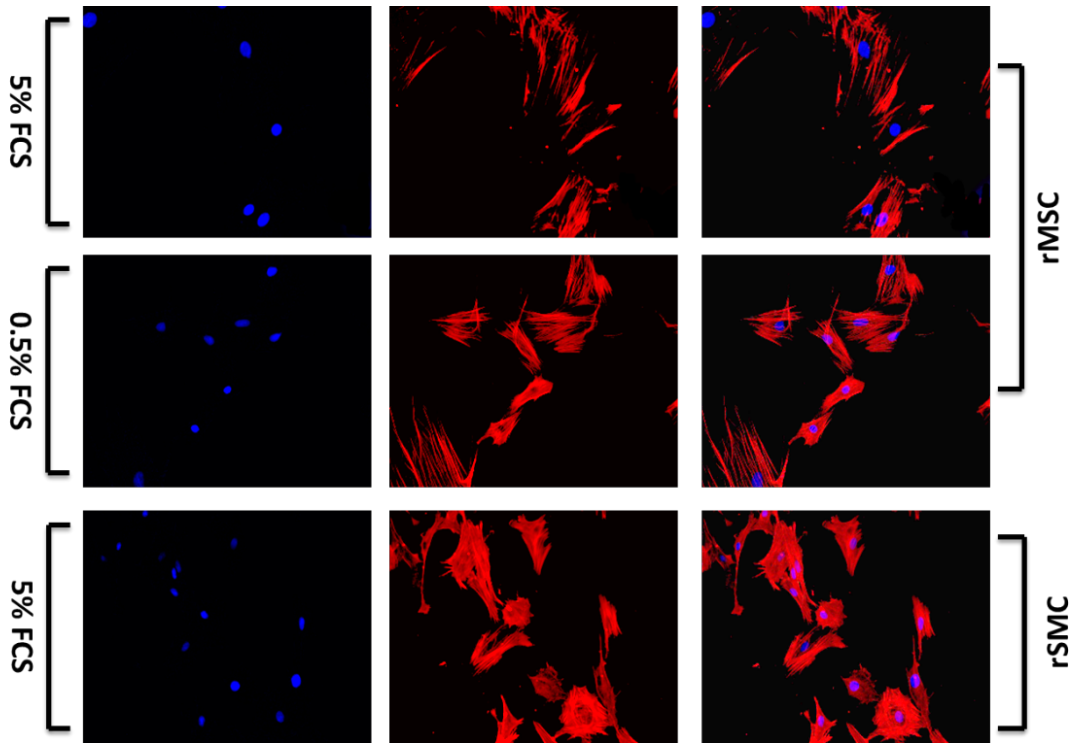


Figure 4.14 *SMA expression in rMSC, rSMC were used as a comparative for SMA expression. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before switched to media containing either 5% or 0.5% FCS for a further 72 hours. The samples were then probed with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

4.8.2 Relative gene expression analysis of A10 and A7r5 cell lines upon quiescence

In order further investigate the MVSC marker expression in the A10 and A7r5 cell lines gene expression analysis was carried out using qRT-PCR. The analysis was completed by measuring the level of expression of both MVSC [Sox10, Sox17 and S100β] and SMC [SMA, CNN1, MHY1, MHY11, SMO] markers using real-time qRT-PCR. A10 and A7r5 cell lines were quiesced in low serum for 24h and then in parallel experiments serum deprived in 0.5% FCS for 72 hours or cells were supplemented with 5% FCS. GAPDH was used as a "house-keeping gene" for both assays.

The repercussions of serum deprivation on SMC differentiation and MVSC marker expression was evaluated by measuring the changes in expression of the

markers associated with each respective phenotype (as described above). In the A7r5 cell line serum stimulation resulted in a significant increase in Sox10, Sox17 and S100β coupled with a decrease in SMC differentiation markers Sm1 and CNN1 while expression levels of SMA and Sm2 increased. The greatest increase was found in S100β *(Figure 4.15)*. In contrast the addition of serum to A10 increased SMC differentiation markers and the same increase was found in neural crest cell markers Sox10, Sox17 and S100β expression in these cells *(Figure 4.15)*.

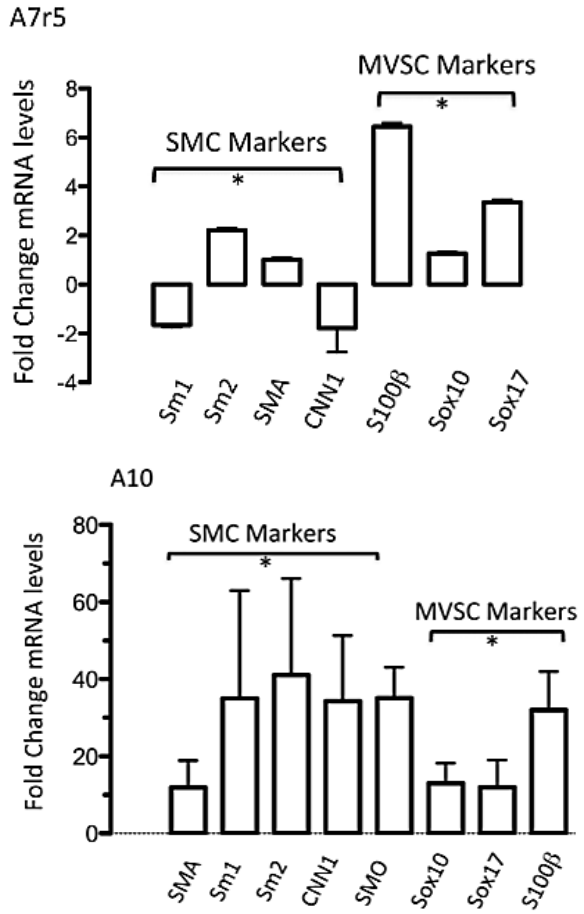


Figure 4.15 *qRT-PCR representative data for A10 and A7r5 cell lines after serum deprivation for 72h. Cells were seeded at 100,000 cells per well and were incubated until they reached confluency. mRNA was prepared as described in the methods section and data was analysed using the Comparative CT method (ΔΔCT), described by Livak and Schmittgen, 2001.*

4.9 Differentiation Potential of A10 and A7r5 Cell lines

The MVSC markers Sox10, Sox17 and S100β that are expressed in A10 and A7r5 cell lines are associated with a stem cell like profile (Tang et al 2012). Their consistent expression in these embryonic vascular cell lines suggests that A10 and A7r5 cell lines may retain some stem cell like properties.

To investigate the multipotent potential of A10 and A7r5 cell lines, they were treated with an induction media to stimulate adipocyte differentiation. Adipocyte differentiation was measured using both classic Oil Red O staining and LipidTOX™ fluorescent staining after 14 d treatment with the induction media. Rat MSCs and rMVSCs were also treated with induction media and these populations served as a positive control *(Figure 4.16)*.

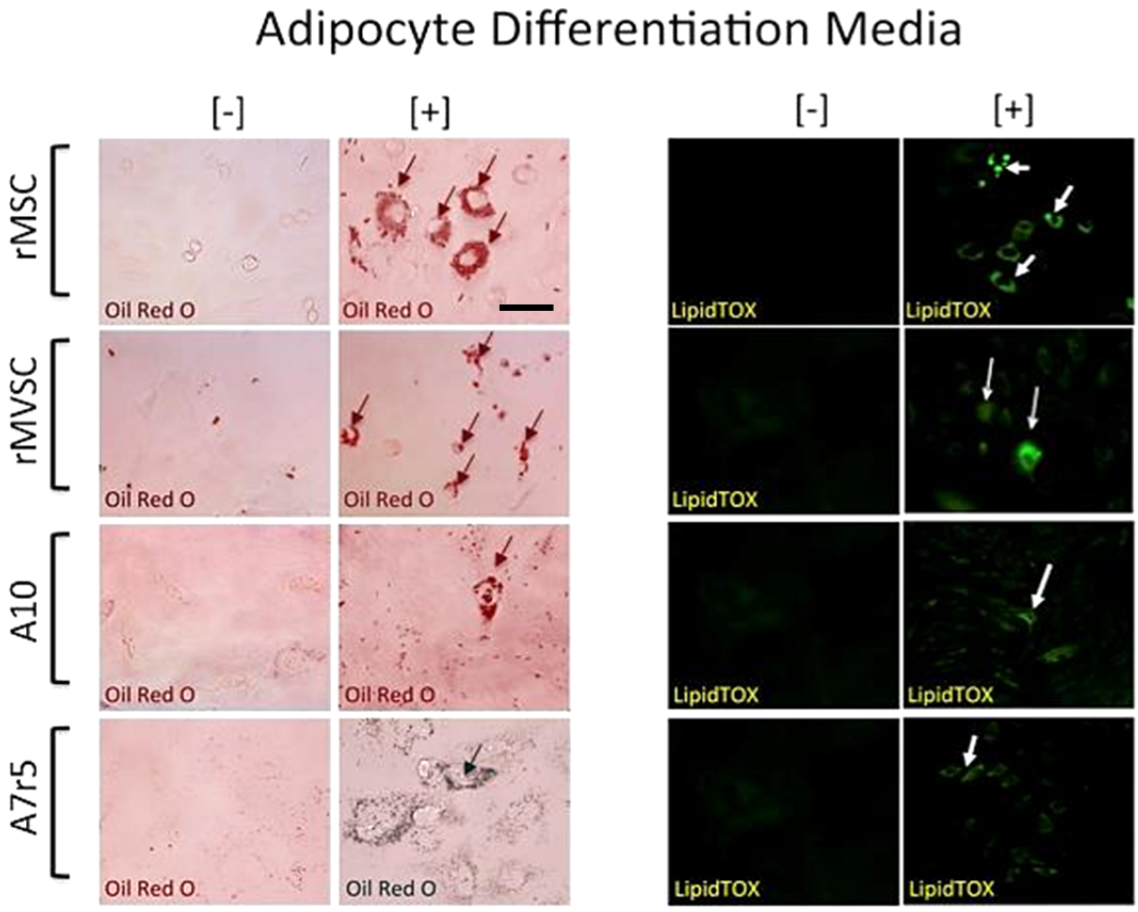


Figure 4.16 *Adipocyte generation in A10 and A7r5 cell lines. Cells were seeded at low density (5,000 cells/well) and treated for 7-14 days with adipogenic induction media. The samples were then stained using Oil Red O dye/LipidTox fluorescent probe to visualise differentiated osteocytes. Scale bar represents 50nm for all images.*

4.10 Adult Smooth Muscle cell cultures

Since these embryonic cell lines displayed properties of multipotent cells it was hypothesized that adult smooth muscle cells in culture may still retain some of these properties. The cells in culture analysed previously in situ expressed high levels of Sox10. It is possible that the majority of cells initially isolated were Sox10 negative but that the Sox10 stem population out grew the negative responders. Protein analysis for of bovine, rat and murine smooth muscle cells was undertaken using western blot and FACS analysis. Representative differentiation markers were used to investigate the presence of both SMC [SM-MHCII, CNN1] and MVSC [Sox10, Sox17] markers. The presence of Sox10, Sox17 and S100β may indicate that smooth muscle cells in culture have been derived from a multipotent vascular stem cell. The fact that the smooth muscle cells present in situ in the vessel display low Sox10 levels and high SM-MHCII levels reinforces this theory (Tang et al 2012, Kennedy et al 2014).

Rat, bovine and mouse smooth muscle cells were analysed using immunoblotting. It was found that all three cell lines expressed SMC markers; SM-MHCII and CNN1, and co-expressed MVSC markers; Sox10 and Sox17 *(Figure 4.17, 4.18)*.

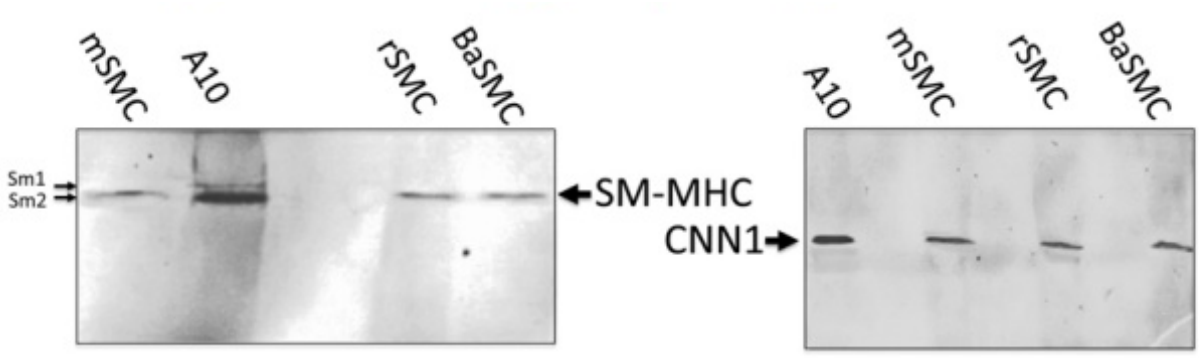


Figure 4.17 *mSMC, rSMC, BaSMC protein immunoblot anlaysis for SMC markers SM-MHCII and CNN1. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis*.

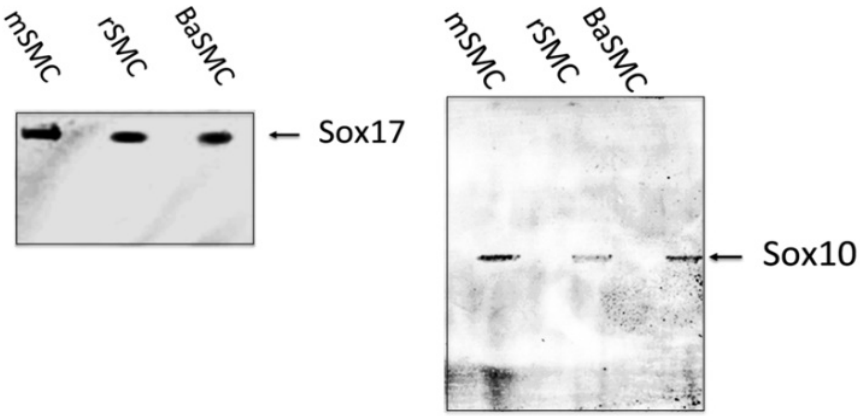


Figure 4.18 *mSMC, rSMC, BaSMC protein immunoblot anlaysis for MVSC markers Sox10 and Sox17. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis.*

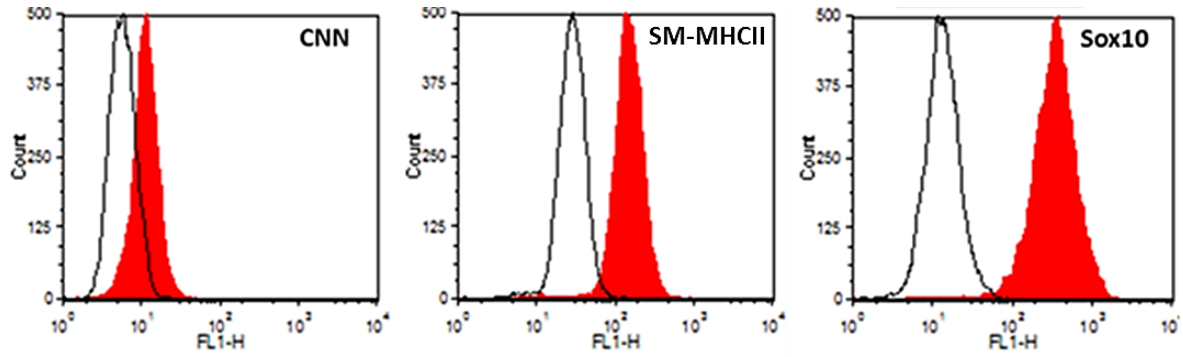


Figure 4.20 *FACS analysis on mSMCs for SM-MHCII, CNN1 and Sox10. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

Immunocytochemistry analysis of the expression of MVSC markers [Sox10, Sox17, S100β] in rat, murine and bovine smooth muscle was undertaken by other members of the lab as described in Kennedy et al. 2014. It was found that rSMCs and baSMCs expressed Sox10 by immunocytochemical methods. These results reaffirm the

results achieved in the study previously mentioned which was undertaken by collaborators of this project. All SMCs used for experiments were checked routinely for markers associated with their phenotype; SMA, CNN1 and SM-MHCII.

4.11 Multipotent Potential of Smooth Muscle Cells

The expression of these MVSC markers shows that the SMCs still retain some multipotent aspects. For this reason rSMCs were treated with osteogenesis induction media. rMSCs were used as positive controls *(Figure 4.21)*. The results indicate that rSMCs have lost their potential to differentiate to osteocytes. The trilineage differentiation capabilities of cells is associated with MSCs, this means that although the rSMCs express phenotypic stem cell like markers they have less mulipotency potential than MSCs have.

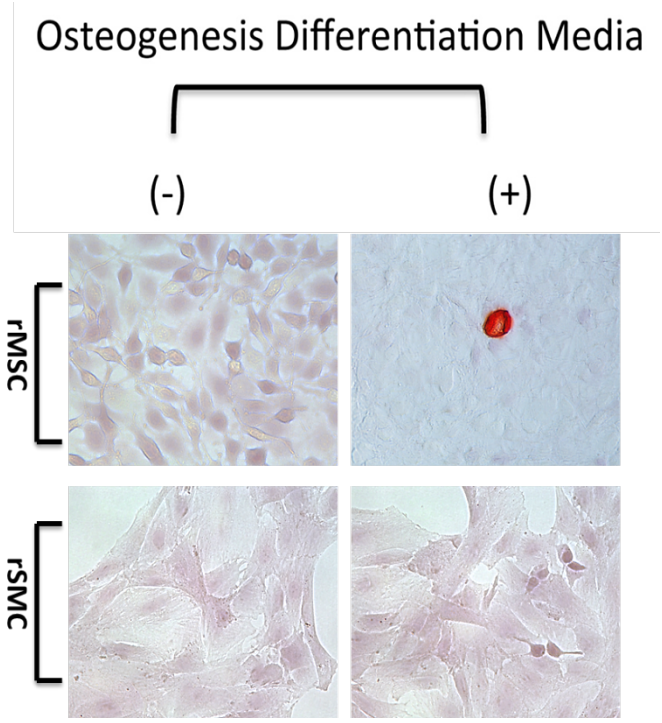
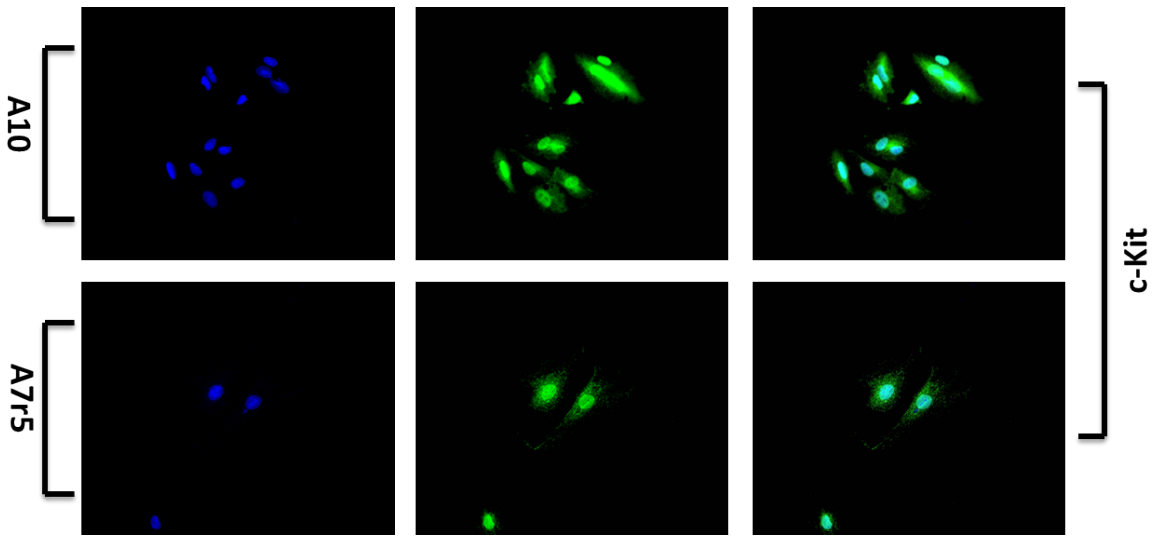


Figure 4.21 *Osteogenesis differentiation of MSC and rSMC. Cells were seeded at low density (5,000 cells/well) and treated for 21 days with osteogenic induction media. The samples were then stained using Alizarin Red dye to visualise differentiated osteocytes.*

4.12 Local expression of Stem associated markers C-Kit and CD133

C-kit (CD117) is a stem cell growth factor receptor associated with the hematopoetic system as previously described (Fazel et al. 2006). The expression of c-Kit was analysed using immunocytochemistry and FACS. Both A7r5 and A10 cells expressed c-Kit *(Figure 4.22)*. MVSC and SMC cells were also screened and the two lines also displayed c-Kit positivity by both protein detection methods *(Figure 4.23, 4.24)*. CD133 is associated with hematopoetic cells and is thought to be involved with angiogenesis. All three cell lines A10, A7r5 and MVSC expressed CD133 *(Figure 4.25)*. This might indicate that these cells could play a role in angiogenesis during embryonic development or post natal injury.



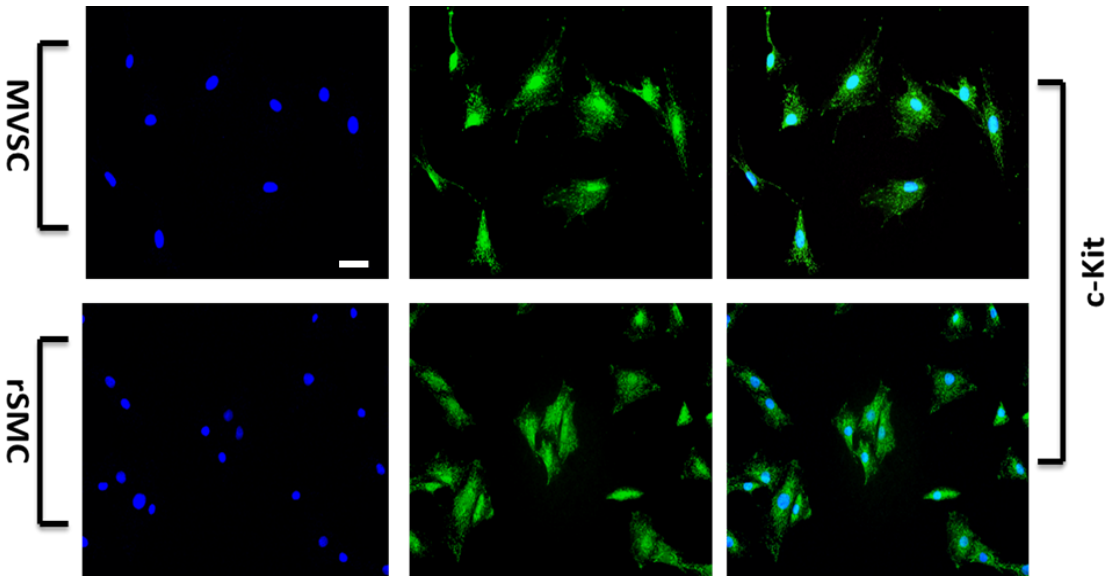


Figure 4.22 *c-Kit expression in A10, A7r5, MVSC and rSMC cells. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody. Scale bar represents 50nm for all images.*

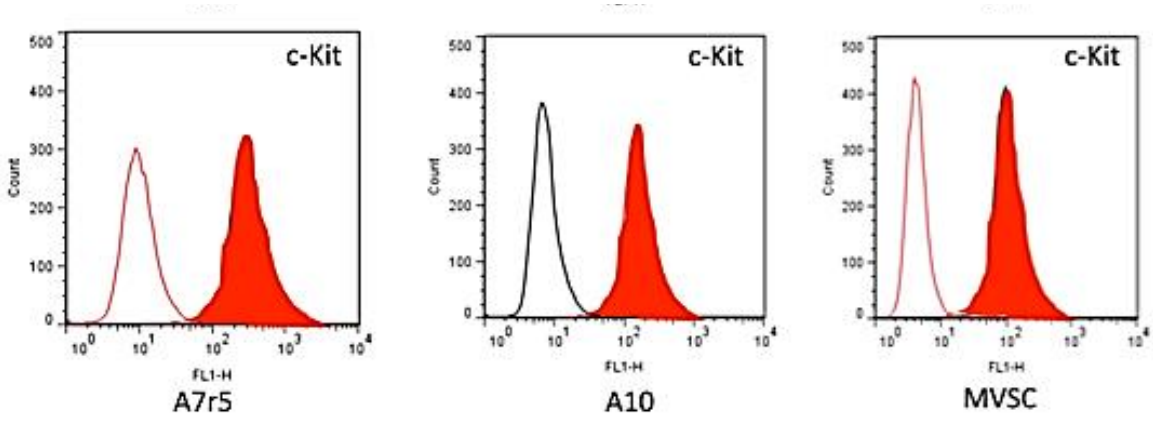


Figure 4.24 *FACS analysis of c-Kit on A7r5, A10 and MVSC cells. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

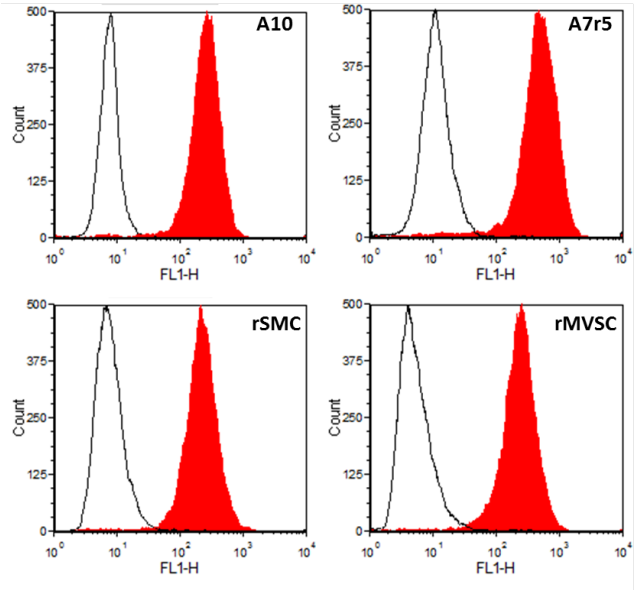


Figure 4.25 *FACS analysis of CD133 on A7r5, A10 and MVSC cells. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

4.13 Local expression of Flt-1/Vascular Endothelial Growth Factor Receptor

Flt-1 or Vascular Endothelial Growth Factor (VEGF) receptor has been shown to influence vascular smooth muscle cell functions (Orlandi & Bennett 2010). A10, A7r5 and MVSC were analysed for the presence of Flt-1 using FACS analysis. Flt-1expression has been found in atherosclerotic lesions, therefore it was of great interest to see the protein analysis of this marker in the cell lines in culture. It was found that all three cell lines were positive for VEGF receptor *(Figure 4.26)*.

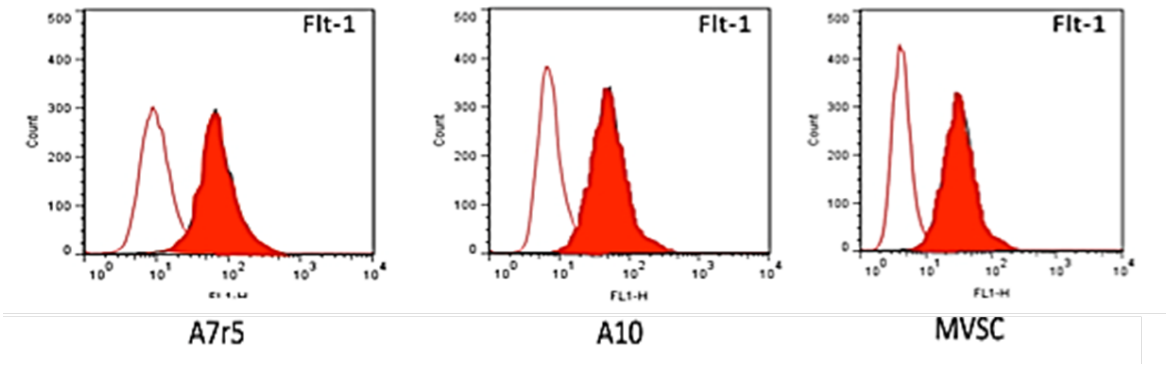


Figure 4.26 *FACS analysis of Flt-1 expression in A7r5, A10 and MVSC cell lines. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

4.14 Growth Profile of MVSC, A10 and A7r5 Cell lines

A proliferation Assay was carried out on MVSCs, A10, A7r5 and rSMC cell lines. This was to compared their growth rate over time. Cells were seeded at equal density to begin with and analysis of the samples was carried out for 12 days. The samples were DAPI stained on days 1, 3, 5, 7, 9 and 12 *(Figure 4.27)*. It was found that A10 and A7r5 had similar growth patterns. After day 8 A7r5 proliferation plateaued but A10 cells continued to grow. MVSCs lagged behind initially and A10 and A7r5 cells had a faster growth rate, however, MVSCs recovered after day 12.

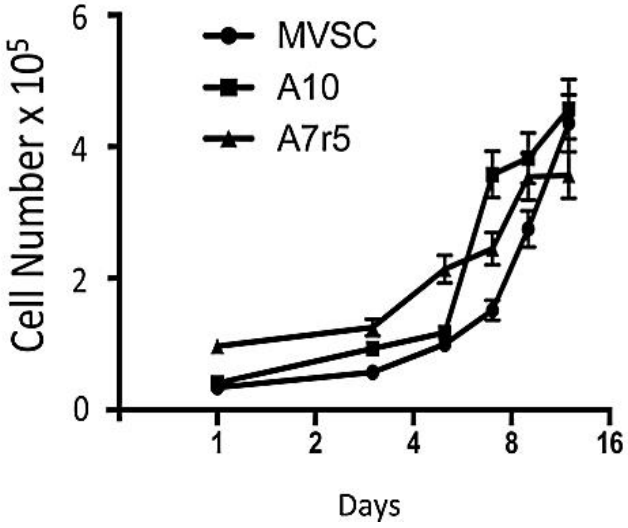


Figure 4.27 *Proliferation assay for A10, A7r5 and MVSC cells. Cells were seeded at low equal seeding density (10,000 cells/well). Multiple samples for each cell line were prepared. Cells were fixed in formaldehyde after 1, 3, 5, 7, 9 and 12 days and stained with DAPI to visualise the nuclei and allow for counting.*

4.15 Summary

- Rat embryonic smooth muscle cell lines A10 and A7r5 were analysed for the presence of stem cell like markers expressed in MVSCs [Sox10, Sox17, S100β]. Protein analysis was carried out using immunocytochemistry, western blotting and FACS analysis. Both cell lines were positive for the neural stem cell markers.
- In parallel experiments the cell lines were analysed for their SMC marker expression, both found to be $SMA^{+}/CNN1^{+}/SM\text{-}MHCII^{+}$, with A7r5 cells displaying reduced level of myosin in comparison with A10 cells.
- A10 and A7r5 cell lines were quiesced for 48h and then either serum deprived for a further 72h in 0.5% FCS or supplemented with media containing 5% FCS. The effects of serum were investigated using immunocytochemistry.
- It was found that the serum deprivation of A7r5 and A10 cells made the cells more smooth muscle cell like, with an increase in actin filaments observed in both cell lines. Calponin filaments were clearer upon serum deprivation with

an overall increase in intensity for both cell lines. There was no apparent change in myosin expression.

- Rat MSCs were used as a comparison for SMA. Serum deprivation in the MSCs caused an increase in the actin filaments and staining pattern was similar to that of actin in SMCs.
- qRT-PCR analysis was used to analyse the gene expression of the effects of serum. In A7r5 cells, serum stimulation caused an increase in Sox10, Sox17 and S100β coupled with a decrease in SMC differentiation markers Sm1 and CNN1 while expression levels of SMA and Sm2 increased. In contrast, serum increased all the expression of all genes in A10 cells.
- A10 and A7r5 cells both showed potential to differentiate into adipocytes.
- Adult smooth muscle cells were examined for stem cell markers by western blot. Rat, murine and bovine SMCs all expressed Sox10, Sox17 and S100β at a protein level.
- Rat SMCs did not retain potential to differentiate down the osteocyte lineage.

Chapter 5:

NE4C as a model for Multipotent Vascular Stem Cells

5.0 Introduction

Another aim of this project was to identify a cell line that could be used routinely in experiments as a representative for the MVSC cell line (with subsequent confirmation of results using the MVSC cells). MVSCs are difficult to isolate and lose multipotency as they are passaged. This would mean a lot of labour to use this cell line continuously. The NE4C cells are an immortalised neuroectodermal cell line. It was proposed that these cells could mimic MVSCs for future experiments. Neural stem cells develop from the neural crest, which is derived from the ectoderm. MVSCs were isolated from the aortic arch which is also of ectoderm origin (Tang et al 2012). For this reaon the NE4C cell lines were chosen as the representative cell line. These cells are characterised by their high levels of Nestin. Neural crest stem cells have also been shown to express Pax6 (Xie et al. 2011b).

For the purpose of this work the NE4C cells were characterised for NCSC markers [Nestin, Pax6] and MVSC associated markers [Sox10, Sox17, S100β] in order to establish how similar these cells were to the isolated MVSC population previously described. Their capability to differentiate to osteocytes and adipocytes was investigated using lineage induction media. In order for these cells to be used as SMC progenitors it is essential that they can differentiate to SMCs after treatment with TGFβ1. The cells were treated as MVSCs were and it was found that the cells did show increased myosin expression immunocytochemically. However, these experiments are preliminary and further investigations need to be undertaken to confirm what was found here.

5.1 Materials and Methods

All materials used were of the highest purity available commercially. Cell culture was carried out as described in Chapter 2. NE4C cells used are a commercially available neuroectodermal stem cell line. The cells were treated using TGFβ1 and PDGFBB for smooth muscle cell differentiation as previously described. Protein analysis was carried out using FACS, western blotting, immunocytochemistry and confocal imaging. The cells were treated with induction media for adipogenic and osteogenic differentiation as previously described.

5.2 Results

5.3 Cell lines and Reagents

NE4C cells are a murine neuroectodermal cell line. The cell line was established from primary brain cultures which were prepared from the fore and midbrain vesicles of 9-day-old mouse embryos lacking the functional p53 genes. These cells have the capability to divide continuously if they are maintained under correct culture conditions. According to the depositary these cells display many characteristics of neural stem cells, such as nestin and SSEA1 immuno reactivity, self-renewal and differentiation into distinct neural cell types upon appropriate induction.

5.4 Expression profile of neural stem cell markers

To verify that these cells express markers associated with the neural crest, the cell line was screened with antibodies against Nestin and Pax6. Immunocytochemistry and FACS were used to analyse protein expression of the two markers. The cell line expressed both stem markers, this allowed for further analysis to be carried out on the NE4C cell line *(Figure 5.0. 5.1)*.

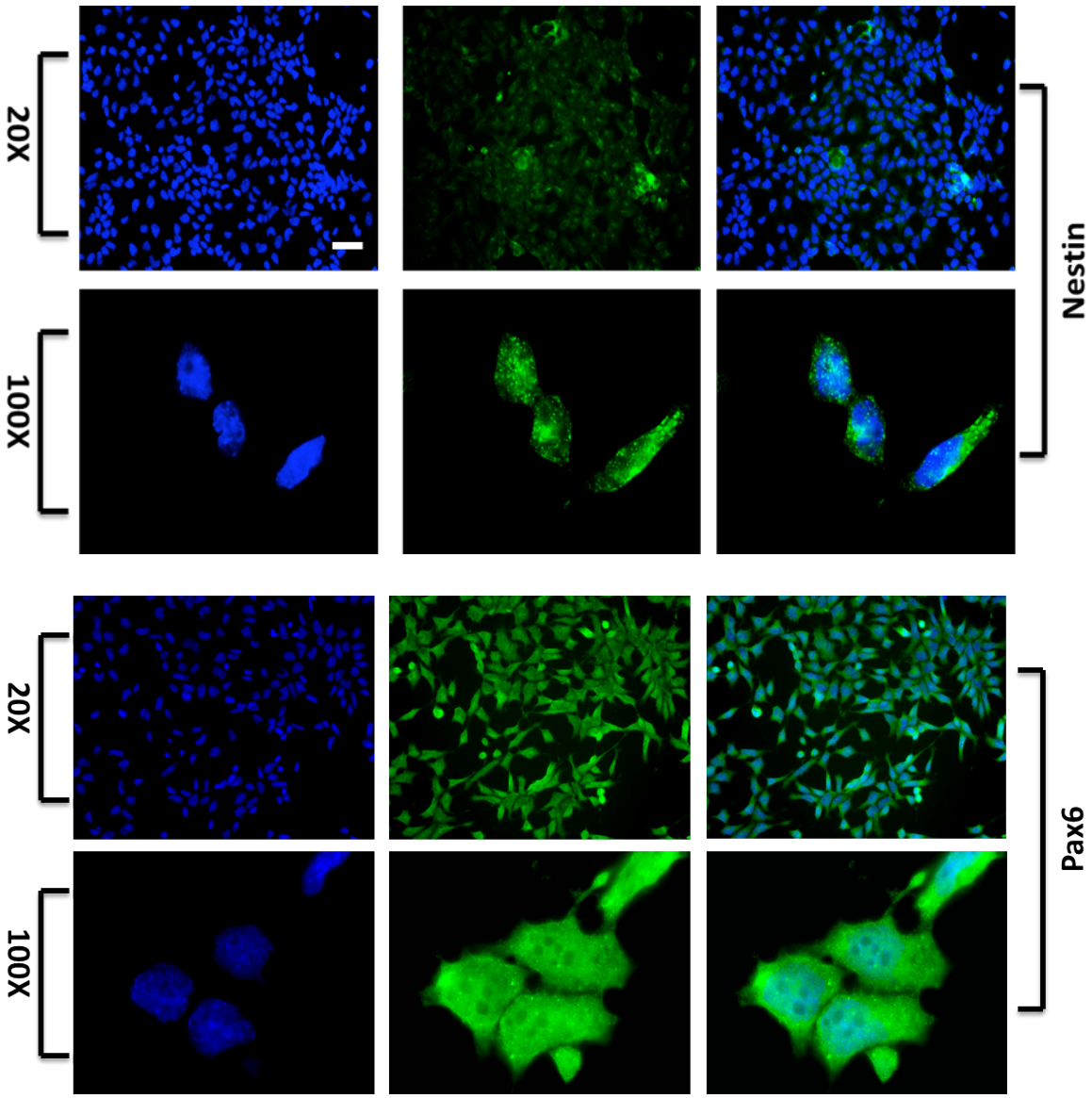


Figure 5.0 *Immunocytochemical data for Nestin and Pax6 on NE4C cells. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody. Scale bar indicates 50nm for all images*

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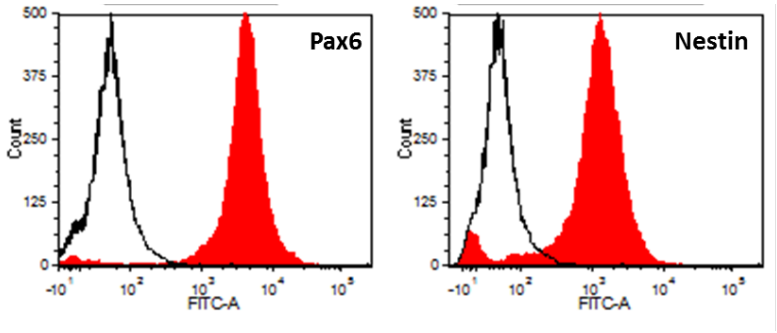


Figure 5.1 *FACS data for Nestin and Pax6 on NE4C cells. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

5.5 NE4C expression of MVSC associated neural and glial stem cell markers

MVSCs isolated were taken from the aortic arch which is also of neuroectodermal origin. For this reason it was hypothesized that NE4Cs could be used as a model for the MVSCs. In order for these cells to be used as an MVSC-like cell, the properties of the cell line had to be examined. This included expression of the stem cell markers Sox10, Sox17 and S100β. NE4Cs were found to be positive for the three MVSC markers using both immunocytochemistry and FACS analysis *(Figure 5.2, 5.3)*.

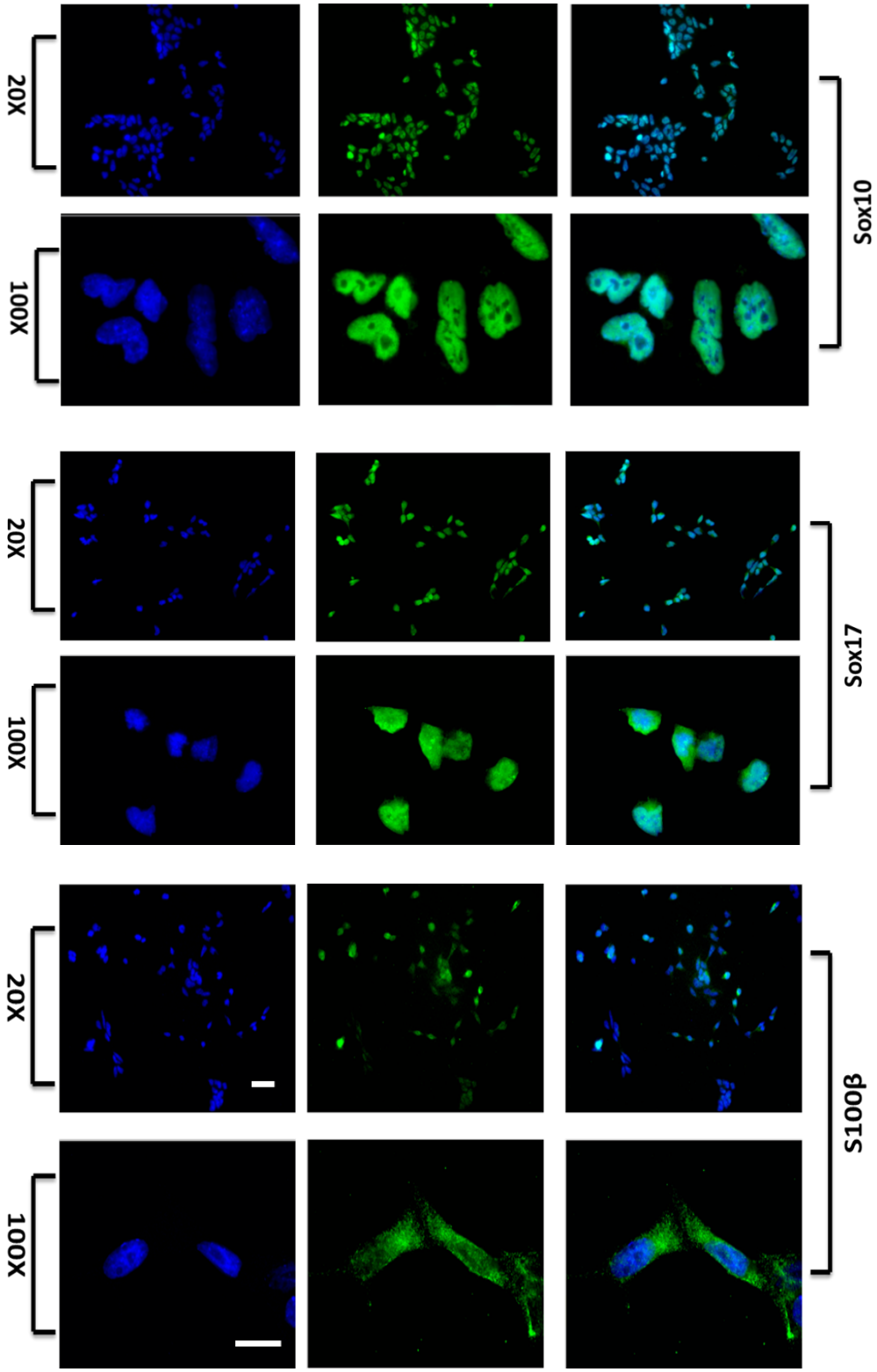


Figure 5.2 *Representative data for Sox10, Sox17 and S100β expression in NE4C cells. Cells were seeded at a low density (5,000 cells/well) and incubated for at least 48 hours before probing with the appropriate primary and secondary antibody. Scale bar indicates 50nm for all images.*

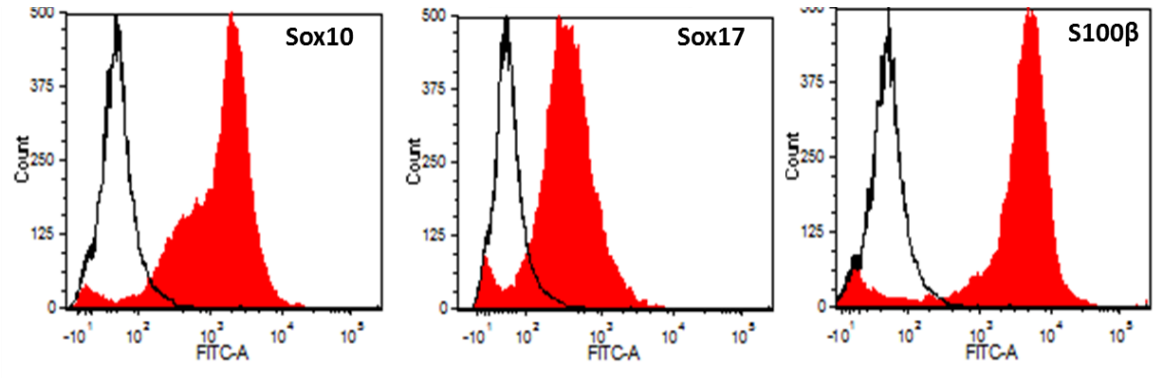
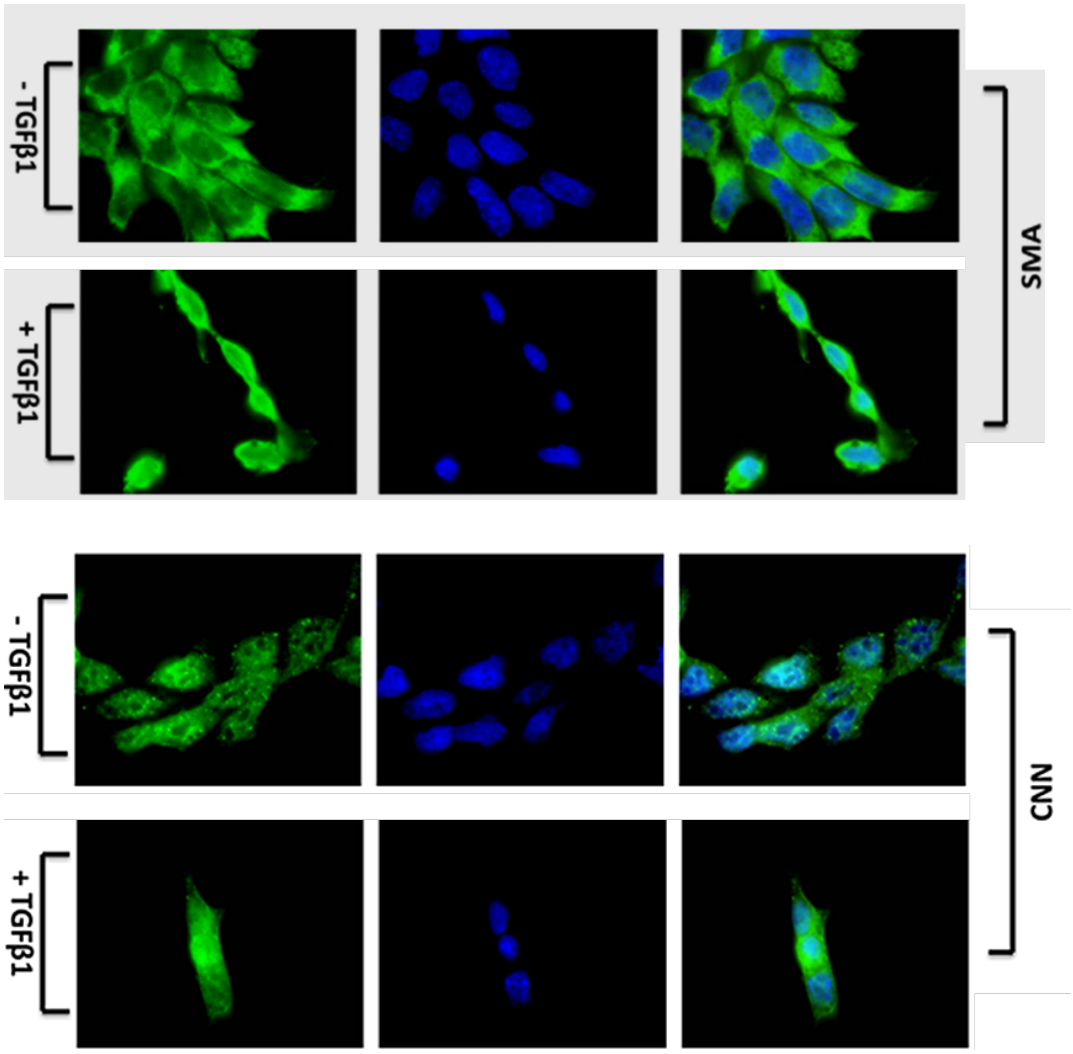


Figure 5.3 *FACS data for Sox10, Sox17 and S100β expression in NE4C cells. Cells were prepared as required for FACS analysis. Protein expression of the markers can be seen in red with secondary controls in black. The shift in peak indicated protein expression.*

5.6 Multipotent potential of NE4C cells

5.7 NE4C differentiation to SMC

MVSCs showed a good response to TGFβ1 activation for SMC differentiation. It was hypothesized that the NE4C cell line would respond in a similar manner to the stimuli. The NE4C cells were treated in the same manner as the MVSCs were, they were treated with an SMC induction media composed of 5% FBS, supplemented with 5ng/ml of TGFβ1 and 10ng/ml PGGFBB. The cells were tested for the presence of SMA, CNN1 and SM-MHCII proteins using immunocytochemistry *(Figure 5.4)*. The cells expressed levels of SMA and CNN1 before administration of the induction media. After the 72 hour treatment there was no noteable increase in SMA expression with no filaments visible. CNN1 expression seemed to increase slightly with regard to fluorescent intensity. The cells were SM-MHCII$^-$ in maintenance media and after treatment an increase in expression was observed but there was no strong visibility of filaments. Lysates for NE4C cells were prepared +/- TGFβ1 treatment. Samples were probed for CNN1 and SM-MHCII *(Figure 5.5)*. There was an increase in both expression markers after treatment.


SMA
- TGFβ1
+ TGFβ1
CNN
- TGFβ1
+ TGFβ1

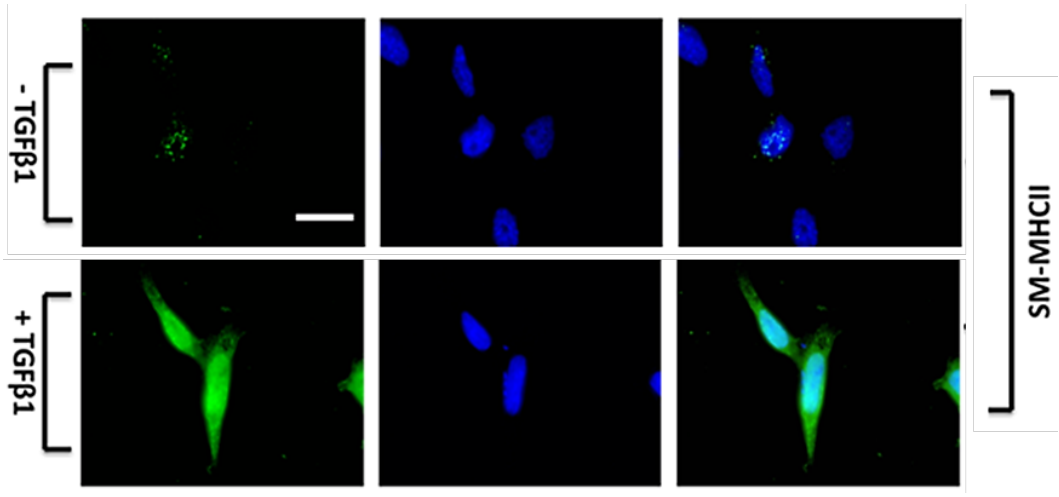


Figure 5.4 *Representative immunocytochemistry data for SMC markers SMA, CNN1, SM-MHCII on NE4C cells in maintenance media or after treatment with 5ng/ml of TGFβ1 and 10ng/ml PGGFBB for 5 days. The cells were then probed with the appropriate primary and secondary antibody. Scale bar indicates 50nm for all images.*

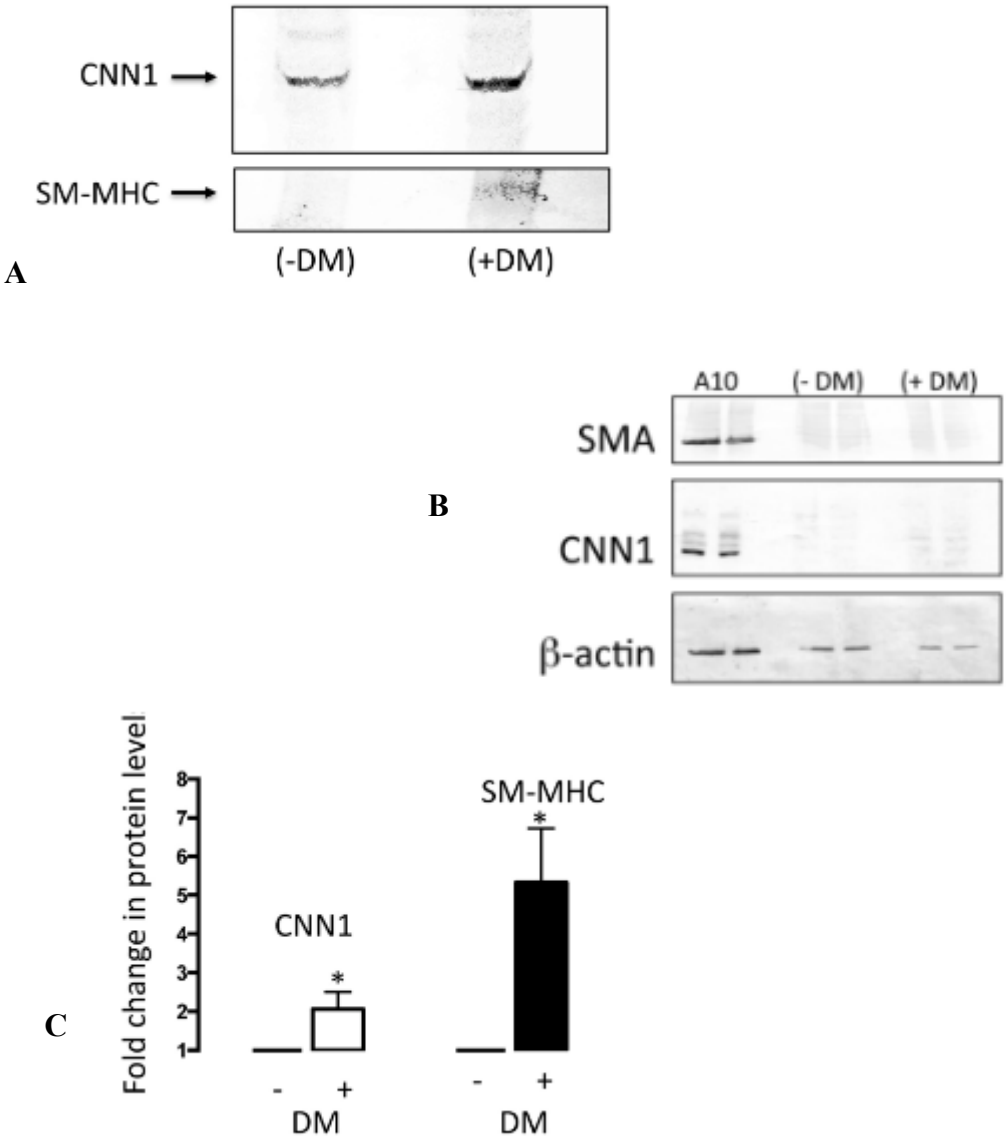


Figure 5.5 *CNN1 and SM-MHCII expression in lysates from NE4C cells. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis*.

5.7.1 Role of Smad2/3 in the induction of SMC differentiation from NE4C cells

The response of TGFβ treatment was not as great as expected and due to the fact that the cells seemed to express some levels of SMA and CNN1 prior to treatment. Therefore, another method of analysis was required. As previously described the TGFβ pathway is Smad2/3 dependent. Hence, before any further analysis was

undertaken it was investigated whether or not the NE4C cells have the TGFβ1 receptor

The activation of Smad2/3 with TGFβ1 would cause a marked increase expression in phosphoSmad2/3. The expression increase in phosphoSmad2/3 would indicate the presence of the TGFβ1 receptor on the cell surface. Western blot analysis was used to determine if the receptor was present. NE4Cs were treated as previously described (+/- TGFβ). Whole cell lysates were used for the detection. Smad3 was used as an internal loading control *(Figure 5.6A)*. PhosphoSmad2/3 was not detected in either maintenance or treated samples *(Figure 5.6B)*. This result indicates that the TGFβ1 pathway was activated (as suggested with ICC data) and is working independently of Smad2/3.

A

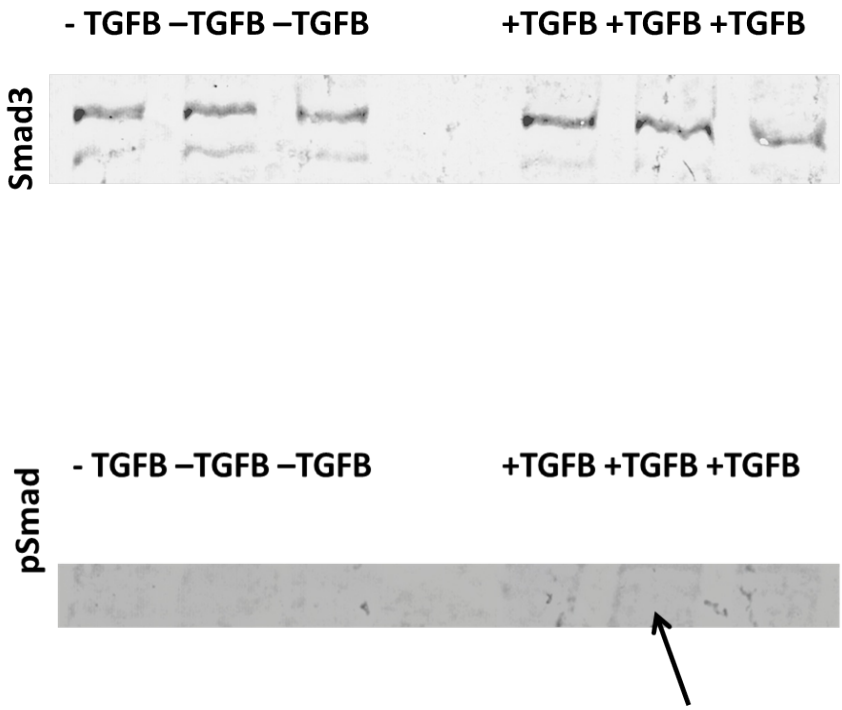


Figure 5.6 *Smad3 and phoshoSmad3 expression in cell lysates after treatment with TGFβ1. Cells were seeded at a density of 100,000 cells per well and left incubated for at least 48 hours before protein isolation was carried out. Samples were probed with appropriate primary and secondary antibodies and finally TMB for colorimetric analysis*.

5.8 Adipocyte and Osteocyte differentiation

In order for a cell type to be referred to as multipotent with respect to a mesencymal stem cell, it must be capable of differentiation to osteocytes, chondrocytes and adipocytes. For the purpose of these experiments two lineages were chosen to investigate the potential of the NE4C cells; Osteocyte and Adipocyte *(Figure 5.7, 5.8)*. Cells were treated in parallel experiments with appropriate induction media and left to undergo differentiation for the specific times required for each lineage. Rat MSCs were used as positive controls for both experiments. NE4Cs were capable of differentiating to an osteocyte.

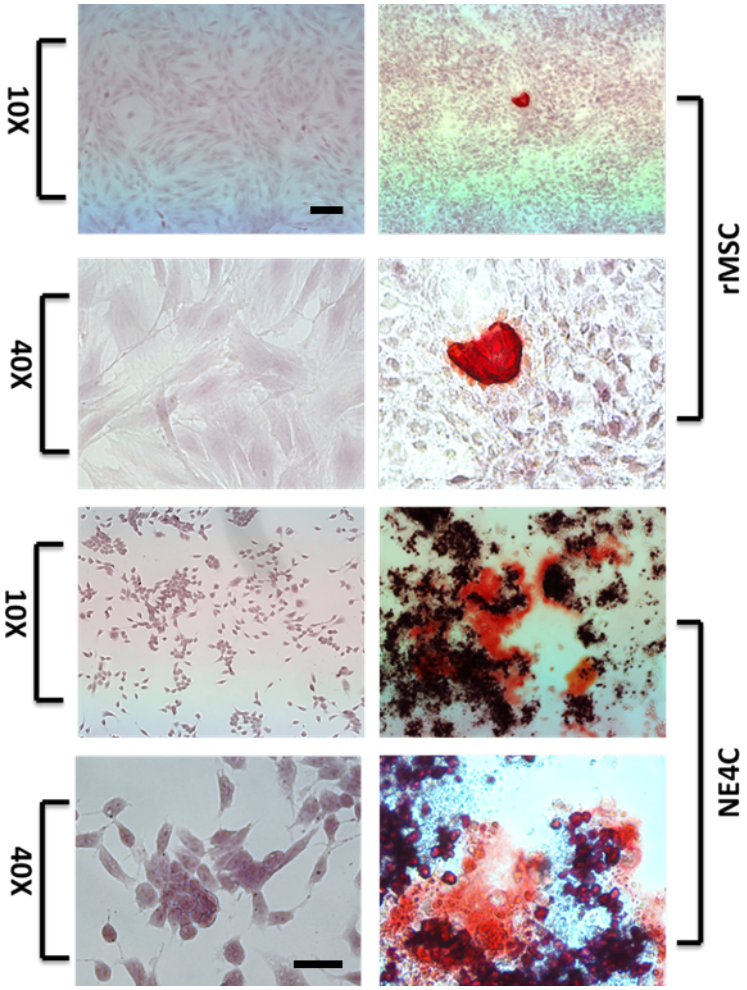


Figure 5.7 *NE4Cs differentiate into osteocytes, images were taken at phase contrast using low power (10X) and high power (40X) magnifications. Cells were seeded at low density (10,000 cells/well) and treated for 21 days with osteogenic induction media. The samples were then stained using Alizarin Red dye to visualise differentiated osteocytes. Scale bar indicates 50nm for all images.*

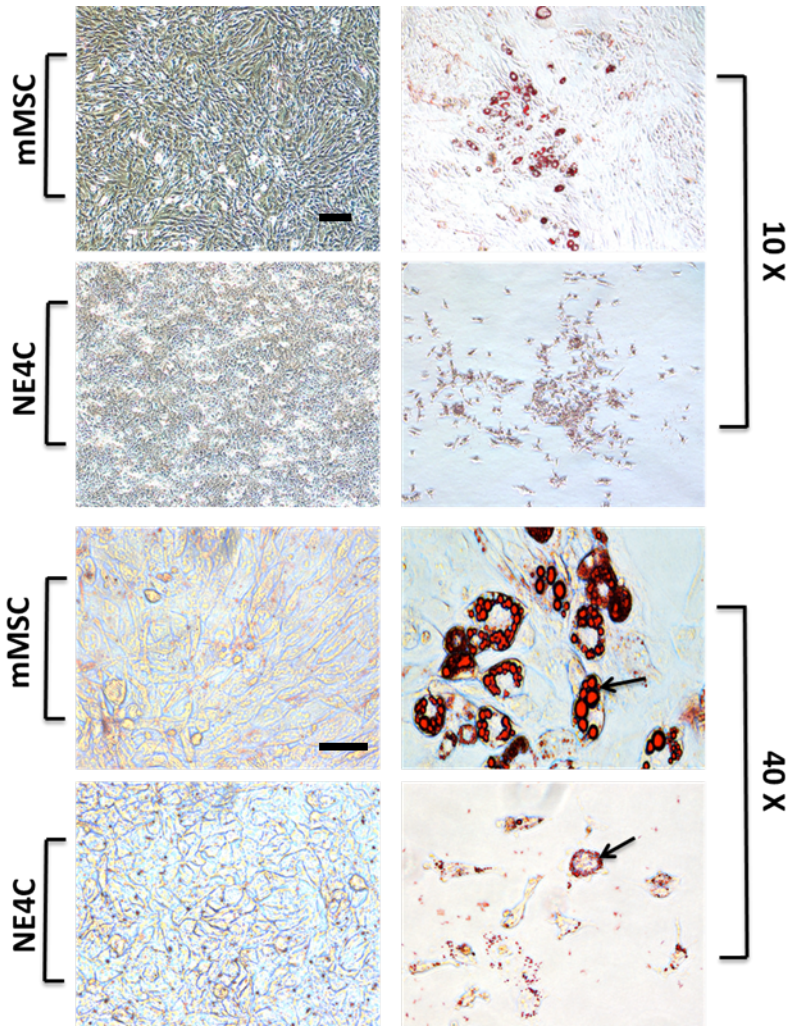


Figure 5.8 *NE4Cs differentiate into adipocytes, images were taken at phase contrast using low power (10X) and high power (40X) magnifications. Cells were seeded at low density (10,000 cells/well) and treated for 7-14 days with adipogenic induction media. The samples were then stained using Oil Red O dye fluorescent probe to visualise differentiated osteocytes. Scale bar indicates 50nm for all images.*

5.9 Summary

- NE4C cells express neural stem cell markers Pax6 and Nestin.
- The cells also express markers associated with the MVSC cell line: Sox10, Sox17, S100β
- The cells were capable of differentiation into both osteocytes and adipocytes, demonstrating that they have the multipotent properties that are associated with MSCs and MVSCs

- NE4C cells showed slight potential for differentiation into SMCs using TGFβ1, with a flourescent increase in SM-MHCII found after treatment.
- The TGFβ1 pathway, appears to be acting SMAD2/3 independent as there was no phosphoSMAD detected in TGFβ1 treated samples
- Further analysis is needed to confirm these results regarding TGFβ1

Chapter 6:

Discussion

6.0 Discussion

6.1 Multipotent Vascular Stem Cell Isolation and Characterisation

Cardiovascular disease is one of the world's biggest threats to mortality at the moment. There are many contributing factors that have caused the increase in death rates associated with this disease. Ireland in particular has a huge rate death associated with this disease with 38% of annual deaths connected to CVD. With new age diets introducing food with high fat content, the fast paced lifestyles that we lead and the huge focus on social side of life there is a real need to address the issue of CVD. There are many types of CVD including stroke, aortic disease, coronary heart disease and peripheral artery disease to name a few. The main focus of this project is on blockages in the main arteries associated with the heart, mainly put down to atherosclerosis. As previously discussed stent implantation is used to combat the blockage and to reduce its size allowing blood to pass freely. However, stent failure rate is high and other side effects can occur as a result of this (Nikolsky et al. 2003). The failure of these stents has been associated in part with the infiltration of cells into the lesion (Chaabane et al. 2013). It was originally hypothesised that the cells involved were smooth muscle cells that had dedifferentiated, but it is now thought that the cells involved are resident stem cells that differentiate into SMCs, bringing about the question, is cardiovascular disease is a stem cell disease?

The stem cell origin of vSMCs within vascular lesions has been very controversial in more recent years (Schober & Weber 2011); (Torsney & Xu 2011; Fukuda & Aikawa 2010b; Orlandi & Bennett 2010; Tang et al. 2012; Bochaton-Piallat et al. 1996). Initial studies were carried out in studies using canine carotid arteries by Holifield et al. (1996). In this study, proliferative SMCs were found in the neointimal and it is suggested that these modulated cells were not derived from differentiated SMCs but are sourced from a medial SMC that is myosin type 2 negative (SM-MHCII).

The original "de-differentiation" of SMC theory has been questioned and more recently this phenomenon has been attributed to stem cells (Nguyen et al 2013, Tang et al 2012, Klein et al 2011). Recent studies undertaken by Tang et al 2012 show data that conflicts with the original theory that smooth muscle cells are not terminally differentiated. Nguyen et al 2012 argue that strong evidence exists

showing that smooth muscle cell plasticity exists and that SMCs are capable of undergoing phenoptyic transition from a contractile state to a less differentiated synthetic state. They also focus on how this process is thought to underlie some vascular occlusive diseases. Nemenoff et al 2011 show evidence that differentiated SMCs can undergo phenotypic modulation and contribute to vascular remodelling using tamoxifen inducible SM MHC-Cre ^{T2}/Rosa26-floxStop/βGal mice, which directly contradicts the findings by Tang et al 2012 (Nguyen et al 2012). Tang et al have argued that it is likely that many of the historic experiments which have been carried out demonstrating SMC plasticity in primary cultures, were carried out cells that were assumed to be derived from SMCs. They highlight the fact that the possibility of a multipotent cell was never considered for these experiments and that previous research should be re-evaluated taking these resident vascular progenitor cells into considerations. Both groups highly emphasize the need for a strong lineage tracing mechanism in order to conclude the origin of the SMC involved with this type of vascular disease (Tang et al 2012, Nemenoff et al 2012).

Since primarty studies, many types of SMC-related progenitor cells have been identified within the tunica media. The cells identified are mainly mesenchymal-like cells displaying some of the multipotent properties of these cells such as adipocyte and/or osteocyte differentiation. Others have identified progenitor cells in the adventitial layer that express Sca-1^{+} and are capable of SMC differentiation (Sainz et al. 2006). These cells with Sca-1^{+} expression were also found to contribute to atherosclerosis of vein grafts in ApoE-deficient mice (Hu et al. 2004; Xu 2008). The three main types mentioned in the literature include resident vascular stem cells, circulating progenitors and bone marrow derived stem cells. (Tanaka et al. 2003) used a murine model with GFP tagged bone marrow cells. After wire injury, GFP tagged cells were found in the intima. (Werner 2002) also reach the same conclusion that vascular lesion damage can be initiated by bone marrow derived cells. In a conflicting study (Hu 2002) provide evidence that the SMCs involved in transplant atherosclerotic lesions are cells which have been derived from the recipient and not from the bone marrow. Another study carried out by (Simper 2002) identified a population of circulating smooth muscle progenitor cells in blood, the research suggests that there is a possibility that circulating progenitor cells were involved in vascular remodelling.

The cell population of interest here is the "resident" stem cell population. Tang et al 2012 have identified a population of MVSCs that are capable of differentiation *in vivo* and *in vitro* into SMCs. One of the aims of this study was to isolate an MVSC population from rat aortic medial tissue. Resident MVSCs were successfully isolated. Once isolated the objective was to fully characterise these cells and to examine them for stem cell like properties, differentiated smooth muscle cell markers and to evaluate their phenotype with respect to mesenchymal stem cell features. An exact profile of MVSC marker expression has not been elucidated, partly due to the differences in the mechanism of how the cells are obtained and subsequently cultured, slight differences in media composition and sub culturing techniques could contribute to this. The heterogeneity of the resident cells is also due to fact that cells can easily gain or lose marker expression *in vivo* and *in vitro*, for example SMCs are known for losing their myosin expression in culture. Stem cells are extremely sensitive and this can cause high variability from lab to lab.

MVSCs were generated using an explant method from the medial layer of aortas retrieved from Sprague Dawley rats. They were the first few cells to migrate off the explant tissue. The appearance of MVSCs under phase contrast differs to that of regular SMCs. MVSCs grow into a star like pattern when they are confluent in comparison with the spindle, elongated shape associated with the smooth muscle cell. Immediately it was clear that the MVSC population isolated was different to regular smooth muscle cells.

Upon isolation MVSCs were successfully characterised for three different subset panels of markers associated with neural/astrocyte cells (Sox10, Sox17, S100β), mesenchymal stem cells (CD29, CD44, CD146) and smooth muscle cells (SMA, CNN1, SM-MHCII). The three collections of markers were chosen in keeping with the theory that MVSCs differentiate to MSCs which in turn differentiate to SMCs, which infiltrate over time after administration of the stent within the aorta. Protein analysis using immunocytochemistry, FACS and western blotting showed that the MVSCs were positive for Sox10, Sox17 and S100β. These results are similar to what Tang et al. discovered in 2012. The cells were also positive for SMA and CNN1. Only SM-1 was detected in MVSCs lysates, no SM-MHCII was presented, this result coincides with studies undertaken by Tang et al. in 2012. FACS analysis showed fluorescence when the SM-MHCII antibody was used.

Immunocytochemistry showed that the cells were negative for the antigen as there were no filaments present which is the indicator of mature SMCs. The slight stain found was attributed to background staining. As FACS analyses a much greater population of cells, the fluorescence detected showing "expression" was also attributed to the non specific binding of the primary antibody. This was confirmed by western blot whereby the SM-MHCII antibody detected both myosin SM-MHCI and SM-MHCII isoforms. Tang et al. who isolated MVSCs in the same manner using different arteries describe their MVSC population to be $Sox10^{+}/SM\text{-}MHCII^{-}$. Studies shown here by this group and also by other members of the team in Rochester University have shown that the Sox10 expression *in vivo* is very low *(Figure 6.0)*, with only a small percentage of cells expressing the marker (10% in the case of Wang et. al). Collaborative researchers at the University of Rochester have shown using lysates from the medial layer to express low levels of Sox10 and high levels of SM-MHCII, which is in keeping with the theory that Sox10 levels *in vivo* are low (Kennedy et al. 2014).

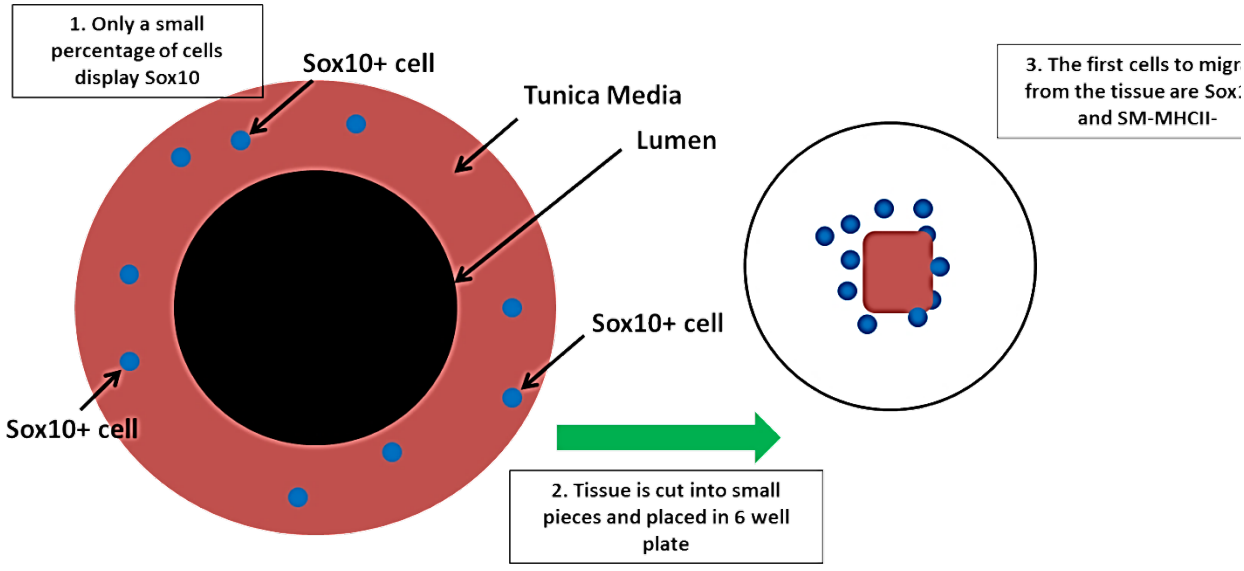


Figure 6.0 *Sox10 expression is low in the vessel and SM-MHCII expression is high. A small minority expressing $Sox10^{+}/SM\text{-}MHCII^{-}$ exists in the vessel wall. This was confirmed by in situ analysis (Wang et al 2012) and from cell lysates isolated by collaborators in Rochester.*

As previously described, a huge controversy exists between different scientist with regard to the origin of SMCs found in neointimal lesions, whether they are derived from resident progenitors, resident mature SMCs or both. However, there is no controversy surrounding the phenotype and characteristics of medial SMCs in normal vessels *in situ* before culture. Tang et al 2012 describe how they prepare SMCs from explant culture whereby SM-MHCII was tagged to green fluorescent protein (GFP). The resulting culture was a population on SMCs that were immunocytochemically GFP negative (therefore SM-MHCII negative) but expressed Sox10, Sox17 and S100β. However, the cells acquired SM-MHCII when sub-cultured *in vitro* or activated by Notch stimulation ligand or by induction using TGFβ1. In contrast medial cells *in vitro* prior to enzymatic dispersal are mainly GFP positive and the expression of Sox10, Sox17, S100β is minimal to none. The profile of SM-MHII^{+}/Sox10^{-} is maintained when the cells are enzymatically dispersed and probed in culture at P0. They also demonstrate how the injured vessel is populated mainly with Sox10^{+}/SM-MHCII^{-} cell type in comparison with a healthy vessel. This data indicates that the cells re-populating the injured vessel are from an MVSC origin (Figure 6.1) (Tang et al. 2013).

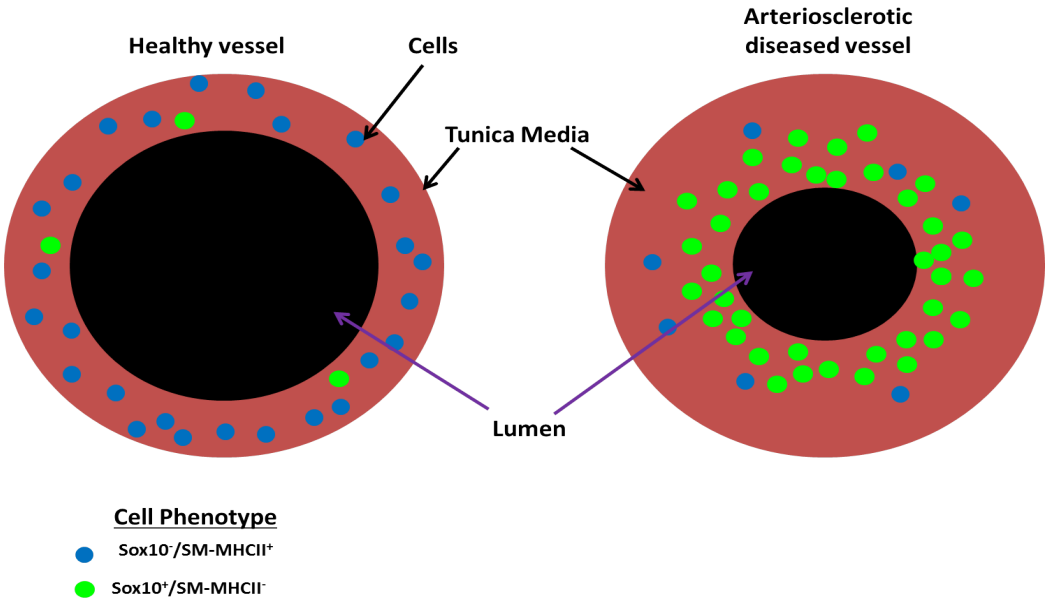


Figure 6.1 *Majority of the cell phenotype changes after vessel injury/diseased state from a Sox10^{-}/SM-MHCII^{+} to Sox10^{+}/SM-MHCII^{-}*

For the purpose of this research, the phenotype of the majority cells in the tunica media (without stem-like properties) of the aorta was investigated. The medial layer of the aorta was enzymatically dispersed in an enzyme cocktail. These cells were seeded at a low passage upon isolation and analysed immunocytochemically for Sox10 and SM-MHCII. It was found that these cells all expressed SM-MHCII, with clear visibility of myosin filaments. There was very little Sox10 expression, some Sox10 was found to be expressed in the cytoplasm but none in the nucleus. The research undertaken by (Rehberg et al. 2002) suggests that Sox10 is an active nucleocytoplasmic shuttle protein, these findings can explain the cellular localisation of the staining in the immunocytochemical analysis. In a parallel experiment MVSCs were seeded at low passage after isolation and as before the cells all displayed Sox10 positivity with no expression of SMC marker SM-MHCII. These studies are similar to what Tang et. al 2012 discovered.

An aim of this study was to define MVSC transition to SMC following treatment with media containing serum and in a parallel experiment to examine their transition following treatment with TGFβ1 and PDGF-BB. MVSCs isolated here were similar to that of Tang et al 2012 whereby when the cells were grown in maintenance media they were SM-MHCII$^-$ but were positive for SM-MHCI, Sox10, Sox17 and S100β. However as the cells were cultured in 10% FBS the results were different. It was found that the expression of Sox10 persisted after culture in 10% FBS for 21 days. Although the expression of SMC differentiation marker SM-MHCII in particular was enhanced when the MVSCs were grown in non-maintenance media (ie differentiation media) an even greater increase in expression was found following stimulation with a differentiation media supplemented with TGFβ1 and PDGF-BB with clear visibility of filaments. MSCs have been shown to differentiate to SMCs with TGFβ1 stimulation, mediated by notch signalling (Kurpinski et al. 2010). TGFβ1 expression has been shown to be elevated in human vascular restenosis lesions (Nikol et al. 1992). The study shows that TGFβ1 plays an important role in the remodelling of the vasculature after injury, it is possible that one of these roles includes stimulating MVSCs to migrate from the medial layer to the intima disguised as newly differentiated SMCs with their newly acquired myosin expression. Furthermore, TGFβ1 inhibition has also been found to prevent injury induced lumen reduction instead, promoting vessel enlargement (Ryan et al. n.d.). In

contrast to Tang et al 2012, MVSCs isolated in this study were Sca-1^{+}, this suggests that Sca-1^{+} may persist in cells other than the adventitial. Tang et al screened their MVSC population in differentiation media, whereas this population were Sca-$^{1+}$ in maintenance media. When the MVSCs form this study were grown in differentiation media and screened they too were negative which coincides with Tang et al 2012. In a similar manner NE4C in neural stem cell media are Sca-1^{+} but when grown in differentiation media they are Sca-1^{-}. There may well be a relationship between progenitors in the media and in the adventitia but the depth of this relationship is unknown. The presence of Sca1 in the MVSCs suggests an adventitial origin for these cells. There could well be a cross over at the adventitial/medial boundary whereby the cells display a similar phenotype. The presence of Sca1 could also indicate that some of the adventitial cells remained on the medial layer within this boundary after the removal of the adventitial layer.

It was important to analyse mesenchymal markers to see if the MVSC population represented any of the intermediate mesenchymal populations. Since MVSCs expressed markers associated with smooth muscle cells it was highly likely that they could express some MSC markers. Similarly a rat MSC cell line was screened for the presence of stem cell markers associated with the MVSC to investigate if properties associated with these markers are retained. It was found that MVSCs expressed CD29, CD44 but not CD146. In comparison MSCs co-expressed Sox10, Sox17, S100β, CD29, CD44 and CD146. MSCs showed slight expression of SM-MHCII and still retained SMA and CNN1 markers as the move closer to the SMC phenotype and become less multipotent (Liu et al. 2013). There was some expression cross over between the two cell lines, indicated in *Figure 6.2*. Klein et al 2011 isolated a population of resident vascular stem cells that expressed CD44 with the ability to differentiate into SMCs and also contribute to new vessel maturation.

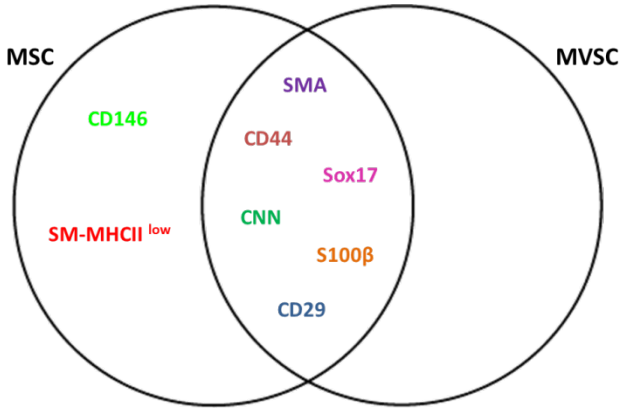


Figure 6.2 *Overlap of expression markers between MSCs and MVSCs*

MSC- like cells described by (Tintut et al. 2003) express CD29 and CD44 similar to what was found in the MVSC population isolated in this study. Tintut et al found that the population they isolated from the artery wall was capable of differentiation into chondrocytes and adipocytes but not osteocytes. In contrast when MVSCs were stimulated with appropriate induction media they had the potential to differentiate into osteocytes and similarly, MSCs in culture up to passage 28 retained the potential to differentiate into both adipocytes and osteocytes. In another study (Crisan et al. 2008), identified a population of perivascular MSC-like cells that were $CD146^{+}$ and still retained the potential to differentiate to osteocytes, chondrocytes and adipocytes.

These cells are thought to become 'modulated' regarding their SMC phenotype, with a decrease in SMC differentiation markers as the cells are passaged over time. In particular SM-MHCII is down regulated in comparison with freshly isolated P0 cultures or cells analysed *in situ*. It is difficult to pin point the exact reason as to why the loss of SM-MHCII occurs if it is not attributed to the fact the cells are stem cells and never expressed myosin to begin with. However, as discussed before it may be due to the de-differentiation of SMCs to a synthetic phenotype, but SMCs are also known for losing SMC differentiation markers over time in culture. The loss could also be due to an environmental factor if the cells are not getting the cytokines *in vitro* that they would be stimulated by *in vivo*. Studies from the 1970's and 1980's focus particularly on the loss of SM-MHCII in culture (American et al. 1979). Other forms of myosin exist and they are not fully

understood, this makes it hard to distinguish exactly what myosin is being expressed by cells and this can cause difficulties for interpretation of results, and conflicting results (Rovner et al. 1986). Antibody variation and specificity from lab to lab can also result in conflicting data.

6.2 Evaluation of Stem Cell markers in Embryonic and Adult SMC lines

The way in which vSMCs behave in culture has long been a subject for investigation (Sainz et al. 2006). In culture, SMCs isolated from different parts of vessels display heterogeneity with respect to what markers they express (Orlandi et al. 1994). The idea that these SMCs in culture have arisen from stem cells opens up questions about the phenotype and origin of the SMCs cells *in vivo* that are involved in neointimal infiltration (Tang et al. 2013; Orlandi & Bennett 2010); Stoneman 2011). Originally these cells were thought to be modulated "de-differentiated" SMCs that had reverted back to a synthetic state, but more recently the idea that these cells *in vivo* and *in vitro* originate from stem cells is the subject of debate. For the purpose of this study A10, A7r5 and smooth muscle cell lines derived from rat, murine, bovine were analysed for SMC and MVSC differentiation makers, growth properties and the multipotency capabilities of the cell lines were assessed.

Both embryonic cell lines A10 and A7r5 and adult aortic SMCs have been shown by researchers to express a panel of well recognised SMC differentiation markers including SMA, CNN1, SM22 and tropoelastin. Although SM-MHCII is the most specific marker to SMCs the expression of this late stage markers is less common in these cultures (Manabe & Owens 2001; Firulli et al. 1998; Bochaton-Piallat et al. 1992). Bochaton-Piallat ML et al 1996 induced intimal thickening (IT) by endothelial injury and subsequently cultured two cell populations from the intimal layer. Two distinct populations were noted, with epithelioid cells dominating the IT and spindle shaped cells more frequent in the media. These epithelioid "pup" cells resembled the phenotype of A10 cells and to some extent A7r5. A10 and A7r5 cells were also similar to new born rat SMCs as they continued to express SMA and SM-MHCII when cultured over time (Bochaton-Piallat et al. 1992). Both A10 and A7r5 cell lines expressed SMC markers SMA and CNN1, the two cell lines had similar growth patterns regarding these two markers (D. C. Graves & Yablonka-Reuveni

2000). When both cell lines were serum deprived the expression intensity profile of SMA and CNN1 increased with noteable enhanced filaments. This increase in contractile protein expression has been noted in other studies (Li et al. 1999). In comparison the expression of SM-MHCII in A10 and A7r5 cells not uniform and the pattern of the expression marker differed (Firulli et al. 1998). A10 cells expressed mainly Sm2 with some level of myofilaments visible under serum and serum deprived conditions. In contrast to A10, A7r5 cells expressed little or no filaments, the staining was attributed to non-muscle SM-MHCII. These cells do however still share several characteristics with neointimal cells including actin and non-muscle SM-MHCII expression (Rao et al. 1997).

As previously discussed the origin of cells present in neointimal cells is still unknown and research continues in this area. Since A10 and A7r5 cell lines represent embryonic SMCs this prompted the investigation of the origin of these cells and their expression of markers associated with MVSCs. The expression of these markers in adult SMC lines was of great interest to see if these cells retained any of the makers associated with MVSCs while co-expressing relevant SMC markers.

Lineage tracing has been used to try resolve whether adult rat SMCs are derived from differentiated mature SMCs from the medial layer (Nemenoff et al. 2011) or from a stem cell population (MVSCs) (Tang et al 2012). Nemenoff et al. 2011 labelled bone marrow cells and showed that they were not involved with neointimal formation but that the cells accumulating in the lesion were resident SMCs. On the other hand, Tang et al showed that resident multipotent vascular stem cells differentiated to SMCs and infiltrated the neointima and that these particular cells were responsible for vascular remodelling – not resident SMCs that had dedifferentiated. SM-MHCII expression plays a large role in understanding what cells are involved in restenosis. Is it the resident MVSCs that are originally myosin negative that infiltrate or is it that a smooth muscle cell can become synthetic and lose its myosin expression? Epigenetic studies have been under taken to track the histone modifications of the MHY11 locus *in vivo* and *in vitro*. These studies back up the research of other groups that suggest SMCs in culture and SMCs involved in vascular remodelling are derived from mature vessel resident SMCs (Gomez et al. 2013) (Gomez & Owens, 2012). When studying loss or gain of marker expression or

change in cell phenotype, epigenetic studies are very useful but in the case of these embryonic cell lines they express both panels of MVSC and SMC markers.

Recent studies from (Ciceri et al. 2012; Liao et al. 2013) have suggested that rSMCs *in vitro* under specific conditions can differentiate to osteogenic and skeletal muscle lineages. This supports the theory that SMCs in culture retain stemness and demonstrate plasticity by acquiring neural cell markers. Similarly somatic cells have been shown to respond to stimulus-triggered acquisition of pluripotency (Obokata, Wakayama, et al. 2014; Obokata, Sasai, et al. 2014).

It is possible that SMC cells in culture (eg A10, A7r5) that express multipotent markers are in fact de-differentiated SMCs that have been derived from differentiated smooth muscle cells. If this were the case it would mean these cells have the capability to revert back to a multipotent/pluripotent state when subjected to certain factors and begin to express stem cell like markers such as the neural crest markers observed in A10, A7r5 and adult SMC cell lines. This study demonstrates that both A10 and A7r5 cells co-express MVSC markers [Sox10, Sox17, S100β] (Tang et al. 2012), SMC markers [SMA, CNN1, SM-MHCII] and also express stem cell antigen 1 which is associated with multipotent progenitor cells (Holmes & Stanford 2007) and resident adventitial progenitor cells (Wu et al. 2014). A10 and A7r5 cell lines still retain some of the differentiation capabilities associated with multipotent cells. With appropriate induction the cells differentiated to adipocytes in the same manner as MSCs and MVSCs did. Although there was a smaller percentage of differentiation in A10 and A7r5 cells in comparison with MSCs/MVSCs, the adipocyte formation was still evident. An experiment was carried out by a member of the lab group using DAPT. DAPT, which inhibits Notch signalling and is fundamental for MSC to SMC differentiation (Doi et al. 2009). The result achieved suggests that DAPT significantly altered both MVSC and SMC differentiation markers in A10 and A7r5 cells promoting neural stem cell phenotypes while increasing differentiation. Notch has previously been shown to promote de-differentiation of SMCs (Morrow et al 2005).

The reasoning behind why MVSC neural stem cell markers are expressed in SMC lines in culture is unknown. It is possible that SMC lines in culture originate from MVSCs and that after time in culture SMCs differentiate from these and out-

grow the de-differentiated SMCs and over time they dominate the culture (Tang et al 2012). Proliferation assays were carried out on MVSCs, A10 and A7r5 cell lines to compare their growth rates. The results suggest that MVSCs take longer to recover initially after being seeded. They lag behind the two embryonic cell lines in the early phases of growth but by day 12 the MVSCs have recovered and the growth rate of the three cell lines is comparable.

A10 and A7r5 cells express Sca-1^+ suggesting that these cells also display some perivascular markers. Vascular Sca-1^+ is mainly attributed to adventitial progenitor cells but has also been identified in a side population of medial progenitor cells that are capable of differentiation down the SMC lineage (Hu et al. 2004; Sainz et al. 2006; Xu 2008). The presence of c-kit and Flt-1 positive cells within A10 and A7r5 cells is associated with SMCs that are derived from large arteries of older vessels and may suggest a stem cell origin (Ferlosio et al. 2012). Interestingly, Hibbert et al. 2004 found that vascular progenitor cells positive for c-kit populated human coronary in-stent restenosis but cells found in patients with primary atherosclerotic lesions were void of c-kit.

The discovery that resident vascular stem cells exist in the vessel wall has raised questions regarding the origin of smooth muscle cells in culture. It is believed that these cells could be derived from MVSCs and still share some of the multipotent properties associated with these stem cells. With regard to this three different smooth muscle cell lines were screened for neural stem cell markers Sox10, Sox17 and astrocyte marker S100β and also for smooth muscle differentiation markers SMA, CNN1 and SM-MHCII. All three cell lines expressed the panel of stem cell associated markers while still expressing SMC differentiation markers. However, the cells had lost their multipotent capability to be induced to other cell types (such as osteocytes). It is still possible that these cells were derived from resident vascular stem cells.

The commercial cell lines that were used for this work were obtained using two different culture methods, either by enzymatic dispersal (rat and murine) or by explant (bovine). The interesting discovery here is that although the culture methods are different all three cell lines expressed the stem cell markers associated with MVSCs in late passage. The murine cell line used was an immortalized one yet still

mimicked the phenotypic profile of MVSCs. In a similar study Tang et al 2012 suggested that the MVSC population they isolated was initially SM-MHCII$^-$/Sox10$^+$ and became SM-MHCII$^+$/Sox10$^-$ after up to 8 weeks in culture. Similarly, Nessy et al. 2010 describe how their NCSC derived MSC cell population lose Sox10 expression after treatment with TGFβ1. In contrast, this study exhibits three different smooth muscle cell populations in culture that clearly show retention of stem cell markers Sox10 with co-expression of three differentiation markers associated with SMCs. The co-expression of Sox10 and SM-MHCII could indicate a proliferative transition between MVSCs and SMCs that display both markers (Tang et al. 2012). It is possible there is a period of time where the cells can express both markers, as they become more smooth muscle cell like, they may still retain stem cell markers but lose the ability to carry out functional stem cell like multipotent activity, such as osteogenesis.

Nestin is associated with muscle and progenitor cells (Huang et al. 2009). Nestin generally is not expressed in healthy vessels but upon injury the marker can reappear (Vaittinen et al. 2001). Huang et al. 2009 demonstrate how Nestin can be expressed by SMCs when grown in serum of stimulated with Thrombin, some Nestin positive cells were found in rSMC cultures but the percentage was low.

The origin of these cells displaying both SMC differentiation markers (SM-MHCII) and stem cell associated markers (Sox10) remains an issue. The ability of the cells to co-express these two panels of markers is clear but the reasoning behind this has not yet been identified. As described previously Gomez at el. 2013 have identified a strategy where they confirmed the origin of early passage cultured SMCs. They used single cell analysis and investigated the epigenetic signature for the Sm-2 locus. However, these SM cell lines show expression of both MVSC and SMC markers in late passages. There is a need for the development of an epigenetic signature that can identify the origin of MVSCs, until this has been developed the origin of cultured SMCs cannot be validated. From these results the exact origin of the SMCs in culture cannot be identified and it is possible that they arise from differentiated SMCs, MVSCs or a combination of both.

The plasticity of smooth muscle cells is an on-going debate in research. It is unclear whether SMCs are terminally differentiated or if they can trans differentiate

into other cell types. Gomez et al 2013 show how SMCs in atherosclerotic lesions have down regulated expression of differentiation markers associated with the contractile SMC phenotype. It has also been shown that SMCs have the ability to differentiate to osteocyte and skeletal lineages (D. C. Graves & Yablonka-Reuveni 2000)(Ciceri et al. 2012; Speer et al. 2009). Bobryshev 2005 showed that SMCs in atherosclerotic plaques displayed features of trans differentiation into chondrocytes. It was found that cells with decreased SMA expression displayed a concomitant expression of Sox9. Similar studies were observed in ApoE deficient mice.

A study that further supports the theory of smooth muscle cell trans differentiation is that mSMCs are capable of differentiation into macrophage like cells after treatment with cholesterol (Rong et al. 2003). When human SMCs are treated with a specific combination of sex steroids they were also capable of trans differentiation into neural stem cells with characteristics typical to that of the MVSC phenotype (Bukovsky 2009). Human Aortic SMCs have been shown to be capable of differentiation into iPS cells using a lentiviral transduction method that contained refined transcription factors such as Sox2, Oct4, Nanog and other factors associated with stem cells (Lee et al. 2010). This group then showed that these cells were capable of differentiation to revert back to the SMC phenotype using a gelatin and a commercially available smooth muscle cell specific medium.

Other *in vivo* factors can influence SMC marker expression behaviour. Often it is very difficult to mimic these in culture even with the great advances in cell culture reagents available. For example, (Brown et al. 2005) showed that "dedifferentiated" SMCs behave differently when they are co cultured with endothelial cells. EC co culture promotes an up regulation in contractile SMC markers, other properties such as changes in cell morphology was also noted. It is possible that some of the unknowns or obscure results associated with SMC in culture are just down to the fact that the cells are not in their natural environment and are not receiving the factors they require to behave as they would *in vivo*.

The trans differentiation capabilities of SMCs are clearly demonstrated by the above examples. It is highly possible that SMCs are capable of reverting back to their stem cell like state but due to the expression of stem cell like markers in SMCs one cannot rule out the possibility that these cells have been derived from a small

population of MVSCs that were present at the initial time of culture and that over time these cells begin to dominate in culture. Further screening of different smooth muscle cell lines derived from different origins would provide more insight into the marker expression of these cell lines. It is increasingly important to identify the mechanisms behind SMC plasticity to create research models that can mimic what processes are occur *in vitro* which would lead to the subsequent development and improvement of novel or specialised drugs that can target the specific cell type.

Because SMCs express a neural stem cell like phenotype in serum it was suspected that the expression of MVSC and SMC markers could change upon exposure to induction media with certain external stimulants including Notch or TGFβ1. This was carried out by another member of the research group and it was found that TGFβ1 stimulation and DAPT treatment failed to have any significant impact on the expression ratio of SM-MHCII and Sox10 in the cells, both remaining positive after induction (Kennedy et al. 2014). TGFβ1 is used to promote the differentiation of different types of stem cells into SMCs including embryonic stem cells, mesenchymal stem cells and iPS cells (Guo et al. 2013; Kurpinski et al. 2010; Bajpai et al. 2012b). It has also been reported to suppress Sox10 expression in neural crest progenitor cells (John et al. 2011). Contrarily to this, DAPT inhibits Notch signalling pathway which is a vital mechanism for mesenchymal to smooth muscle differentiation (Kurpinski et al. 2010; Tang et al. 2012; Kane et al. 2011). Sox 17 which is another stem cell like marker expressed by the MVSC population has been shown to act upstream of the Notch pathway and downstream of the canonical Wnt system and is involved in orchestrating arterial specification, therefor may be critical for MVSC transition to MSC in vitro (Corada et al. 2013). Interestingly Wang et al 2012 found that the Sox17 expression in their MVSC population disappeared after 3 weeks in culture with 10% FBS, whereas Sox17 expression persisted in the MVSC population isolated here, after 3 weeks incubation in regular differentiation media.

A10 and A7r5 embryonic cell lines retained the multipotent potential to differentiate into adipocytes and osteocytes. However, it was found that the SMCs did not retain the differentiation potential associated with MSC or MVSC cell lines in that they could not differentiate to osteocytes or adipocytes after induction with specific media.

6.3 NE4C as a model for Multipotent Vascular Stem Cells

As previously described, NE4C cells are a neuroectodermal stem cell line isolated from the fore and midbrain vesicles of 9 day old murine embryos lacking the functional p53 gene. This cell line would serve as a good model for MVSC cells. Both cell lines are of neuroectodermal origin and display stem like characteristics including the expression of Sox10, sox17 and S100β as well as trilineage differentiation capabilities. Preliminary studies were undertaken on this cell line to identify certain characteristics. The cells were screened for markers associated with the neural crest and they were found to be Nestin and Pax6 positive. In contrast to this MVSCs in maintenance media displayed a $Nestin^{+}/Pax6^{-}$ phenotype (Tang et al. 2012). Despite these differences in marker expression it was thought that these cells could be used routinely for experiments investigating SMC differentiation as well as using the MVSC cell line for confirmation of results.

One of the objectives of this work was to determine whether the NE4C cell line mimicked the multipotent properties and phenotypic characteristics of MVSCs. The NE4C cells were examined for the expression of MVSC markers Sox10, Sox17 and S100β. The cell line was positive for all three markers, this gives a further indication of the similarities between these cells and MVSCs. The cells must be deemed multipotent in order to suffice as a model for MVSCs. Like MSC and MVSCs the NE4C cells were capable of differentiation into osteocytes and adipocytes after treatment with appropriate induction media. (Billon et al. 2007) have also shown that quail neural crest cells are capable of adipocyte differentiation. Similarly, *in vitro* studies have shown that Sonic hedgehog can promote the differentiation of neural crest cells into many different cell types including osteocytes (Achilleos & Trainor 2012).

Studies have indicated that neural crest stem cells have the capability to differentiate into SMCs under the correct conditions. The differentiation of both NCSCs and SMCs is associated with activation of the TGFβ1 pathways. In a model using Monc-1 cells Chen & Lechleider 2004 showed that TGFβ1 could be used to indcue SMC marker expression and characteristics within this pluripotent neural crest stem cell lines. This research also suggests the involvment of SMAD2/3

activation for the induction of the SMC lineage. An earlier study on the same cell line undertaken by Shah et al. 1996 also showed that TGFβ1 is an inducer of the SMC lineage. More recent studies have suggested that TGFβ1 can have different effects on neural crest cells that arise at different axial levels. For example trunk neural crest cells assume a SMC fate when they are induced with TGFβ1, in comparison, in cranial neural crest cells this fate is suppressed by TGFβ1 induction. Most of the studies involving NCSCs have been carried out in culture and not animal models, therefore the results cannot be completely validated as there are multiple different environmental factors that separate *in vitro* from *in vivo* studies. However, there is still evidence suggesting a link between NCSC differentation to SMCs. DiGeorge Syndrome is the most common micro deletions syndrome in adults, Wurdak et al. 2005 found that inactivation of TGFβ1 leads to multiple defects that are reminiscent of this disease. In a more recent study, Hirota et al. 2014 showed that serotonin induced the differentiation of a stem cell line to Smooth Muscle – rat bone marrow stromal cells, interestingly they found that TGFβ1 did not mediate SMC differentiation.

One of the aims of this study was to identify the NE4C transition to SMC lineage following treatment of TGFβ1 and PDGF-BB (exact same as MVSC treatement). SMC marker expression was analysed in the same manner as MVSCs were. Results indicated that the cells already expressed a basal level of SMA and CNN1 before stimulation with differentiation media. There was a slight increase in CNN1 after induction. SM-MHCII was not detected pre stimulation, after the treatment there was a clear indication of the presence of myosin. However, there were no visible filaments noticed as with the MVSCs. A western blot analysis was also carried out and an increase was observed in both CNN1 and SM-MHCII after treatment, but not as significant as predicted. Because the response was not as robust as expected, it was investigated if the cells display the receptor for TGFβ1. This was done by checking if Smad2/3 was being activated. No phosphoSmad was detected in samples that had been treated with TGFβ1. The original data suggests that TGFβ1 differentiated NE4C cells to SMCs with myosin expression presented, but the fact that the Smad2/3 pathway was not activated could mean that in this case TGFβ1 works in dependently of the Smad2/3 pathway. The data presented provides a case

that NE4C cells may be capable of differentiation into SMCs but further experimental analysis is required before this can be concluded.

Smad pathways in general are known as the central mediators of signals form receptors for TGFβ1. Smad4 is the central mediator of TGFβ1/BMP signalling, a study undertaken by Ko et al. 2007 suggested that Smad4 is not required for the migration of cranial neural crest cells but it is required for the development of the cardiac outflow tract. There is evidence that supports the theory that an alternative non-Smad pathway can participate in TGFβ1 signalling. Yue & Mulder 2000, review this and the study predates the discovery of Smads. Cell differentiation, cell proliferation, apoptosis and cell migration that are induced by TGF-β1 frequently uses abundant MAPK modules and alternatively non-Smad effectors, as described by Moustakas & Heldin 2005. Embryonic fibroblasts that were isolated from Smad4 knockout mice still retained the capability of a number of cellular genes and responses that are associated with TGFβ (Sirard et al. 2000). An interesting study from Hagedorn et al. 2000 suggests that minor concentration gradient differences can have different effects on neural progenitor cell differentiation, it was found that lower doses of TGFβ promoted neurogenesis and a slight increase in the dose predominaltly induced apoptosis. Efficient NE4C differentiation to SMC could well be a case of optimising the concentration of TGFβ1.

7.0 Conclusion

In conclusion, these results suggest that multipotent resident vascular stem cells exist in the medial layer of the aorta, based on a rat model. The cells were deemed multipotent due to their expression of MVSC associated markers Sox10, Sox17 and S100β (Tang et al 2012) and by their potential to differentiate into SMCS, adipocytes and chondrocytes, similar to the way in which mesenchymal stem cells can produce these cell types. The cells were SM-MHCII negative upon isolation and did not begin to express this late stage marker until they were grown in differentiation media containing 10% FBS in DMEM or else media containing TGFβ1 and PDGFBB. The cells expressed SMA and CNN1 in maintenance media, a slight increase in CNN1 protein expression was observed after TGFβ1 treatment.

Currently, the origin of cells that are involved with vascular remodelling is highly debated (Majesky et al. 2011; van Oostrom et al. 2009; Cheung et al. 2012). It has been suggested that smooth muscle cells are not terminally differentiated and that they can de-differentiate and contribute to lesion formation (Gomez et al 2012). The SM-MHCII negative population has been associated with this de-differentiated cell phenotype for many years. More recent research has suggested that cells may be derived from resident stem progenitors that reside in the vasculature and that these cells contribute to the remodelling and lesion formation in vascular disease. Some of these populations identified are progenitor cells with similar characteristics to what was found here (Tang et al. 2012; Klein et al. 2011; Hu et al. 2004).

Another aim of this study was to investigate the stem cell properties of embryonic A10 and A7r5 cell lines and adult SMCs from three sources; bovine, rat and murine. The expression of neural crest and glial cell markers in A10 and A7r5 cell lines suggests that these embryonic cell lines may be smooth muscle cells that are derived from an MVSC progenitor, a de-differentiated SMC or a combination of both. A10 and A7r5 cells express $Sca1^{+}$ which suggests that these cells could also originate from the adventitial mesoderm. The re-evaluation of these cells lines with regard to MVSC markers means that these cells could be useful in determining the role of SMCs in disease. The cells present a phenotype that represents a neonatal/neo-intimal SMC population known to be derived from MVSC following vascular injury (Tang et al 2012). The results suggest that adult smooth muscle cell lines in culture are derived from an MVSC source by expression of neural stem cell markers. Primary dispersed smooth muscle cells in culture express low levels of MVSC markers in comparison with primary explanted MVSCs. In situ protein analysis has shown that very little MVSC marker expression exists (Kennedy et al 2014). It is clear that the SMC lines acquire MVSC markers by de-differentiation or they may be outgrown by a small population of MVSC present in initial cultures. Further studies such as lineage tracing mechanisms would provide more insight into the developmental origin of these cell types.

The origin of smooth muscle cells in culture is also challenged by the data. When SMCs in the vessel are analysed they are highly positive for SM-MHCII and express relatively no Sox10. This data is consistent with Tang et al 2012 using in situ analysis of tissue sections. A small population of these cells displayed a $Sox10^{+}$/SM-

MHCII$^-$ phenotype. When this tissue was cultured and cells explanted away from the tissue the cells first off the explants were of the latter phenotype. When regular adult smooth muscle cells and embryonic smooth muscle cells were analysed in culture they displayed a Sox10$^+$/SM-MHCII$^+$ phenotype, sometimes showing a low response for SM-MHCII. This indicates that the cells in culture do not represent the same smooth muscle cell phenotype that is present in vivo. It is possible that when the smooth muscle cells initially were isolated that the minority MVSC population in the culture eventually outgrew the other SMCs. This would reason as to why we see the Sox10 expression in our SMC lines. Another possibility is that the vascular SMC is a stem cell and not fully differentiated, capable of de-differentiation into a progenitor cell. In vivo, it is possible that these Sox10$^-$/SM-MHCII$^+$ cells of the vessel wall are capable of differentiating into the Sox10$^+$/SM-MHCII$^-$ cells that infiltrate into the lesion. The cells present in the lesion of injured vessels (Tang et al 2012) are Sox10 positive. Smooth muscle cells in culture here present the Sox10 antigen and therefore represent the diseased tissue in vivo.

Finally, neural crest stem cell line NE4C showed some potential to differentiate into SMCs via TGFβ treatment. Similarly to MVSCs, the cells had the ability to differentiate into osteocytes and adipocytes. The results suggest that the NE4C differentiation to SMC via TGFβ pathway works independently Smad3. Further experiments are required to fully conclude data for the NE4C cell line.

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Appendices

A. Antibodies

Antibody	Company	Cat #
CNN (FACS)	Bioss USA	bs-0095R-A488
CNN (ICC)	Sigma	C2687
αActin	Sigma	A5228
SM-MHCII	Santa Cruz	Sc79079
SM-MHCII	Abcam	Ab 683
SM-MHCII	Sigma	M7786
Sox10	Abcam	Ab 155279
Sox17	Millipore	09-038
S100β	Millipore	04-1054
CD 29	Millipore	04-1109
CD 44	Abcam	Ab 24504
CD 146	Millipore	04-1147
SCA 1^{+}	Miltenyi	130-039-222
VEGF (Flt1)	Abcam	Ab 32152
SMAD3	Abcam	Ab 40854
CD 133	Biobryt	orb10288
c-kit	Santa Cruz	C-19
Nestin	ThermoScientific	MA1-110
Pax6	Abcam	Ab 5790

Table 3.0 *Antibody Cat. Number*

B. Secondary antibody only controls (no primary) for Immunocytochemistry

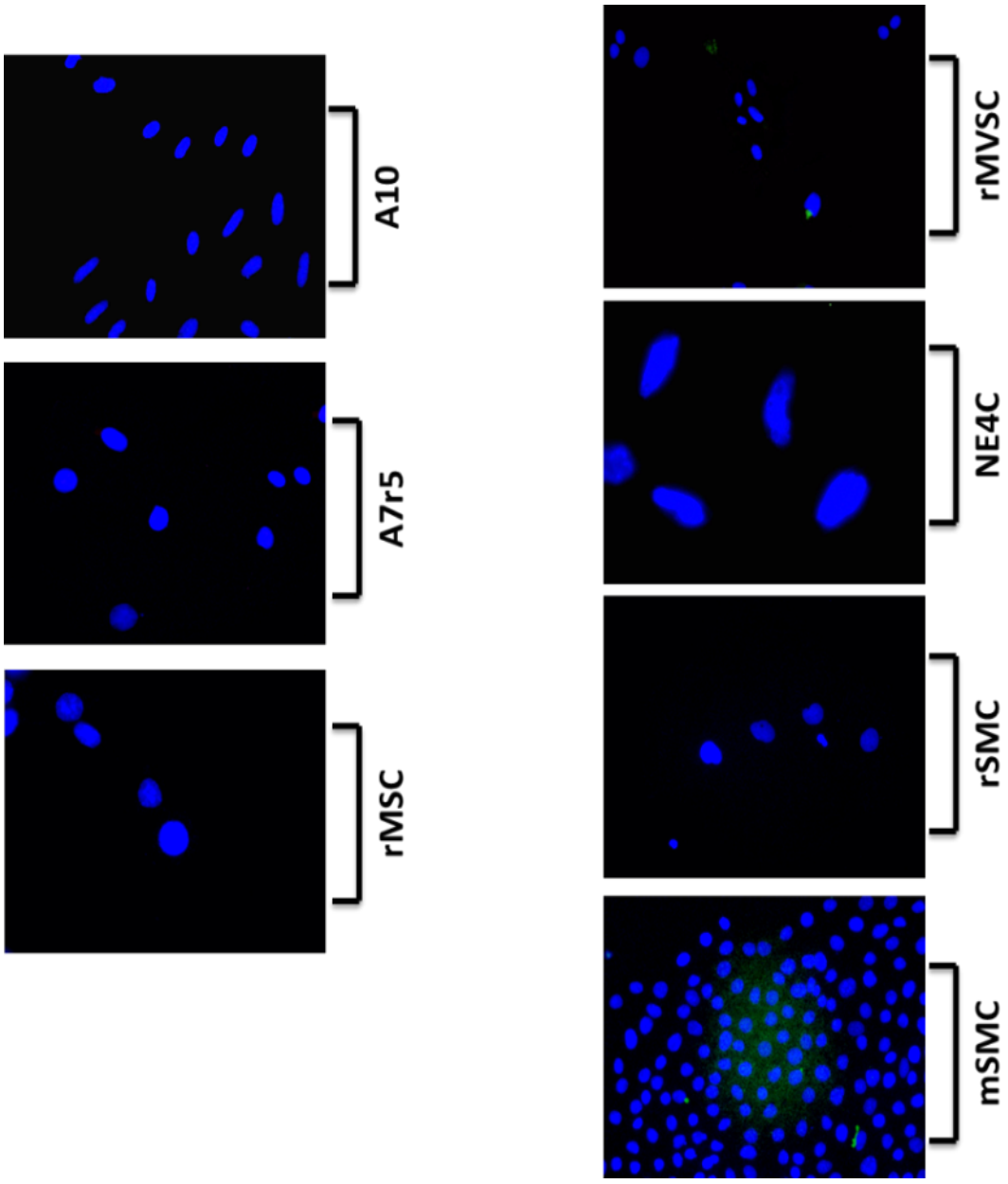

Figure *7 Sample images of secondary antibody only controls for immunocytochemistry using Alexa 488. All cell lines (A10, A7r5, rMSC, rMVSC, NE4C, rSMC, mSMC) were treated in the same way as regular samples but the samples were incubated in block buffer overnight rather than primary antibody. All samples were treated in the same manner from this point onwards.*