The Role of Notch1 Receptor N-glycosylation in the transition of Stem Cells to vascular Smooth Muscle Cells

A dissertation submitted for the degree of Ph.D.

By

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September 2018

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Declaration

I hereby certify that this material, which I now submit for assessment on the programmeof study leading to the award of.....**Ph.D.**..... 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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Dedication

This study is wholeheartedly dedicated to my family: my husband Adedeji, without his patience, understanding and support, the completion of this work would not have been possible and my children Ameera, Hameeda and Tareef (PhD baby), who have been my source of inspiration, gave me strength when I thought of giving up and taught me about patience especially during the writing period.

To my parents Mufutau Olayinka and Idayat Idowu Olayinka, who have raised me to be the person I am today.

And lastly, I dedicate this thesis to Almighty God, who made this journey possible, thank you for the guidance, knowledge, strength, and good health to complete this work.

Acknowledgements

I would like to express my sincere gratitude to my supervisor and advisor Professor Paul Cahill for encouraging me to pursue a PhD in the first place. His expertise, understanding, support, and guidance made it possible for me to work on a topic that was of great interest to me. It was a pleasure working with him.

I also would like to thank my secondary supervisor Dr. Brendan O'Connor for his advice, guidance and continuous support from his group on the Enzyme Linked Lectin Assay technology.

I am also grateful to Dr Paula Meleady and Mr Michael Henry in National Institute for Cellular Biotechnology (NICB) for facilitating the proteomic analysis presented in this report.

Many thanks to Dr Barry Byrne for his help with the HPLC procedure and Mr David Cunningham for his assistance in training and troubleshooting.

I want to thank my colleagues, from the Vascular Biology and Therapeutics Lab, past and present, who have contributed in some way to this thesis, whether through direct input or through advice and guidance, including Mariana Di Luca, Claire Molony, Roa Bashmail, Eoin Corcoran, Denise Burtenshaw, Dorota Kozlowska, Emma Fitzpatrick, Roya Hakimjavadi and Joseph Mackle.

Lastly, I would like to thank my parents again, my brothers and sisters for their moral support and encouragement. And my husband for being so understanding and supportive throughout the project.

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Abbreviations

aa	Amino acid
AAL	Aleuria Aurantia lectin
ACN	Acetonitrile
APS	Ammonium Per Sulfate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Cnn1	Calponin 1
Con A	Concavalin A
CO_2	Carbon dioxide
CVD	Cardio Vascular Disease
DAPI	4,6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DES	Drug Eluting Stents
DLL-1	Delta like ligand 1
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethylsulfoxide
DANN	Deoxyribose Nucleic Acid
EDTA	Ethlyenediamine Tetracetic Acid
EGF	Epidermal Growth Factor
ELLA	Enzyme linked lectin assay
EMEM	Eagle's Minimum Essential Medium
ER	Endoplasmic reticulum

FA	Formic acid
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GlcNAc	N-acetyl glucosamine
GalNAc	N-acetyl galactosamine
Hes	Hairy enhancer of split
Hey	Hairy enhancer of split related with YRPW motif
HRP	Horseradish peroxidase
h	Human
ICC	Imunocytochemistry
IgG	Immunoglobulin G
IL	Interleukin
Jag-1	Jagged ligand 1
LCA	Lens Culinaris
LC	Liquid chromatography
Lfng	Lunatic Fringe
mAb	Monoclonal antibody
Mfng	Manic Fringe
mRNA	messenger RNA
MAL	Maackia Amurensis lectin
MS	Mass spectrometry
MSC	Mesenchymal Stem Cell
MVSC	Multipotent Vascular Stem Cell
Myh11	Myosin heavy chain 11
NECD	Notch Extracellular Domain

NICD	Notch Intracellular Domain
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pofut1	Protein O-fucosyltransferase 1
Poglut1	Protein O-glucosyltransferase 1
PNA	Peanut agglutinin
PVA	Polyvinyl Alcohol
RNA	Ribonucleic Acid
RIPA	Radioimmunoprecipitation Assay
RT-PCR	Reverse Transcription PCR
SBA	Soybean agglutinin
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
SiRNA	small interfering RNA
SMC	Smooth Muscle Cell
αSMA	Smooth muscle alpha actin
SM22α	Transgelin 2
TBST	Tris Buffered Saline + Tween 20
TFA	Trifluoroacetic acid
TGF-β1	Transforming growth factor beta
vSMCs	Vascular smooth muscle cells
WGA	Wheat germ agglutinin

Units

cm	Centimetres
d	Days
°C	Degrees Celsius
g	Grams
kDa	Kilo Dalton
ml	Millilitre
ng	Nanogram
nl	Nanolitre
µg	Microgram
µl	Microliter
µM	Micromoles
U	Enzyme units
h	Hours
mAU	milli-Absorbance Units
mg	Milligram
min	Minutes
m	Mouse
m	Molar
nm	Nanometer
x g	G force
rpm	Revolutions per minute
s	Seconds

Manuscripts

Hakimjavadi, R., **Olayinka, A**., Di Luca, M., Guha, S., Walls, D., Redmond, E. M., and Cahill, P. A.2017. Pathogenetic Mechanisms in Alagille Syndrome. DOI: 10.1002/9780470015902.a0021440.pub2

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Presentations: Poster

Olayinka, A., O'Connor, B. and Cahill, PA. (2015) Detection of N-glycans on the Notch1 receptor using an Enzyme Linked Lectin Assay (ELLA) in vitro. Annual Notch Meeting, 4-8th October, Athens, Greece.

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Presentations: Oral

Olayinka. A., Cahill, PA. (2015). The role of N-Glycosylation in the transition of stem cells to vascular smooth muscle cells. Transfer to Ph.D., 18th August, Dublin City University, Dublin, Ireland.

Olayinka. A., Cahill, PA. (2015) N-Glycosylation, Notch1 and Vascular Stem Cell Function. Annual Research day, 30th January, School of Biotechnology, Dublin City University, Dublin, Ireland.

Olayinka. A., Cahill, PA. (2014) N-Glycosylation, Notch1 and Vascular Stem Cell Function. Science Foundation Ireland (SFI) Audit, November, Dublin City University, Dublin, Ireland.

Abstract

The Role of Notch1 Receptor N-glycosylation in the transition of Stem Cells to vascular Smooth Muscle Cells

Abidemi Olayinka

Cell fate decisions within the vasculature are crucial to the pathogenesis of vascular diseases, including, arteriosclerosis, atherosclerosis and restenosis after angioplasty. Notch signalling is involved in regulating cell fate in vasculature development during embryogenesis resulting in altered cell fate decisions leading to vascular disease. The Notch signalling pathway is highly regulated by a number of mechanisms including glycosylation, a post-translational modification.

The main objective of this research work was to define a putative role for N-glycosylation of Notch1 receptor in controlling resident vascular stem cell fate *in vitro*. Utilising ligand Jagged1 (Jag-1)-induced Notch signalling assay, Quantitative RT-PCR, immunocytochemistry, ectopic expression of Notch1 receptor, siRNA knockdown, pharmacological inhibition and enzyme linked lectin assay (ELLA), alterations in N-glycan decoration of the Notch1 receptor were assessed before the effects on Notch signalling and Notch ligand promotion of myogenic differentiation were evaluated.

N-glycosylation of the Notch1 receptor was assessed using ELLA and confirmed the presence of N-glycans on the receptor, an effect that was abrogated following inhibition of glycosyltransferase activity with tunicamycin and Lunatic Fringe (Lfng) knockdown. Jag-1-induced Notch activation increased Notch target gene expression and promoted myogenic differentiation of bone-marrow derived mesenchymal stem cells and resident vascular stem cells. Selective knockdown of the Notch1 receptor in stem cells resulted in a decrease in Jag-1 stimulated Hey1 Notch1 target gene expression concomitant with a reduction in myogenic differentiation due to decreased smooth muscle differentiation marker expression (Cnn1 protein level and Myh11 mRNA level). Inhibition of N-glycosylation with tunicamycin lead to a down regulation of smooth muscle differentiation marker, Myh11 independent of a reduction in Notch target gene expression. Lfng knockdown lead to a similar significant reduction in Jag-1 induced myogenic differentiation (reduced Myh11 mRNA expression). Collectively, these results suggest that N-glycosylation of Notch1 receptor is involved in Notch signalling leading to altered resident vascular stem cell fate.

Chapter 1:

Introduction

1.1 Cardiovascular disease

1.1.1 The Vascular system

The vascular system is composed of several blood vessels and their primary function is to supply blood to all parts of the body. It is made up of a series of vessels known as veins and arteries. The arteries circulate blood from the heart to different parts of the body transporting oxygenated blood and nutrients to targeted cells and tissues. If there is no adequate blood supply to these cells and tissues, they may become damaged or necrotic; therefore, it is important to ensure that the vascular system is functioning properly. The primary role of most veins is to transport oxygen depleted blood from the tissues towards the heart. There are 3 different layers of cells that form the arteries as illustrated in Figure 1.1.

The tunica intima/endothelium layer; this is the innermost section, made up of a thin monolayer of endothelial cells and essential in maintaining and regulating the vessel (Gimbrone et al. 2000). There is a connection between these cells and a basal lamina, an extracellular membrane layer composed of collagen, proteoglycans and glycans. The endothelium promotes blood circulation and possesses an anti-thrombotic/anti-inflammatory effect or surface (Gimbrone et al. 2000). The cells are smoothly aligned thus preventing friction and reducing turbulence during blood circulation. The porous structure of the elastic membrane on the inside allows the flow of blood through the vessel wall. There are small valves in veins, connected to the endothelium to stop blood from flowing back (Gimbrone et al. 2000).

The tunica media layer; this is the middle section of the vessel where vascular smooth muscle cells, pericytes, elastin and proteoglycans are located. The manner in which the cells and proteins are aligned and the elastic features of the medial layer contributes to its fibrous nature. The tunica media is known to be the primary source of the vascular smooth muscle cell (Zipes and Braunwald 2005). The tunica adventitia layer is the outer section of vessel wall and is separated from the tunica media by a thin layer of elastin. It is composed of connective tissue, fibroblasts, stem cells and has less fibrous structure compared to medial layer (Seeley 2011; Kennedy et al. 2014b).

1.1.2 Vascular Disease

Cardiovascular disease (CVD) is the leading cause of death in the western world. Globally cardiovascular related disease was responsible for approximately 18 million deaths in 2015, representing 31% of all global deaths According to World Health Organisation (WHO), almost 23.6 million people will die from CVDs by 2030 (WHO facts, 2017). CVD affects the arteries and/or the veins by restricting or completely blocking blood circulation. This obstruction results in lack of nutrients and oxygen reaching vital organs, causing irreversible damage that could lead to stroke, hypertension, arteriosclerosis and atherosclerosis. The discussion of cells involved in this obstruction has been a controversial topic in the context of vascular stem cells (Tang et al 2012, Nguyen et al. 2013).

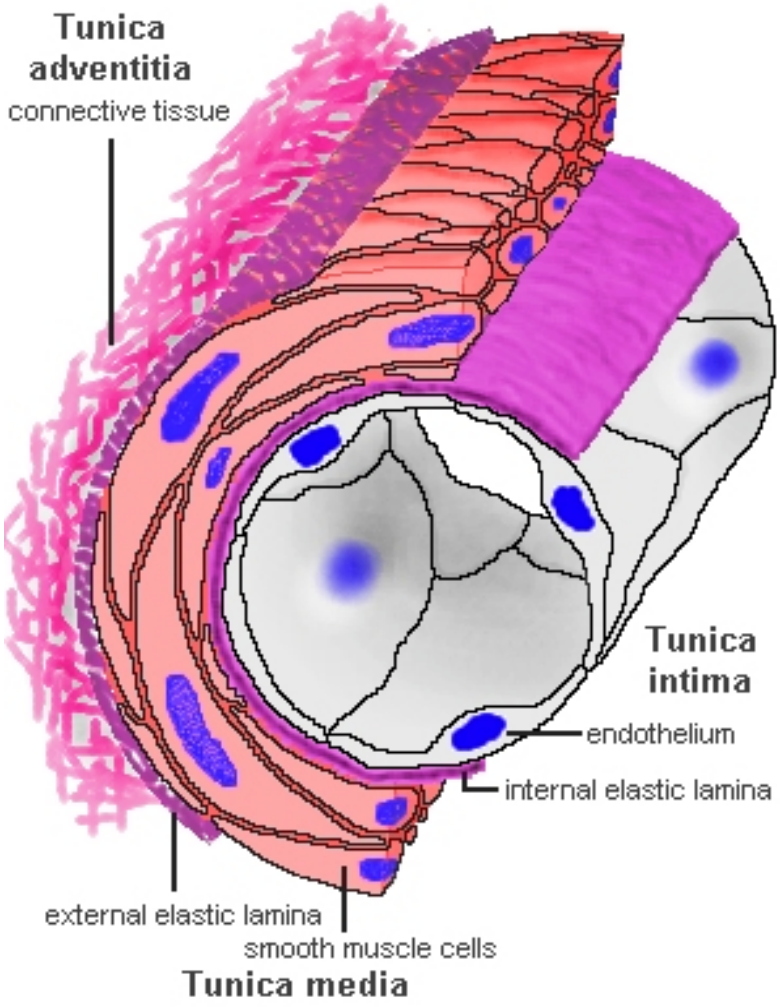

Figure 1.1 The arterial structure. Representative image of the layers of the arteries, illustrating the tunica media, intima and adventitia and the cell types found within each layer. (Image adapted from SIU Med 2015, http://www.siumed.edu/~dking2/crr/cvguide.htm).

1.1.3 Arteriosclerosis

Arteriosclerosis is a thickening (symmetrical or asymmetrical) of the vessel wall resulting from neointimal thickening or inward vessel remodelling. It is primarily attributed to transplant arteriosclerosis, angioplasty-induced restenosis, vein graft atherosclerosis and spontaneous atherosclerosis. This condition restricts blood flow to vital organs and can remain undiagnosed for a long period of time (Fishbein and Fishbein 2009).

1.1.4 Atherosclerosis

Atherosclerosis is a form of asymmetrical vascular remodelling and a sub form of arteriosclerosis, which can develop from many contributory factors such as fat build up, cholesterol and plaque formation and as a result, the vessel becomes obstructed and blood flow is restricted (Fishbein and Fishbein 2009). There are controversial debates surrounding the cells responsible for this obstruction (Tang et al. 2012, Nguyen et al. 2012), with several studies demonstrating a putative role for the de-differentiation of contractile SMC, stem cells-derived myogenic progeny and SMCs following endothelial-mesenchymal transition (EndMT) (Yuan et al., 2017).

1.1.5 Current treatment for cardiovascular diseases

Treatment may include lifestyle changes, medication and/or surgery depending on the severity of the disease and is generally the same for both men and women. The treatment of lifestyle changes is applied mainly as a preventative measure to relieve CVD symptoms, reduce risk factors, lower the risk of blood clots and is only effective in early-diagnosed patients. In the event that lifestyles changes are insufficient, medicines and or by-pass surgery may be required. The medicines can help to relieve symptoms, reduce the risk of sudden death, decrease LDL cholesterol and blood pressure in patients and prevent or delay surgery (Mensah and Brown, 2007). The last resort when lifestyle changes and medication do not suffice is then surgery such as angioplasty and stent deployment.

Vascular remodelling generally develops from coronary atherosclerosis whereby the arteries become narrowed and hardened as a result of an excessive build-up of plaque around the wall of an artery. The techniques applied to remove the

atherosclerotic plaque are invasive, such as percutaneous transluminal coronary angioplasty (PTCA) and stenting (Haery et al. 2004).

Angioplasty is a procedure that requires inflation of a tiny balloon inside a narrowed or blocked artery that crushes the plaque and widens the artery to increase the flow of blood (Haery et al. 2004). The insertion of bare-metal stent (BMS), a tiny metal mesh about 18mm in size, in the narrowed vessel can be performed to prevent elastic recoil and reduce the incidence of restenosis (Panyam and Labhasetwar 2003). Still, there are several drawbacks and side effects associated with coronary stenting. In-stent restenosis can occur from these procedures when neointimal cells accumulate within the vessel wall and become narrow again via a different mechanism than the original injury (Yin, Yang and Wu 2014).

Stents may cause permanent physical irritation and present the risk of long-term endothelial dysfunction or inflammation. Stents may also cause thrombosis and prevent the vessel from remodelling and functioning properly (Yin, Yang and Wu 2014). All these can make future bypass surgery and non-invasive imaging difficult to perform. Drug-eluting stents (DES) have since been developed that release anti-proliferative and anti-mitotic drugs (Sirolimus and Paclitaxel) to combat the accumulation of neointimal cells (Yin, Yang and Wu 2014). It is a local drug delivery system to injured blood vessel where the drug that is released from a polymer coating or loaded directly onto the stent (Van et al. 2005). Drug is released at the site of vascular injury for a fixed duration; this enables the delivery of effective dose of the drug while simultaneously avoiding systemic toxicity.

Although the first-generation DESs have been proven to have better success rate than BMSs in reducing vascular restenosis through inhibition of smooth muscle cell

(SMC) proliferation, there are still safety issues such as suboptimal polymer biocompatibility, delayed stent endothelialisation which can lead to late and very late thrombosis, and local drug toxicity. These might arise due to the non-selective inhibitory effect the DES anti-proliferative drugs have on both endothelial cell (EC) and SMC proliferation (Tan et al. 2013; Yin, Yang and Wu 2014).

1.2 Smooth Muscle Cell Origin

Advanced research in vascular biology has brought about interesting debate on the origin of smooth muscle cell and its development. The origin of SMCs can differ within blood vessel. Vascular SMCs are derived from approximately five different sources during embryogenesis. These include; neural crest, proepicardium, serosal mesothelium, secondary heart field and somites. The neural crest cells of the ectoderm give rise to the SMCs found in the aortic arch and the mesoderm cells gives rise to the SMCs in the ascending aorta (Seidelmann, Lighthouse and Greif 2014; Xie et al. 2011).

Vascular smooth muscle cells (VSMCs) control pressure in the vasculature. They contract and relax to aid the transport of blood to regions where tissue oxygenation and nutrients are required (Steinbach and Husain 2016). SMCs are known to maintain their plasticity better than other cell types in order to fulfil various roles in contraction, proliferation and extracellular matrix synthesis (Alexander and Owens 2012). Depending on their environmental stresses, SMCs are purported to easily switch between two phenotypes, de-differentiate from a contractile and quiescent state into a synthetic and proliferative type (Liu, Leslie and Martin 2014; Daniel and Sedding 2011). SMCs with contractile features express cytoskeleton and contractile proteins such as smooth muscle α-actin, smooth muscle myosin heavy

chain (Myh11), calponin (Cnn1) and transgelin (SM22a). These proteins are required to maintain the contractile function of SMCs in the vasculature (Wang et al. 2015). The phenotypic modulation to synthetic type results in increased production of extracellular matrix and down regulation of the contractile proteins (Daniel and Sedding 2011). The apparent ability of SMC to readily switch is beneficial when repair is necessary following vascular injury.

There can be complications when there is an imbalance, such that the synthetic type is predominant, this may lead to vascular diseases such as atherosclerosis and aneurysms (Liu, Leslie and Martin 2014). It is therefore important that the origin of SMCs during arterial remodelling is clarified in order to understand how SMC accumulate at sites of plaque formation or neo-intima progression (Daniel and Sedding 2011).

From as early as 1973, Ross and Glomset proposed the hypothesis that arterial smooth muscle cells contribute to the development of atherosclerosis. They are the primary cells that accumulate and undoubtedly form the extracellular matrix components of atherosclerotic lesions; they are supposedly responsible for accumulation of intracellular lipid and deposition of lipid in the extracellular matrix. Although the mechanisms involved in these changes could not be established, there is some indication that endothelial injury might be involved (Ross and Glomset 1973).

As vSMCs originate from several mesodermal lineages, there is a conflicting view about whether the SMC-like cells in lesions are heterogeneous or multipotent vascular stem cell-derived progeny that differentiated into various subpopulations with specific functions (Nguyen et al. 2012; Tang et al. 2012).

1.3 Stem / Progenitor cells contribute to Smooth Muscle Cells accumulation

There is accumulating evidence to suggest that stem cells and/or progenitor cells are involved in the development of arteriosclerosis, including transplant arteriosclerosis, angioplasty-induced restenosis, vein graft atherosclerosis and spontaneous atherosclerosis. Stem cells are thought to exist within the three layers of the vessel wall and research groups are inconclusive regarding the phenotypic markers of the stem cell populations resident within the different layers. Presence of populations of stem cells is indicated within the tunica media layer (Tang et al. 2012, Sainz et al. 2006, Tintut et al. 2003, Hu et al. 2002). The cells isolated by Tintut et al (2003) have similar multipotent potential as mesenchymal stem cells (MSC).

It is speculated that MVSC become mesenchymal stem cell (MSC)-like while transitioning into smooth muscle cells (Tang et al. 2012). MSCs are a heterogeneous group of pluripotent stem cells with intrinsic ability to self-renew and capacity to differentiate towards a wide range of cell lineages. They have been demonstrated to differentiate into osteocytes, adipocytes, myocytes, epithelial cells, chrondocytes, fibroblasts and neurons. MSC were originally associated with the bone marrow but they have since been identified in several other tissues including adipose tissue, periosteum, synovial fluid and umbilical cord. This lead to the conclusion that MSC-like cells resides in nearly all organs and tissues, though their roles are not understood. There is accumulating evidence to suggest that the perivascular niche is residence to these MSC-like cells located within the various tissues (Liu, Zhuge and Velazquez 2009).

MSC play a role in repair by differentiating into connective tissue cells in response to stimuli from trauma, inflammation and fracture within the body (Pountos and Giannoudis 2005). They tend to migrate from the residence such as bone marrow to the injured areas and differentiate in situ to contribute to the repair and regeneration. Therefore, MSCs are desirable candidates for therapeutic use in regenerative medicine field (Liu, Zhuge and Velazquez 2009; Pountos and Giannoudis 2005).

Stem cells derived from the recipient in a transplant procedure may also be used to reconstruct injured endothelial cells of arteries. However, there are several controversial issues surrounding the procedure such as whether the bone marrow-derived stem cells differentiate into smooth muscle cells to form neointimal lesions of the vessel wall (Xu 2008; Cooley et al. 2014).

1.3.1 Stem cells in the Adventitia

Hu et al. (2004) suggested that the SMCs that accumulate in atherosclerotic lesions might have been derived from vascular progenitor cells, but the source of these progenitor cells is not clear. To investigate the hypothesis of the existence of vascular progenitor cells in adults, an extensive study was carried out on various tissues from ApoE-deficient mice. The resulting data suggests that progenitor cells that have stem cells markers (e.g. Sca-1^+) were present in the adventitia but none expressed the SSEA-1^+ embryonic stem cells marker. Isolated Sca-1^+ cells are capable of differentiating into SMCs in response to PDGF-BB stimulation *in vitro* and a large population of vascular progenitor cells exists in the adventitia that can differentiate into SMCs, which contribute to atherosclerosis. These findings

indicate that these progenitor cells may have a significant role in cellular, genetic, and tissue engineering approaches to vascular disease (Hu et al. 2004).

A study by Xu (2005) also demonstrated that stem/progenitor cells existing in the blood and adventitia influence endothelial repair and smooth muscle cell accumulation. There is an indication that the progenitor cells in circulation and in the adventitial layer migrate into the intima where they proliferate and differentiate into neo-SMC (Xu 2005).

A recent study by Majesky et al (2016) in mice demonstrated that a subpopulation of adventitial Sca1$^+$ progenitor cells is generated *in situ* from differentiated SMCs. Using an *in vivo* cell-fate-mapping strategy with a SMC epigenetic lineage trace, they established that the vascular adventitia contains subpopulations of progenitor cells expressing SMC with a distinct phenotype. There is an indication that each subpopulation originated from differentiated SMCs. These SMC-derived AdvSca1$^+$ cells displayed multipotent features with capacity to differentiate *in vivo* into mature SMCs, resident macrophages and endothelial-like cells. Upon injury, SMC-derived Sca1$^+$ cells proliferate and contribute significantly to adventitial remodelling (Majesky et al. 2016).

Kramann and colleagues conducted a lineage tracing study of Gli1$^+$ perivascular cells in mice. Gli1, a zinc finger transcription factor is a specific marker for MSC-like adventitial cells and is a known hedgehog target gene. To investigate the role of Gli1$^+$ cells in vascular disease and remodelling, they subjected Gli1-CreER: tandem dimer Tomato (tdTomato) transgenic mice to various vascular injuries. Red fluorescent protein tdTomato expression was induced by tamoxifen injection. In the

wire-injured model, they found tdTomato cells expressing smooth muscle actin and Cnn1 localized in the media and neo-intima of the femoral artery. They conclude that these MSC-like adventitial $Gli1^{+}$ cells upon injury, migrate towards the tunica media and neo-intima and subsequently exhibit SMC-like phenotype and differentiate into osteoblasts and that these cells were involved in the significant calcification of arteries (Kramann et al. 2016; Kramann et al. 2015).

1.3.2 Stem cells in the Media

Seminal studies by Tang et al. (2012) characterised a population of medial progenitor stem cells that display phenotypic markers related with neuroectoderm stem cells and that these cells are able differentiate into SMCs following injury and as a result contribute to intimal thickening of the artery and may be partly responsible for causing vascular disease. They concluded that that multipotent progenitor cells are involved in restenosis and that the "de-differentiated" SMC do not play any role in the process (Tang et al. 2012).

1.4 Signal Pathways involved in Smooth Muscle Cell differentiation

The dynamic nature of the vessel wall allows for the existence of various stem cells. It is now becoming evident that vascular stem cells become activated following injury, transition to vSMC and subsequently differentiate into smooth muscle like-cells that play a significant role in vascular regeneration and remodelling. They can serve as a possible source of smooth muscle cells for regenerative medicine applications such as constructing vascular grafts (Tang et al. 2012, Gomez and Owens 2012, Xu et al. 2008). Key signalling pathways including transforming growth factor-beta (TGF-beta), the Notch pathway (Kurpinski et al.

2010), and hedgehog signalling (Erbilgin et al. 2013) have been determined to influence vascular SMC differentiation.

1.4.1 Notch Signalling Pathway

The Notch signalling pathway is highly conserved and plays a significant role in embryonic development and adult life, and controls various mechanisms in the vasculature such as stem cell proliferation, cell-cell interaction and differentiation state (Guruharsha, K.G., Kankel, M.W. and Artavanis-tsakonas 2015). Dysregulation of the Notch signalling pathway contributes to a variety of human diseases, and modulating the Notch receptor signalling can serve as a tool for regenerative medicine for vascular disease (Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010). Notch receptors (approx. 300kda) have large extracellular domains (NECD) composed of various epidermal growth factor (EGF) – like repeats. Mammals have Notch receptor prologues (1-4). Both the receptor and its ligands, Delta and Serrate (referred to as Jagged (Jag-1) in mammals) are large single-pass transmembrane proteins. The ligands expressed in adjacent cells bind and activate the Notch receptor (Figure 1.2), promoting a series of proteolytic cleavage events (Guruharsha, K.G., Kankel, M.W. and Artavanis-tsakonas 2015). This activation causes the translocation of the intracellular domain of the receptor towards the nucleus (Figure 1.3), where the downstream specific genes are expressed (Table 1.1). Although the mechanisms involved in Notch signalling pathway are similar in most Notch-dependent processes, the regulatory factors do vary (reviewed in Bray 2006).

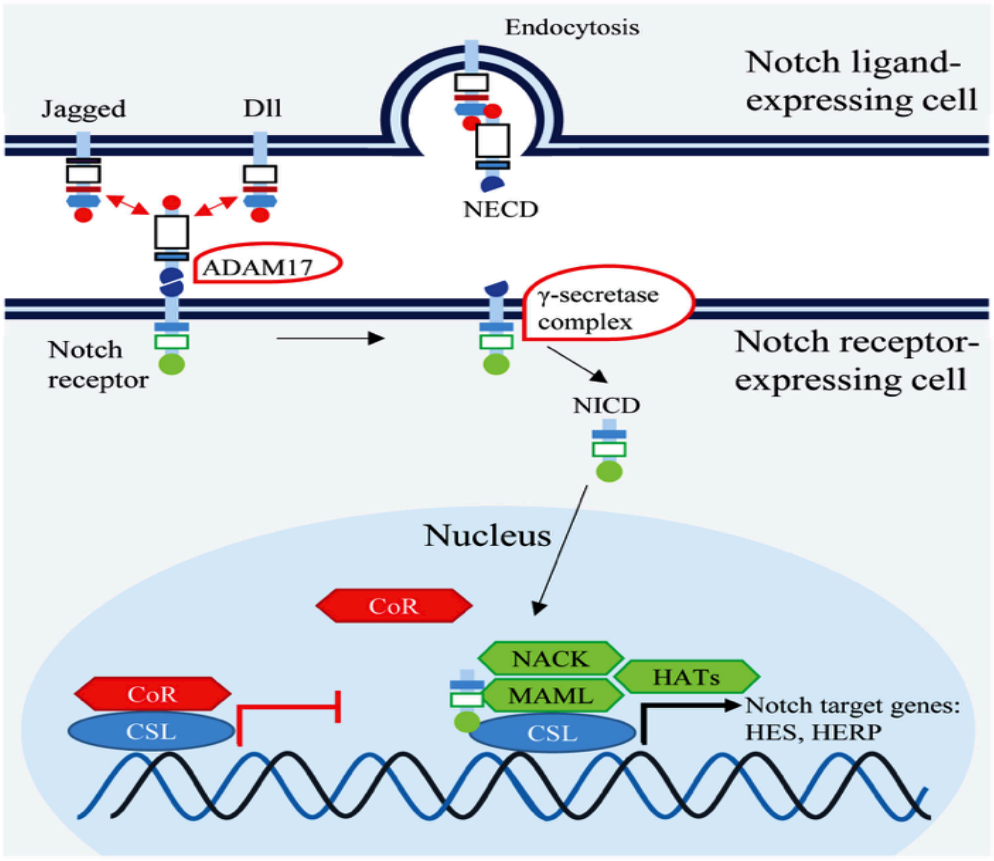

Figure 1.2. Notch signalling in cells. Notch receptor and ligands are trans-membrane proteins. Ligands expressed in adjacent cells activate the receptor, which undergoes successive proteolytic cleavages. The Notch intracellular domain (NICD) translocates to the nucleus where it associates with CSL/RBP-Jk. The NICD-RBP-Jk form a transcriptional complex which activates the expression of the primary Notch target genes, hairy enhancer of split (HES) and HES-related repressor protein (HERP) families that act as Notch effectors (Iso, Kedes and Hamamori 2003).

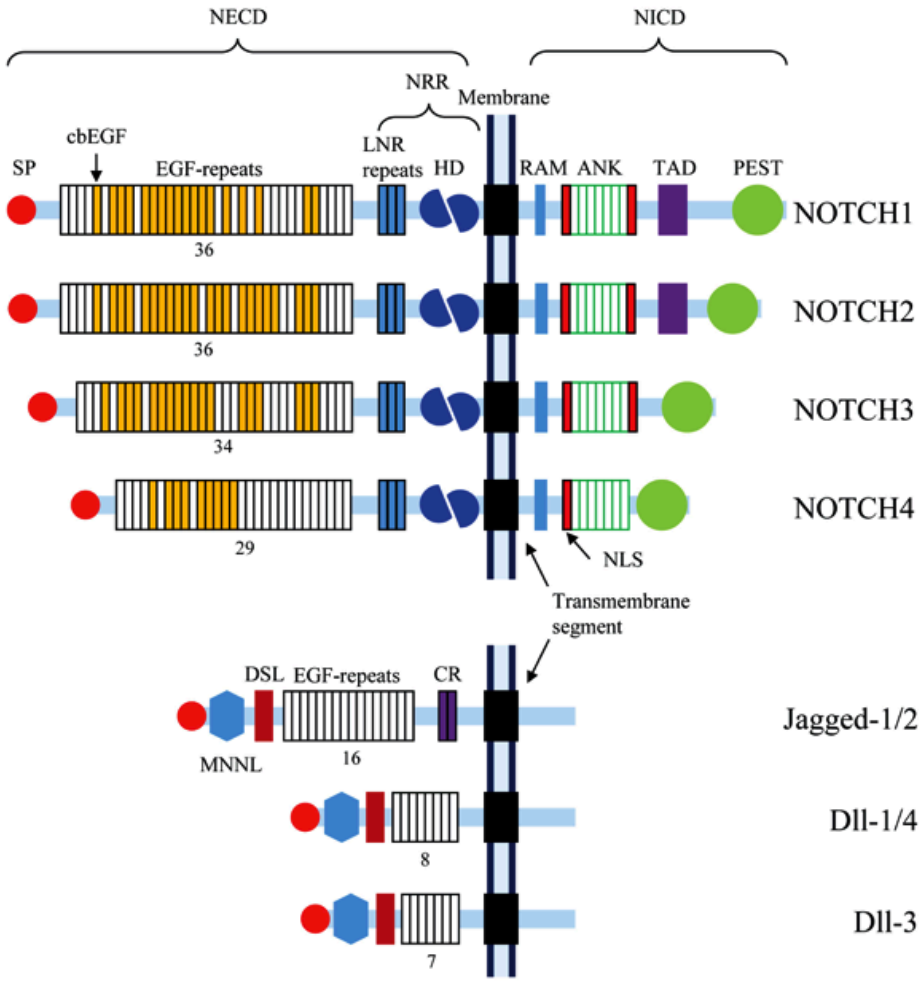

Figure 1.3. Structure of Notch receptors and their ligands. The NECD is mainly composed of EGF repeats and the Notch Negative Regulatory Region (NRR). The NICD contains mo tifs that mediate translocation to the nucleus. The Notch ligands, Delta like ligand (DLL 1,3,4) and Jag-1 (1,2) all consists of multiple EGF repeats on their extracellular domain.

Table 1.1. HERP family. The proteins are identical homologue of other species. (Adapted from Iso, Kedes and Hamamori 2003).

Abbreviations	Full name	Species	Function
HERP1 HERP2 HERP3	HES-related repressor protein	Mouse, rat, human	Transcriptional repressor
Hesr1 Hesr2 Hesr3	Hairy/E(spl)-related	Drosophila, mouse, human	Transcriptional repressor
Hey1 Hey2 HeyL	Hairy/E(spl)-related with YRPW	Drosophila, chicken, mouse, human	Transcriptional repressor

1.4.2 Notch signalling control of Smooth Muscle Cell differentiation

The Notch signalling pathway plays an important role in the differentiation and function of vascular smooth muscle cells. Studies have shown that Notch activation promotes myogenic differentiation but inhibits SMC differentiation in others. Over expression of constitutively activated form of NICD in human aortic smooth muscle in culture increased the expression of smooth muscle marker genes (SM-MHC and SM22α) (Doi et al. 2006) and Jag-1 induced Notch activation upregulated contractile proteins expression in human SMCs (Boucher et al. 2011; Wang et al. 2015). In contrast, Active Notch signalling has been shown to inhibit SMC differentiation (Morrow et al. 2005; Proweller, Pear and Parmacek 2005) and contractile phenotype of adult SMCs (Havrda et al. 2006). However, it universally promotes SMC differentiation in stem cell populations. MSC have been stimulated to become smooth muscle like via Jag-1-induced Notch activation (Kurpinski et al.

2010; Doi et al. 2006). Though the reports are contradictory, the generally supported view is that Notch signalling is an inducer of SMC differentiation in undifferentiated or poorly differentiated cells.

1.4.3 Regulation of Notch signalling

Glycosylation is largely involved in regulation of Notch receptor activity (Takeuchi and Haltiwanger 2014). The EGF repeats on the large NECD are heavily glycosylated and a lot of research has been undertaken to determine the effect of this post-translational modification on Notch signalling pathway (Sala et al. 2012).

1.5 Glycosylation

Glycosylation is a process where carbohydrate groups (glycans) attach to lipids and proteins to form a dense glycocalyx coating on the membrane of all cells, including stem cells. These glycans are the main component of the glycocalyx and since they form the outermost layer, they generally act as recognition sites making them the first cellular molecule to come in contact with hormones, antibodies, pathogens and other cells (Lanctot, Gage and Varki 2007). Glycans play a significant role in correct folding and transportation of proteins intracellularly and but also control stem cell communication with its environment and are essential in regulating the growth and differentiation of stem cells *in vitro* (Hamouda et al. 2013).

Glycosylation is the most common form of post translational modification (PTM), a distinct process mediated by specific enzyme(s) during which carbohydrate side chains are covalently bound to either the side chain of asparagine (N-linked) (Figure 1.4) or serine/threonine (O-linked). The carbohydrate component of glycosylated proteins (glycoproteins) generally have a significant influence on the

recognition, signalling, and interaction activities within and between cells and proteins, and often play a major role in correct folding and defining the structure of the protein (Lipscomb et al. 2005). It is a process that takes place in the endoplasmic reticulum and Golgi apparatus whereby carbohydrate groups are attached to proteins by covalent bonds through several reaction mechanisms that are catalysed by various glycosyltransferases. The four main types of protein glycosylation in eukaryotic cells are N-linked glycosylation, O-linked glycosylation, C-linked mannosylation and glypiation with the N- and O- linked being the most common (Wang, Peterson and Loring 2014).

UDP-GlcNAc or UDP-GalNAc are important high energy nucleotide sugars utilized as substrates for glycosyltransferases in the processing of two main categories of glycosylation in the mammalian systems, N-linked and O-linked (Elmouelhi and Yarema 2008).

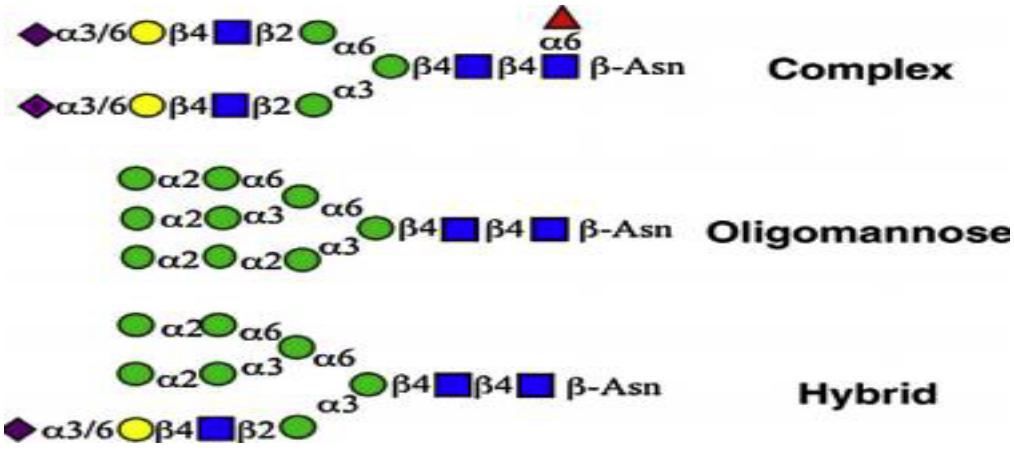

Figure 1.4. N-glycan types in mammals. High Mannose N-glycan, complex N-glycan. Combination of complex and high mannose N-glycans form the hybrid type. Blue squares=N-acetyl-D-glucosamine, green circles =D-mannose, red triangles=L-fucose, yellow circles=D-galactose, Purple diamonds=N-acetyl-D-neuraminic acid (Neu5Ac)(Adapted from Corfield and Berry 2015).

1.5.1 N-linked Glycosylation

N-linked glycosylation involves the attachment of carbohydrates to the amide mitogen of asparagine residues (Luther and Haltiwanger 2009). In eukaryotes, the vast majority of secreted and membrane-bound proteins are N-glycosylated (Haltiwanger and Lowe 2004) with approximately 90-95% of glycosylated proteins having N-linked glycans in their structure. The process of N-glycosylation is initiated in the cell cytoplasm, advances to the endoplasmic reticulum (ER) and concludes in the Golgi apparatus. A dolichol-linked heptasaccharide ($Man_3GlcNAc_2$-P-P-Dol) is assembled in the cytosolic face of the ER in a stepwise manner (Elmouelhi and Yarema 2008). Dolichol is an isoprenoid lipid involved in the transportation of monosaccharides and oligosaccharides within the cell and across the cell membrane (Aebi 2013). The dolichol-linked heptasaccharide is subsequently modified in the luminal side of the ER by the addition of 3 glucose residues and 4 mannose residues to form a 14-sugar oligosaccharide ($Glc_3Man_9GlcNAc_2$-P-P-Dol) which is then transported to the polypeptide by oligosaccharyl transferase (Elmouelhi and Yarema 2008). This oligosaccharide is linked via N-Acetylglucosamine (GlcNAc) to the amide residue of unique Asparagine in the consensus sequence Asparagine-X-Serine/Threonine where X can be any amino acid except proline (Gloster 2014). The linkage of the hydrophilic oligosaccharide to the polypeptide controls the biophysical properties and folding of the resulting protein (Aebi 2013).

A trimming process is carried out to remove terminal glucose, the 2 glucose adjacent to the terminus and a mannose by the enzymes α-glucosidase I, α-glucosidase II and α-mannosidase respectively. This process serves as a critical

quality control for proper protein folding and transportation. Subsequent transfer to the Golgi involves additional trimming of three mannose residues to form $Man_3GlcNAc_2$, which can be further trimmed or elongated (Elmouelhi and Yarema 2008). N-glycans are highly diverse structures composed of several monosaccharide sub-units and there are generally greater than seven (Haltiwanger and Lowe 2004). The three main types of N-glycans are high mannose, complex and Hybrid (Figure 1.4). They tend to have a conserved trimannosyl core of $Man_3GlcNAc_2$ in common, but differ based on the further glycan modifications outside of this core; (i) High mannose type: 5-9 additional mannoses are added to the trimannosyl core (ii) Complex type: with no further addition of mannose but branching antennae is composed of various sugars and (iii) Hybrid type: composed of both high mannose and complex antennae structure (Haltiwanger and Lowe 2004).

1.5.2 O-linked Glycosylation

O-linked glycosylation is the attachment of sugar chains to oxygen of a hydroxyl group in serine, threonine or hydroxyl-lysine residues (Luther and Haltiwanger 2009). In contrast to the collective transfer of the lipid-linked oligosaccharide to protein during N-glycosylation, O-glycosylation involves enzyme mediated sequential transfer of monosaccharides. O-glycosylation is the broad term used to address various types of glycan modifications, generally categorised based upon the first monosaccharide linked to the protein (Zauner et al. 2012). Mucin-type O-glycosylation is a common form where N-acetylgalactosamine (GalNAc) residue is attached to the hydroxyl group of the Serine/Threonine with no specific consensus sequence which is subjected to further elongation and branching (Li et al. 2015;

Christiansen et al. 2014). The mucin proteins are a group of proteins generally located on mucosal surfaces that form a protective barrier (Moremen, Tiemeyer and Nairn 2012). Up to 20 N-acetylgalactosaminyltransferases have been identified in human, each having specificity for different protein and tissue. Malignancies are typically identified with modifications in the mucin-type O glycosylation (Zauner et al. 2012). The less common types of O-glycosylation include O-fucosylation and O-glucosylation, which will be discussed in greater detail given their importance in Notch signalling.

1.6 Glycosylation of Notch receptor

The earliest studies of the Notch protein indicate the presence of N-glycans (Kornfeld, Reitman and Kornfeld 1981) confirmed by interaction with N-glycan binding Lentil lectin (Johansen, Fehon and Artavanistsakonas 1989). In addition to *N*-glycosylation, three unusual types of *O*-linked glycosylation have been defined on the EGF-like repeats of the Notch proteins: *O*-fucosylation, *O*-glucosylation and *O*-GlcNAcylation (Moloney et al. 2000b); Matsuura et al. 2008) (Figure 1.5). Evidence from several reports has indicated the significance of glycosylation of the Notch receptors in regulating Notch signalling (Stanley 2007; Okajima, Matsuura and Matsuda 2008; Luther and Haltiwanger 2009). Several other components involved in Notch signalling pathway, including the Notch ligands, the γ-secretase complex component Nicastrin, and the glycosyltransferase Pofut1 are also significanlty glycosylated (Panin et al. 2002; Tomita et al. 2002; Loriol et al. 2007).

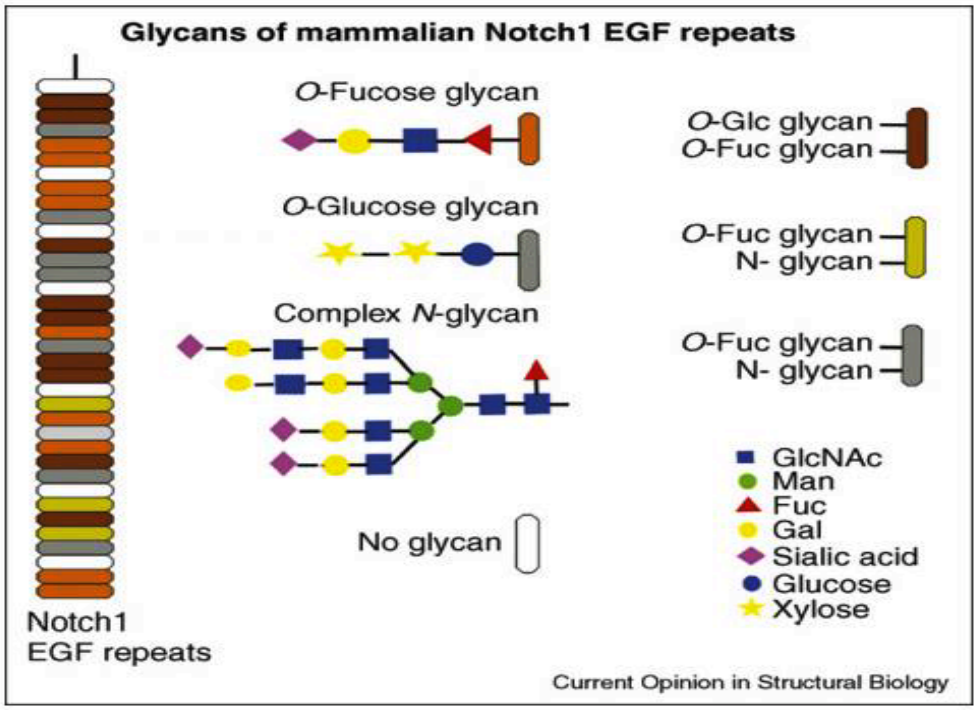

Figure 1.5. Glycosylation of the EGF repeats. O-fucose glycans, O-glucose glycans and complex N-glycans are attached to the Notch receptor EGF repeats. Protein O-fucosyltransferase (Pofut 1 in mammals) adds fucose to the glycosylation sites on the EGF-like repeats and acetylglucosaminyltransferases of the Fringe family (Lunatic, manic and radical Fringe in mammals) elongates the carbohydrate chain by adding GlcNAc (N-acetyl glucosamine) residues. All Notch receptors have consensus sequence being regulated by these O and N-glycans. The largest O-fucose glycan observed to date is sialic acid α(2,3)Galβ(1,4)GlcNAcβ(1,3)Fuc-O and the largest O-glucose glycan is Xylα(1,3)Xylα(1,3)O-Glc-O. (Adapted from Stanley 2007; Sala et al. 2012; Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010).

1.6.1 O-fucosylation of Notch

The specific enzymes responsible for the attachment of *O*-fucose to EGF-like repeats and its elongation have been well charactersied. The addition of fucose residues to the hydroxyl group of serine and threonine takes place in the endoplasmic reticulum (ER) and is driven by Pofut1 (Ofut1 is *Drosophila*) (Wang et al. 2001; Luo and Haltiwanger 2005; Okajima et al. 2005), with GDP fucose acting as a substrate donor of fucose. The importance of this modification has been addressed in gene-knock out experiments, where the depletion of Pofut 1 resulted in

embryonic lethality in mice (Takeuchi and Haltiwanger 2014). It is believed that Pofut 1 plays a functional role in all Notch homologs as Pofut 1-deficient embryos displayed greater severity in phenotype than that of any Notch mutant (Haltiwanger and Lowe 2004). The addition of GlcNAc to the *O*-linked fucose then takes place in the Golgi, by Fringe (Fringe in *Drosophila*; Lunatic, Manic and Radical Fringe proteins in mammals) (Bruckner et al. 2000; Moloney et al. 2000b; Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010).

As previously mentioned, Protein O-fucosyltransferase 1 (Pofut 1) initiates the addition O-fucose to Ser/Thr which takes place within the consensus sequence C^{2} – X-X-X-X (S/T)-C^{3} (where X is any amino acid) between the second and third cysteine in the EGF repeats. This sequence is more common and conserved within the Notch family than any other protein family (Takeuchi and Haltiwanger 2014a). O-fucose is further elongated by the addition of N-Acetyl glucosamine (GlcNAc) (Moloney et al. 2000b; Bruckner et al. 2000) followed by galactose and sialic acid respectively (Xu et al. 2007).

Fringe is a glycosyltransferase (Table 1.2) known to regulate Notch signalling, though the exact mechanism is not fully known (Moloney et al. 2000b). Fringe mediated addition of GlcNAc to O-fucose has been shown to have no impact on protein configuration, however, the modification has been linked to enhanced interaction between Notch receptor and delta like 1 ligand (DLL-1). While other studies have found that Fringe inhibits Jag-1-induced Notch signalling. There is a possibility that the regulatory effects observed could be due to direct ligand interaction with the modified glycans (Moloney et al. 2000b, Takeuchi and Haltiwanger 2014).

Previous studies on Chinese hamster ovary (CHO) cells have identified the role of Beta-4-galactosyltransferase-1 (β4GalT-1) and Alpha-2, 3-sialyltransferase in the elongation of the *O*-linked disaccharide on the mammalian Notch1 to its longest form, the sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc-α-*O*-Ser/Thr tetrasaccharide (Stanley 2007).

1.6. 2. O-glucosylation of Notch

The NECD is also modified with O-glucose glycans. O-glucose is linked to the serine residue within the consensus sequence C^1-X-S-X-P-C^2 between the first and the second cysteine of a specific EGF (Moloney et al. 2000a; Wang et al. 2001). Drosophila Notch is O-glucosylated by a protein O-glucosyltransferase, Rumi. Three Rumi homologs have been identified in mammals POGLUT 1, KDELC1 and KDELC2, though only POGLUT 1 functionality is known. Depletion of POGLUT 1 in mice results in embryonic lethality and defect in phenotypes (Takeuchi and Haltiwanger 2014) indicating that O-glucosylation, similar to O-fucosylation, is required for the functionality of Notch.

Protein O-glucosyltransferase (Poglut 1) initiates the addition of O-glucose to the EGF repeats and takes place in a similar manner to O-fucosylation. In mammalian Notch, the O-glucose glycans are elongated with the addition of two alpha 1, 3 linked xyloses, a process initiated by specific xylosyltransferases (Moloney et al. 2000a; Whitworth et al. 2010; Takeuchi and Haltiwanger 2014). They use UDP-xylose as a donor substrate. O-glucose consensus sequences are located on the EGF repeats of NECD with trisaccharides the primary structure identified on each site. It is not clear how O-glucosylation affects Notch signaling. There is speculation that

this form of carbohydrate modification may be involved in the correct folding, stabilisation and transport of the Notch receptor, therefore regulating its activity (Takeuchi and Haltiwanger 2014). A report from Luther and Haltiwanger successfully demonstrated that mutations in Rumi in Drosophila produced a temperature sensitive Notch receptor. This suggests that O-glucosylation might have an important role in protein configuration and possibly in stabilising Notch in its active form (Luther and Haltiwanger 2009).

Table 1.2. Common mammalian Notch glycosyltransferases. Inactivation of these Notch specific glycosyltransferases has been shown to negatively impact Notch signalling suggesting that they play an important role in its activity (Adapted from Takeuchi and Haltiwanger 2010, 2014)

Gene	Subcellular localization	Effect on Notch
Pofut1/Ofut1	ER	Essential
Lunatic Fringe	Golgi	Notch activation*
Manic Fringe	Golgi	Notch activation*
Radical Fringe	Golgi	Notch activation*
Poglut1/Rumi	ER	Essential

*Fringe activates Notch signalling from delta ligands but typically inhibits signalling from serrate/Jagged ligands (Takeuchi and Haltiwanger 2010, 2014).

1.6.3 O-GlcNAcylation of Notch

Recently, O-GlcNAc modification has been identified on both Notch receptor proteins and ligands. In mammals, the enzyme involved in this process is an EGF-specific O-GlcNAc transferase known as Eogt1 but the exact mechanism is not known. However, a rare autosomal recessive disorder has been linked to mutations in human Eogt1 suggesting that it is possible that the O-GlcNAc modifications can impact Notch function (Takeuchi and Haltiwanger 2014).

1.6.4 O-N-Acetylgalactosamine (O-GalNAc) modifications of Notch

In addition, a mucin type O-GalNAc glycan found outside the NECD EGF repeats has been discovered to modify Notch signalling activity (Boskovski et al. 2013). The addition of the O-GalNAc to the EGF repeats is aided by a group of proteins N-acetylgalactosaminyltransferases (GALNT) that is composed of 20 isoforms in human (Bennett et al. 2012). This type of modification has been shown to also control Notch activity. However, there is no known consensus sequence for this form of glycosylation, yet the addition of O-GalNAc to a threonine residue has been demonstrated to promote proteolytic cleavage event (Takeuchi and Haltiwanger 2014).

1.6.5 N-Glycosylation of Notch

There is limited information on the N-glycosylation of Notch. What is known is that there are several consensus sequences in NECD for the addition of complex type N-glycans to the EGF repeats, Asn-X-Ser/Thr (where X is any amino acid except proline). Very little is known with regards to the function of N-glycosylation

on the Notch receptor activity and this project seek to establish a role for N-glycosylation in Jag-1-induced Notch signalling.

1.7 Stem cells and surface glycans

Stem cells are specialized cells with the ability for self-renewal through cell division and capability of differentiating into multiple lineages. They are categorized as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (Ullah, Subbarao and Rho 2015). Several stem cell bio markers have important carbohydrate epitopes which control many stages of stem cell biology such as proliferation and development (Lanctot, Gage and Varki 2007), the composition of the glycans and their lineage-specific nature can be very helpful in determining their role and function during stem cell differentiation and can provide a suitable means for identification (Lanctot, Gage and Varki 2007; Varki 2006; (Spiro 2002). It is important to be able to monitor and control their differentiation state during tissue repair. This can be achieved through study of their biomarkers, however it can be problematic as they are non-specific. An alternative means is required to study the glycosylation markers for precise labelling and tracing of those cells, which will make them a good candidate for cell-based therapeutic applications such as in cardiovascular diseases.

Surani published one of the first reports on the role of N-glycans in embryonic development in 1979. They used tunicamycin, a mixture of homologous nucleoside antibiotics that block the UDP-GlcNAc: dolichol phosphate acetyl glucosamine -1-phosphate transferase. Thus, inhibiting the formation of N-glycans caused severe abnormalities in the early stages of mouse embryonic development. (Surani 1979; Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010). Several groups have since

been able to identify the specific enzymes responsible for the addition and elongation of these glycans in model organisms through advanced genetics and molecular biology, biochemical identification and analysis of carbohydrates (Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010).

Many studies have identified specific stem cell markers for embryonic, hematopoietic and neural stem cells through their respective membrane surface glycans (Hamanoue et al. 2009; (Yagi et al. 2012; Kumar et al. 2013). Particularly N-Glycan lineage specific epitope Lewis X/SSEA-1 is an active regulator of Notch signalling pathway in maintaining stemness of neural stem cells (NSCs) (Yagi et al. 2012).

1.8 Current methods for identification and characterisation of glycosylated proteins

The two most common and widely researched forms of glycosylation on proteins are *N*- and *O*-type, which are determined by how the polypeptide backbone links with the glycan structure. Detection and identification of the glycan structure offers the possibility of understanding the role of glycans in cellular activities. Due to the complex nature of glycan structures, glycome profiling is generally more challenging than the proteome or genome's where respective subunits are linearly linked (Bertozzi & Sasisekharan, 2009). There can be multiple isoforms of glycans found within a homogeneous cell populations, therefore it is crucial that glycosylation is accurately determined to gain insight in the role of glycosylation in relation to the proteome and genome.

1.8.1 Detection of glycosylated proteins

The two techniques often used to detect glycosylated proteins are staining and affinity based. The most basic means of establishing the glycosylation state of a protein is to resolve it on SDS-PAGE and stain the gel using Periodic Acid Schiff (PAS) reaction. However, affinity based tools are more specific and enable the determination of the glycosylation type (Roth, Yehezkel and Khalaila 2012). Lectins are carbohydrate-binding proteins that interact with sugar groups based on their affinity and no enzymatic activity is involved. Commercially available lectins are usually plant-derived (Vandenborre, Smagghe and Van Damme 2011), some carbohydrate binding proteins are identified in mammals where they play a role in cell adhesion and immune recognition (Varki et al., 2009a). Commercial lectins are well characterised and can offer a simpler means of identifying the basic sugar groups on a glycoprotein in place of more sophisticated techniques such as mass spectrometry. Labelled lectins have previously been used to stain cells in microscopy and flow cytometry analysis (Varki and Lowe 2009; Roth 2012; Wang et al., 2008) or to detect glycosylation on glycosylated proteins (Thompson et al. 2011).

Once protein glycosylation has been confirmed, the glycan structure can be characterised further by chromatography and mass spectrometry. The analysis could be done on glycan attached to the protein or following its release (reviewed in Roth, Yehezkel and Khalaila 2012). Analysis of intact glycoprotein is less reliable than that of released glycans. Depending on the nature of the glycan, it can be released from the protein by enzymatic or chemical means (reviewed in Roth, Yehezkel and Khalaila 2012).

Enzymatic cleavage of glycans: The majority of the commercially available glycan-cleaving enzymes are only suitable for N-glycans with limited availability for O-glycans. Glycoamidase F (PNGase F) is the most effective for releasing N-glycan fragment. It does this by cleaving the bond between GlcNAc and an Asparagine residue. PNGase F removes most form of N-linked oligosaccharides, high mannose, complex and hybrid type including oligosaccharides with sulfate substituted residues. However, a much higher concentration of PNGase F may be needed for native proteins (O'Neill 1996).

Chemical realease of glycans: β-elimination and hydrazinolysis are the two main methods for chemical release of glycans from protein, generally more applicable for O-glycans. β-elimination expose the glycoprotein to alkaline environment, this causes the release of glycan. In hydrazinolysis, the glycan is release by hydrolysis reaction at a specific temperature, this involves the addition of anhydrous hydrazine to a salt free lyophilized glycoprotein (reviewed in Roth, Yehezkel and Khalaila 2012).

Following the removal of glycan from the glycoprotein, free glycans are typically in a solution that may contain salts, detergents, proteins, peptides, amino acids etc. It is necessary to remove these contaminants before further analysis can be performed. Some common methods applied are graphitized carbon desalting method and dialysis. Following purification, two techniques may be considered for analysis of glycan structure, chromatography and Mass spectrometry. These methods could be applied separately or coupled (Packer et al. 1998).

1.8.2 Glycan analysis by Chromatography

In chromatography, fluorescent labelling of the glycan fragment is generally desirable to improve detection. Common fluorescent molecule, 2-aminobenzamide (2-AB) is linked to the glycan reducing end by reductive amination (Bigge et al. 1995). The traditional chromatographic techniques employed for separation of glycan are weak anion exchange (WAX), size exclusion chromatography (gel filtration) and normal phase high performance liquid chromatography. Weak anion exchange and size exclusion chromatography generally require large amounts of oligosaccharides. Chromatographic methods allow for relative glycan quantification, the peak area can be used to evaluate the percentage of a specific type of oligosaccharide out of the total glycans and the same oligosaccharide can then be quantified relatively across several samples (reviewed in Roth, Yehezkel and Khalaila 2012).

1.8.3 Glycan analysis by Mass Spectrometry

Analysis of free glycans can be performed using two standard mass spectrometric soft ionization techniques, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Though highly sensitive analysis (pmol-fmol order) can be achieved with MALDI-MS, it is not sensitive for detection of sialylated and sulfated glycans. ESI MS is desirable for the analysis of sulfate and sialic acid residues (Yamada and Kakehi 2011). Mass spectrometry (MS) can be useful in gaining information about protein, peptide sequences and glycan structures. For sample preparation, enrichment of glycoproteins is usually necessary and this can be done by lectin chromatography (Hamanoue and Okano 2011). It requires specialized training, access to instrumentation and correct sample preparation.

Summary

From all the reports, it is evident that myogenic differentiation of resident and/or circulating stem cells plays a major role in vascular development and remodelling. Our group hypothesised that vascular SMC accumulation following vascular injury or restenosis is due in part to differentiation and transition of resident vascular stem cells to SMC under the control of a Notch pathway contrary to the original hypothesis that de-differentiated SMCs were exclusively responsible. However, the distinct mechanism involved in these signalling pathways in the differentiation of stem cells is yet to be fully determined.

Glycans play major roles in cell interaction, as they are usually the first points of contact between cells. They facilitate many interactions both in cis (on the same cell) and in trans (on different cells). The glycan covering (glycocalyx) that surrounds the cell surface can control the binding of canonical protein ligands to their cell surface receptors by either enabling it or inhibiting it, as well as mediating ligand-independent receptor clustering and activation (Bishop, Schukz and Esko 2007; Coles et al., 2011; Haines and Irvine, 2003; Rogers et al., 2011). Researchers are only beginning to understand the essential roles of cell-surface glycans in controlling cellular signalling events (Griffin and Hsieh-Wilson 2016).

Several reports have shown the important role of O-glycan modifications in regulating Notch signalling in both vertebrate and invertebrate (Haltiwanger 2002, Haltiwanger and Lowe 2004; (Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010). Preliminary studies by Johansen's group, as far back as 1989 reported Notch receptor as N-glycosylated protein, however it is not clear how this form of glycosylation control Notch activity.

The overall purpose of this research project is to study the putative role of N-glycosylation of Notch1 receptor and the influence on resident vascular stem cell fate. Ligand-induced Notch signalling assays will provide insights into how alterations in the N-glycans of the Notch 1 regulate Notch signalling. Studying the glycosylation profile of Notch1 and role of the N-glycans in Notch signalling will enable us to better understand the impact of N-glycosylation in stem cell differentiation towards vascular smooth muscle cells and the findings could serve as a means of regulating myogenic differentiation in vivo during disease progression.

1.9 Aim and Objectives of the study

Aim: To establish a role for N-glycosylation of Notch 1 receptor in the transition of resident vascular stem cells to vascular smooth muscle cells *in vitro*.

Objectives :

- To establish platforms for measuring N-glycosylated proteins using liquid chromatography and enzyme linked lectin assay (ELLA).
- To determine the glycosylation state of Notch 1 receptor and determine the effects of Jag-1 activation of the Notch1 receptor on resident vascular stem cell fate.
- To examine the role of N-Glycans on the extracellular domain of the Notch 1 receptor in controlling Jag-1 promotion of myogenic differentiation.
- To study the N-glycosylation profile of resident vascular stem cell populations before and after myogenic differentiation.

Chapter 2:

Materials and Methods

2.1 Materials

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

Cell lines

Table 2.1: Cell lines used in the study

Cell lines	Description
CHO DP-12	CHO DP-12 clone is an adherent cell line that was selected in methotrexate and engineered to express human IgG1 mAb against IL-8 (anti-IL-8 mAb). Cells were a kind gift from Dr. Niall Barron (NICB, Dublin City University).
mMSCs	GIBCO® Mouse (C57BL/6) or S1502 Invitrogen are an adherent cell line derived from bone marrow isolated from C57BL/6 mice at ≤ 8 weeks of gestation through mechanical and enzymatic digestion.
mSMCs	They are primary vascular aortic smooth muscle cells isolated by collagenase - elastase digestions. Murine aortic SMCs were obtained from ATCC, Rockville (MOVAS (ATCC CRL-2797))
mMVSCs	The cell line was obtained from explant cultures of thoracic region of a mouse aorta (procedure carried out by a member of the lab (Kennedy et al., 2014b).

Bacterial strains used in the study

Table 2.2: Bacterial strains used in the study

Bacterial strain	Genotype
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15].

Expression and Reporter constructs

Table 2.3: Plasmids used in the study

Plasmid	Source	Description
pEGFP-N1	Dr. Ronan Murphy Dublin City University	Green fluorescent protein. Fusions to the N terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein in vivo. pEGFP-N1 can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker)
pPGKpuro	Dr. Dermot Walls Dublin City University, Ireland	Puromycin resistance plasmid (Tucker et al., 1996).
pCS2 Notch1 Full Length-6MT	Prof. Raphael Kopan (Addgene plasmid No. 41728	pCS+ NFL contains the Notch-1 protein up to amino acid 2184 fused to a hexameric Myc tag at the carboxy terminus (Schroeter, Kisslinger and Kopan 1998).
pCS2 Notch1 ICv-6MT	Prof. Raphael Kopan (Addgene plasmid No. 41730	Notch1 intracellular domain, contains murine Notch-1 protein beginning at Valine 1744 (aa 1744-2184) fused to a hexameric Myc tag at the carboxy terminus (Schroeter, Kisslinger and Kopan 1998).

Small (or short) interfering RNA (siRNAs)

Table 2.4: Small (or short) interfering RNA (siRNAs) used in the study

siRNA	Source/Code	Description
TriFECta kit	IDT, MMC.RNAI.N008714.12	Target-specific duplexes against Notch 1 gene and three control sequences including a TYE™ 563-labeled Transfection Control RNA duplex, a scrambled universal negative control RNA duplex and a positive control Dicer-Substrate RNA duplex (HPRT-S1 DS Positive Control)
Lunatic Fringe (Lfng)	Qiagen, SI01089347	Target duplex against Lfng, sequence CAGCCGAGGGCTGGCCCTAAA

Primer sets

Table 2.5: Primer sets used in this study. PrimeTime ® qPCR primers were obtained from Integrated DNA Technologies (IDT) and Quantitect Primer Assay from Qiagen.

Target gene	IDT	Qiagen
HPRT	Mm.PT.58.198117239 Mm.PT.58.198117115	QT00166768
Notch1	Mm.PT.58.28794468	-
CNN1	Mm.PT.58.12652862	QT00105420
Hey1	Mm.PT.58.31332652	QT00115094
MYH11	Mm.PT.58.11741166	QT01060843
Lfng	-	QT00105742

Antibodies

Table 2.6: Antibodies used in the study and dilutions. Primary and secondary antibodies for immunocytochemistry studies and western blotting were diluted in 1-2.5% BSA.

Primary Antibodies	**Code**	**Dilution**
mouse monoclonal Anti-Myc (Origene)	TA150121	ICC: 1/100 WB:1/500
rabbit monoclonal Anti-Notch 1 (Abcam)	(EP1238Y) ab52627	WB:1/1000
rabbit monoclonal Anti-Sox 10 (Abcam)	(EPR4007) ab155279	ICC:1/250
mouse monoclonal Anti-smooth muscle myosin (Myh11) (Abcam)	ab683	ICC:1/250
rabbit monoclonal Anti- Cnn1 (Abcam)	(EP798Y) ab46794	ICC:1/250
mouse monoclonal Anti- Nestin (Abcam)	ab11306	ICC: 1/250
mouse monoclonal Anti-Beta Actin (Sigma)	A2228	WB:1/5000
mouse monoclonal Anti- smooth muscle Actin (Sigma)	A5228	WB:1/5000
Secondary Antibodies	**Code**	**Dilution**
HRP-Conjugated Anti-mouse IgG (Sigma)	A5278	WB:1/5000
HRP- Conjugated Anti-rabbit IgG (Sigma)	A0545	WB:1/5000
Alexa Fluor® 546 Goat anti-mouse IgG (Invitrogen)	A-11030	ICC:1/1000
Alexa Fluor® 546 Goat anti-rabbit IgG (Invitrogen)	A-11035	ICC:1/1000
Alexa Fluor® 488 Goat anti-rabbit IgG (Invitrogen)	A-11008	ICC:1/1000
Alexa Fluor® 488 Goat anti-mouse IgG (Invitrogen)	A-11001	ICC:1/1000

Chemical and Molecular Biology Reagents

Table 2.7: Chemical and Molecular Biology Reagents used in this study

Supplier	Product/Code
Abcam	Rabbit Monoclonal (EP1238Y) antibody to Notch 1, ab52627 Anti Sox10, ab 155279 Anti- smooth muscle Myh11, ab683 Anti-Cnn1 antibody, ab46794 Anti-Nestin antibody, ab11306
ATCC	Fetal Bovine Serum, embryonic qualified, ATCC-SCRR-30-2020
Bio-rad	4 x SDS Laemmli Buffer, 161-0747
Fisher Scientific	Protein A columns, 10615575
Invitrogen	Gibco MEM alpha media, 1418345 MSC Fetal Bovine Serum, 1627842 Gentamycin, 1386826 TrypLE dissociation Reagent, 1697614 Alexa Fluor® 488 Goat anti-rabbit (H+L) (A-11008) Alexa Fluor® 546 Goat anti-rabbit (H+L) (A-11035) Alexa Fluor® 488 Goat anti-mouse (H+L) (A-11001) DEPC treated water, 750024 OPTI-MEM reduced serum medium, 11058021 NuPAGE® MOPS SDS Running Buffer (20X), NP0001 NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, 1.0 mm, 10 well, NP0321BOX Invitrogen Purelink Midiprep kit, 518-02-0402
MSC	Magcore cartridges total RNA Cultured Cells 610, MRC-01 // MRC-02 Sensi-fast SYBR No-Rox one step kit, BIO-72005)
Mirus Bio	TransIT - X2 delivery system, MIR6000
New England Biolabs (NEB)	PNGase F, P0704S

Novus Biological	Notch1 Overexpression Lysate (Native) 0.1 mg, NBL1-13725
Origene	Anti-Myc (9E10) Magnetic beads, TA150044 Anti-Myc Monoclonal Antibody, TA150121
Pierce/Thermo scientific	Dnase I, 89836 BCA Assay kit, 23227 Page ruler ladder, 26620 Mem-PER Plus Kit, 89842 Recombinant Protein G, pro-402-10mg
Promega	JM109 Competent Cells, L2005
Qiagen	Rotor-Gene SYBR® Green RT-PCR Kit. 204174 Rnase free Dnase I, 79254
R & D systems	Recombinant rat Jag-1 Fc, 599-JG Recombinant Human IgG1 Fc, 110-HG Recombinant Human Notch-1 Fc Chimera, 3647-TK-050
Sigma Aldrich	Sterile Phosphate Buffered Saline (PBS) Solution with Calcium and Magnesium, D8662 PBS tablets, P4417 DPBS without Calcium and Magnesium, D8537 Dulbecco's Minimum Essential Medium. D5796 Trypan Blue, T8154 4', 6-Diamidino-2-phenylindole (DAPI), D9542 Formaldehyde, F8775 Polyvinyl Alcohol (PVA), P8136 Trizma base, T1503 Sodium Chloride, S7653 Hydrochloric Acid, H1758 Manganese Chloride, M1787 Calcium Chloride, 21114 Magnesium Chloride, M1028 L-glutamine, G7513 Ponceau S solution, P7170 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate for Membranes, T0565 Notch1 ELISA kit, RAB0384 Kanamycin sulfate, K4000

	Puromycin, P8833 Ampicillin sodium salt, A9518 c-Myc peptide, M2435 Monoclonal Anti-Actin, α-Smooth Muscle antibody produced in mouse, A5228 Serum free media, 14360C HRP-labelled anti-biotin labelled antibody, A5278 Sodium Citrate, W302600 Citric Acid, C2404 TMB Tablets, T3405 Hydrogen Peroxide, 18312 Sulphuric Acid, 32,050-1 RIPA lysis buffer, R0278 Protease Inhibitor, P8340 LB Broth, Sigma L3022 Isopropanol, I9516 Ethanol, E7023 Penicillin/Streptomycin, D4333 Fetal Bovine Serum, F9665 Bovine Serum Albumin, A4503 Tunicamycin, T7765 Deoxymannojirimycin Dimethyl sulfoxide, 41639 Triton X-100, T8787 Tween 20, P1379 Glycerol, G5516 Agar, A1296 Minimum Essential Medium, 56416C Sodium Pyruvate solution, S8636 Sodium Bicarbonate solution, S8761 Glycine, G8898
Tocris	1-deoxymannojirimycin hydrochloride, 73465-43-7
Vector Laboratories	Biotinylated Lectins: Concavalin A, B1005 Soybean Agglutinin, B1015 Aleuria Aurantia, B1385 Maackia Amurensis lectin I, B1315 Peanut Agglutinin, B1075 Lens Culinaris, B1045 Wheat Germ Agglutinin, B1025

2.2 Cell Culture methods– Cell Lines

All cell culturing techniques were carried out under sterile conditions using 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 Nikon Eclipse TS100 phase contrast microscope. The cells were cultured in 175, 75, 25 cm^2 flasks and six well plates and were routinely fed every 3-4 days. Cultured cells are generally quiesced in low serum (0.5% FBS) media for 24-48 h before treatments unless otherwise stated. Cell lines were checked routinely for Mycoplasma contamination using the MycoAlert^{TM} Mycoplasma Detection Kit, a selective biochemical test that exploits the activity of mycoplasmal enzymes which are found in all six of the main mycoplasma cell culture contaminants and the vast majority of 180 mycoplasma species.

CHO DP-12 clone cell line

The CHO DP-12 cells were initially cultured in 50:50 DMEM / Ham's nutrient F12, supplemented with 1% Fetal Calf Serum or Fetal Bovine Serum, 1% Penicillin Streptomycin and 400nM Methotrexate (MTX). They were then adapted to grow in serum free media supplemented with 8mM L-Glutamine, 1X ITS and 200nM MTX.

Mouse Mesenchymal stem cells (mMSCs)

GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells (Invitrogen, Catalog no. S1502-100) were cultured in MEM alpha supplemented with 10% MSC-Qualified FBS and 5μg/ml gentamicin.

Mouse Aortic Smooth muscle cells (mSMCS)

A murine SMCs cell line (MOVAS (ATCC® CRL-2797™) was cultured in DMEM supplemented with 10% FBS and 1% Penicillin Streptomycin.

Mouse multipotent vascular stem cells (mMVSCs)

Murine mMVSCs were isolated from aortic explants and cultured in EMEM supplemented with 10% FBS and 1% Penicillin Streptomycin as described previously (Kennedy et al., 2014b).

2.2.1 Subculturing and Expanding

Once the cells reached about 90% confluence, they were split accordingly. Old media was removed from the flask and cells were rinsed with 1 X Phosphate buffered saline (PBS) or Dulbeccos Phosphate buffered saline (DPBS). Appropriate volume of pre-warmed 1X trypsin or 0.25X tryple solution was added to the flask of cells, and placed in a 37°C incubator for 2-5min. Flask was then gently agitated and observed under the microscope to check if cells have detached from bottom of flask. Pre-warmed complete growth medium containing serum was then added to the flask to deactivate trypsin or tryple. The cell suspension was placed in a 15ml centrifuge tube and cells spun down at 1500 rpm for 5 min. Following centrifugation, the supernatant was carefully removed and pellet was re-suspended in 3ml of complete growth medium.

2.2.2 Cryopreservation of Cell Lines

Cells were re-suspended in 1ml of freezing medium containing 10% of dimethylsulfoxide (DMSO), 30% of serum and 60% appropriate non-supplemented media and placed in cryogenic vials. Samples were kept overnight at -80°C before being transferred into liquid nitrogen for long-term storage. When required for use,

the sample of frozen cells was rapidly thawed in a water bath at 37°C and washed once with 7ml of appropriate complete medium, added gently drop-wise to prevent cell death. After centrifugation at 1500 rpm for 5 min at RT, the cell pellet was re-suspended in appropriate complete medium and grown at 37°C in the 5% CO_2, 95% humidified incubator.

2.2.3 Viable Cell Count

Viability and the cell count were determined by using a 0.4% trypan blue solution. Cell suspensions were diluted 1:1 with trypan blue and viable cells were counted on a haemocytometer (Figure 2.1) under a light microscope. Trypan blue does not usually enter healthy live cells but dead cells do take in the dye. The content of the tube was then transferred to both counting chamber of the haemocytometer.

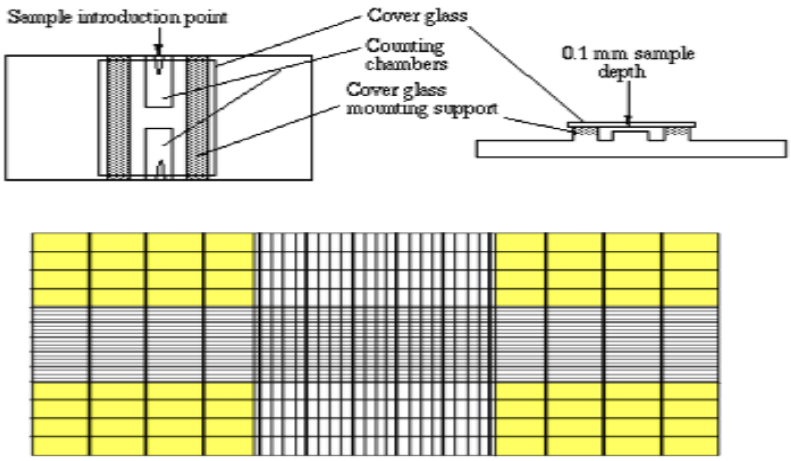

Figure 2.1. Schematic of a haemocytometer. The four quadrants are highlighted in yellow.

The cells were observed under a microscope with 10 X magnification and cells counted manually using a counter. From this, the cell density and viability are determined. The cell density represents the number of cells per ml. This was

determined by dividing the total numbers of cells counted by 4 since there are 4 quadrants, then multiplied by 2, the dilution factor, then multiplied by 10^4. Cell viability was determined using the below formula: (# of viable (live) cells / total # of cells (live + dead)) x 100.

2.3 Cell counting using Nuclear Staining

Cells were washed 3 times with room temperature PBS, then fixed by adding 3.7% paraformaldehyde in PBS and incubated for 20 min at room temperature. The 3.7% paraformaldehyde was aspirated and each well washed 3 times with PBS for 5 min with gentle agitation. Cell nuclei were stained using 2μg/ml DAPI (diluted in PBS) for nuclear visualisation, incubated for 10 min at room temperature, protecting from light. Each well washed once with PBS for 5 min with gentle agitation, protecting the plate from light. An Olympus microscope was used for imaging; all images (minimum of 5) were collected with the same software settings. The cells were counted using Fiji software package and the total number of cells extrapolated.

2.4 Immunocytochemistry

Cells were washed 3 times with room temperature PBS and fixed by adding 3.7% paraformaldehyde in PBS and incubated for 20 min at room temperature. The 3.7% paraformaldehyde was aspirated and each well washed 3 times with PBS for 5 min with gentle agitation. For intracellular staining, cells were permeabilised by adding 0.025% Triton X-100 and incubated for 10-15 min at room temperature. The washing step was then repeated. 1ml of blocking buffer (composed of 5% BSA and 0.3M Glycine in 1% PBST solution) was added to each well and incubated for 1 h at room temperature. The cells were incubated with appropriate antibody (diluted in

PBST containing 1-2.5% BSA) overnight at 4^{o}C protected from light. The wells were washed using 1% PBST solution 3 times for 10 min with gentle agitation. The secondary antibody (fluorophore-conjugated) was diluted accordingly in blocking buffer, added to the each well and incubated for 1 h at room temperature, protected from light. The washing step with PBST was repeated. Cell nuclei were stained using 2μg/ml DAPI (diluted in PBS) for nuclear visualisation, incubated for 10 min at room temperature, protecting from light. Each well washed once with PBS for 5 min with gentle agitation, protecting the plate from light. A drop of fluoroshield mounting medium was placed onto the microscope slide. The coverslips were carefully removed from the wells and blotted to remove any excess liquid and then mounted with the cells facing towards the microscope slide. The edges of the coverslips were sealed with clear nail varnish. An Olympus DP-50 fluorescent microscope was used for imaging (excitation 460-490nm, emission 515-565nm; all images were collected with the same software settings.

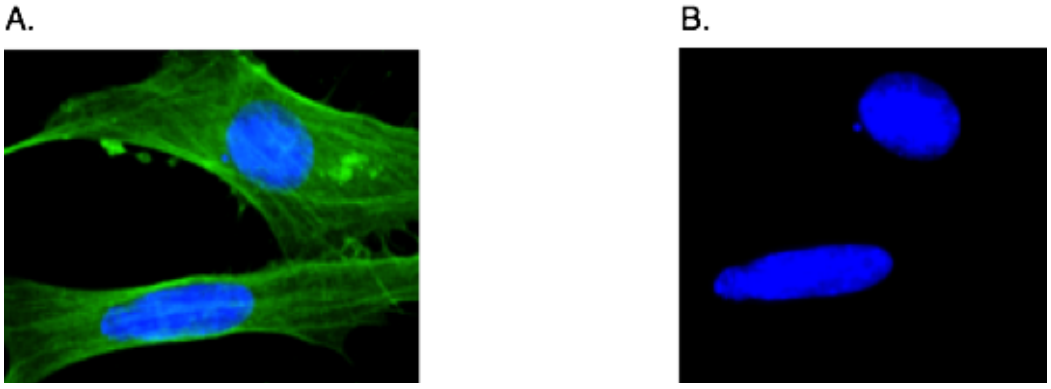

Figure 2.2. Sample immunocytochemistry image. mSMCs expressing smooth muscle Actin, (A) Cells probed with DAPI, primary and secondary antibody (B) cells probed with DAPI and Alexa fluor dye conjugated secondary antibody only (primary antibody control). DAPI stain used for nuclear visualisation.

2.5 DNA Manipulations

2.5.1 Transformations

JM109 competent cells were purchased from Promega and used for Transformation. A 100ng sample of plasmid DNA was added into 50μl aliquot of the competent cells in a sterile microcentrifuge tube. The contents of the tube were gently mixed, and then the mixture was placed on ice for 30 min, before being heat shocked at 42°C for 45 s. The tube was put on ice again for 2 min. 500μl of sterile SOC medium (20% tryptone, 5% yeast extract, 0.5% NaCl, 1% 0.25M KCl, 2% 1M glucose) without antibiotics was then added to the tube, and the content was incubated at 37°C for 45 min. All of the transformation was plated onto an LB agar plate containing the appropriate antibiotics (100μg/ml Ampicillin or 50μg/ml Kanamycin). Non-transformed competent cells were also plated as a negative control. Plates were incubated at 37^{o}C overnight. Only transformed colonies contain the ampicillin or Kanamycin resistance gene, and therefore grow on the ampicillin or Kanamycin-containing agar plates. To prepare a primary culture, a single colony was isolated and transferred to a 20ml sterile tube containing 5ml of LB broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl) supplemented with the appropriate antibiotic. This primary culture was agitated for 8h at 37^{o}C, 1.2ml of this culture was then transferred to a sterile conical flask containing 150ml broth with appropriate antibiotic and incubated overnight at 37^{o}C with agitation of 200 rpm. Glycerol stocks were prepared from the remainder of the primary culture.

2.5.2 Qiagen™ Plasmid DNA Purification

Plasmid DNA purification was carried out on the secondary culture using the QIAGEN-tip HiSpeed® system following the manufacturer's instructions for plasmid purification of animal cells. DNA was then quantified by spectrophotometric analysis as described below.

2.5.3 DNA Quantitation and Storage

To determine the amount of DNA in a sample obtained from the Qiagen plasmid midi kit, the sample was analysed using a NANODROP 1000 Spectrophotometer (Thermo scientific) blanked with TE buffer. The sample was read at wavelengths of 260 and 280nm (Figure 2.3), and the concentration of the DNA in the samples was calculated as follows; Abs260nm x dilution factor x 50 = concentration of DNA (μg/ml). The purity of the DNA was determined by calculating the ratio of absorbance at 260nm to 280nm, with ratio value of approximately 1.8 generally accepted as pure. All samples were tested in triplicate and were kept on ice at all times during the experiment. DNA samples were then stored at 20^{o}C for use in transient transfections.

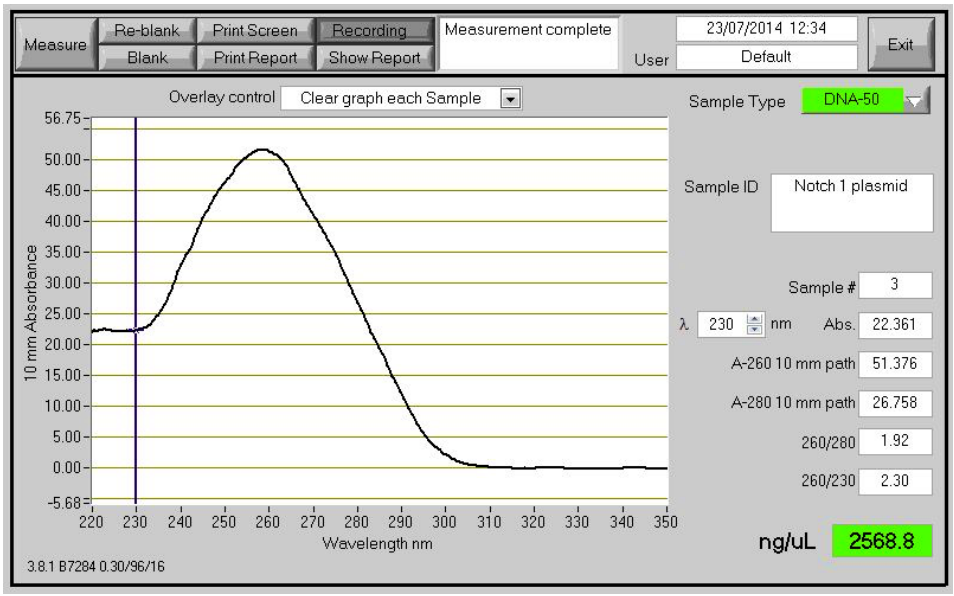

Figure 2.3. Sample Nanodrop read out for DNA Quantification

2.6 Transient Transfections

Trans IT-X2 ™ Dynamic Delivery System (Mirus, supplied by MSC) was employed for the transfection procedure. It is an advanced transfection method, non-liposomal polymeric system that enables high transfection efficiency in many cell types, including primary cells. Trans IT-X2 ™ Dynamic Delivery System can be used for DNA delivery, siRNA delivery or simultaneous delivery of DNA and siRNA.

Approximately 18-24 h before transfection, cells were seeded at an appropriate density (Table 2.8 and 2.9) in complete growth medium. Cells were allowed to grow overnight in the incubator to reach about 80% confluent at the time of transfection. On the day of transfection, spent media was replaced with fresh complete growth media. *Trans*IT-X2 transfection reagent was warmed to room temperature and vortexed gently before use. Plasmid DNA or siRNA or

combination of both DNA and siRNA was added to Opti-MEM I reduced serum medium in a sterile tube and gently mixed. *Trans*IT-X2 was added to the mixture and incubated at room temperature for 30 min to allow sufficient time for complexes to form. The complexes were added in drop-wise to different areas of the culture vessel and then rocked back and forth and side to side for even distribution. The cells were incubated at 37°C from between 24-72 h and the cells were then harvested and assayed as required. Cells were routinely transfected with a green fluorescent protein (GFP) encoding plasmid or with a TYE 563-labeled siRNA control as a means to determine approximate levels of transfection. Transfection efficiency was monitored by fluorescence Microscopy and Western blot.

Table 2.8. Conditions used for DNA transfections.

	6well plate	**T25 flask**	**T75 flask**
Cell density	2×10^5	5×10^5	2×10^6
Complete growth medium	2.5ml	5ml	19.7ml
Serum free medium	250μl	630μl	1911.4 μl
DNA (1μg/μl)	1μl	5μl	7.6 μl
Trans IT-X2	7.5μl	19μl	57 μl

Table 2.9. Conditions used for DNA and siRNA co-transfections.

	siRNA Transfection	**DNA and siRNA co-transfection**
	6 well plate	T25 flask
Cell density	2×10^5	5×10^5
Complete growth medium	2.5ml	5ml
Serum free medium	250μl	630μl
siRNA (10μM stock) 10nM final	2.7μl	6.7μl
DNA (1μg/μl)	-	5μl
Trans IT-X2	7.5μl	19μl

2.6.1 Puromycin selection

Cells were co-transfected with pPGK-puromycin plasmid, which offers puromycin resistance to any transfected cells. Non-transfected cells were selected out 48 h post-transfection with treatment in complete growth media with 10% FBS containing 2μg/ml Puromycin. The cells were incubated for 48h and then harvested and assayed as required.

2.6.2 Immunoprecipitation

MSCs or SMCs were transfected with Notch1 plasmid, tagged with a c-Myc epitope (peptide sequence EQKLISEEDL) to which anti-Myc antibody has high affinity and as a result enables easy isolation (pull down) of Notch1 during Immunoprecipitation. Crude lysates of transfected cells containing Notch1 or non-transfected cells (negative control) were prepared and applied to magnetic beads with pre-immobilised anti-Myc antibody (anti-Myc (9E10) overnight at 4°C with

gentle agitation, allowing the target antigen to bind to the immobilised antibody. After incubation, beads were washed extensively with lysis buffer (RIPA). The beads were washed several times and the Myc tagged Notch1 was then eluted from the beads. The washed beads were pelleted with magnet separator, and then mixed with 20µL 4x SDS sample buffer. The mixture was boiled at 95°C for 10min and the supernatant was loaded onto a SDS-PAGE gel for fractionation. The fractionated IP samples and negative control were blotted on nitrocellulose membrane and detected with anti-Myc antibody. If the eluted c-Myc tagged protein will be used for ELLA, the protein was eluted with 0.1M Glycine buffer (pH 2.0) and neutralized with 1M Tris buffer (pH 9.0) and immediately used or stored at -80^{o}C until use.

MVSCs were transfected with myc tagged Notch1 or empty plasmid. The cells were harvested, washed with PBS and lysed with RIPA : protease inhibitor cocktail. For the immunoprecipitation procedure, Thermo Scientific™ Anti-c-Myc Agarose was used. It is an immunopurification and immunoprecipitation resin specific for c-Myc-tagged proteins expressed in cell lysates. The anti-c-Myc antibody coupled to the resin is a high affinity mouse IgG_1 monoclonal antibody that recognizes the c-Myc- epitope tag (EQKLISEEDL). The agarose was used with microcentrifuge tubes, binding and elution capacities of the resin are based on a 26kDa c-Myc tagged protein.

A 20-100μl sample of Anti-c-Myc Agarose slurry was added to a tube and pelleted at 12,000 x g or 11,297 rpm. The liquid was discarded and the resin washed with one resin volume of TBS, centrifuged at 12,000 x g or 11,297 rpm for 5-10s and liquid discarded. The lysate was added to the tube bringing total volume to 200μl

with TBS. The content of the tube was incubated at 4°C overnight with gentle end over end mixing. The resin was pelleted at 12,000 x g or 11,297 rpm for 5s and the resin was washed with 500μl of TBST (TBS with 0.05%Tween-20 Detergent) 3 times.

Depending on the downstream analysis, the bound proteins were either eluted with 0.1 M glycine pH 2.0 (ELLA) or 1 X SDS-PAGE loading buffer (western blotting). To elute the bound protein, one bed volume of 0.1 M glycine, pH 2.0 was added to the resin then pelleted at 12,000 x g or 11,297 rpm for 5-10s. The eluate was collected in a fresh tube and neutralised with appropriate volume of 1M Tris pH 9.5 or 1 bed volume of 1X SDS-PAGE loading buffer was added, boiled at 95^{o}C for 5min and 20μl loaded onto appropriate wells of a gradient pre-cast gel.

2.7 Protein isolation and quantification

2.7.1 Isolation of anti-IL-8 mAb glycoprotein

The N-glycosylated protein expressed by CHO DP-12 was isolated from the supernatant using a protein affinity chromatography purification method (Huhn et al. 2009) (Figure 2.4). The supernatant was recovered and clarified by centrifugation at 1500 rpm for 5 mins. To concentrate the sample, it was placed in a Millipore ultracentrifugation device and centrifuged at 200 x g or 1458 rpm for 25mins at 20^{o}C. The concentrated samples were either stored at -20^{o}C, adding sodium azide as preservative or immediately purified by protein A affinity chromatography. The purification procedure of IgG1 antibodies was adapted from the method reported by Kim et al (2010). This technique separates protein of

interest based on a reversible interaction between proteins and a specific ligand attached to a chromatography matrix. Protein A HP Spin Trap column (GE lifesciences, Ireland), which are designed for rapid small-scale antibody purification, was employed for this part.

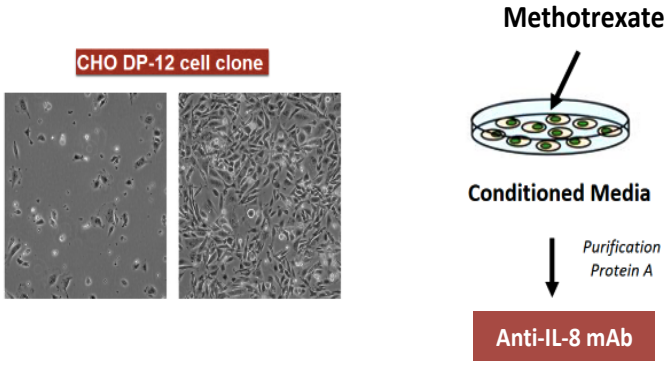

Figure 2.4. Schematic of anti-IL-8 mAb purification by affinity chromatography.

The columns are composed of Protein A Sepharose High Performance medium with a high protein binding capacity. The columns were used with a standard micro centrifuge and the purification procedure takes less than 20 min. Cell culture supernatants were directly applied to the column without prior clarification. Each sample's pH was checked and noted to be around 7 before applying to a spin column. This is important because IgG from many species has a medium to strong affinity for Protein A at approximately pH 7.0.

To equilibrate the column and remove residual storage solvent 600μl Binding buffer was added; placed in a 2ml micro centrifuge and centrifuged at 100 x g or 1031 rpm for 30s. 600μl of sample was added and incubated for 10 min at room

temperature while gently mixing using the stack and mix equipment. The column was centrifuged at 100 x g or 1031 rpm for 30 s to remove liquid. Several sample applications can be performed making sure not to exceed the capacity of the column. The column was washed twice with 600μl binding buffer and centrifuged at 100 x g or 1031 rpm for 30 s to remove liquid. To elute the IgG1, it was necessary to lower the pH to about 2.5 to 3.0. The total IgG1 antibody was eluted by adding 400μl elution buffer to the column. The column was placed in a 2ml micro centrifuge tube containing 30μl of neutralising buffer, and centrifuged for 30s at 100 x g or 1031 rpm. This step was repeated collecting the subsequent elution in a fresh 2ml micro centrifuge tube. In order to keep the sample pH neutral and preserve the activity of purified IgG, the addition of 1 M Tris-HCl, pH 9.0 was required. All samples were then stored at -80^{o}C. To regenerate the column, it was washed with two-column volume of elution buffer. The column was then re-equilibrated with 10 CV of binding buffer. After 2 elution steps, most of the bound antibody is assumed to have been eluted. The column was washed with 2 column volume (CV) of elution buffer. The column was then re-equilibrated with 10 CV of binding buffer.

2.7.2 Isolation of cellular protein

Cellular proteins were harvested using radioimmunoprecipitation assay (RIPA) lysis buffer (composed of; 20mM Tris, 150mM NaCl; 1mM Na_2EDTA; 1mM EGTA; 1% Triton X-100 (v/v); 2.5mM sodium pyrophosphate; 1mM-glycerophosphate; 1mM sodium orthovanadate; 1µg/ml leupeptin) supplemented with protease inhibitor (1/100 dilution of each, Sigma Aldrich). Growth media was removed from cells, then washed 2-3 times with PBS. A cell scraper was used to

detach the cells from the plate surface. The cells were collected and centrifuged for ten min at 1500 rpm 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 cell lysate was freeze thawed to obtain maximum yield of protein from the cells. The lysate was spun at high speed, supernatant was then transferred to a fresh tube. Samples were either stored for long term at -80°C, for short term at -20°C or analysed immediately using BCA assay to determine the overall protein concentration.

2.7.3 Extraction of membrane protein

The Thermo Scientific™ Mem-PER™ Plus Membrane Protein Extraction Kit is designed for the enrichment of integral membrane proteins and membrane-associated proteins from cultured mammalian cells or tissue. It uses a mild detergent-based approach, cells or tissue are permeabilised with a mild detergent to allow the release of soluble cytosolic proteins. A second detergent then solubilizes membrane proteins. All buffers used are supplied in the kit and supplemented with protease inhibitor before use.

For adherent cells, cells were scraped into growth media and suspension centrifuged at 300 × *g or* 2061 rpm for 5 min. The cell pellet was washed twice with cell wash solution and centrifuged at 300 × *g or* 2061 rpm for 5 min. Permeabilisation Buffer was added to the cell pellet, vortexed briefly to obtain a homogeneous cell suspension and incubated for 10 min at 4°C with constant mixing. The permeabilised cells were spun for 15 min at 16,000 × g or 13,265 rpm to pellet cells. The supernatant containing cytosolic proteins was carefully removed to a new tube.

For soft tissue, 20-40mg mouse Aorta was placed in a microcentrifuge tube. The tissue was washed with copious amounts of cell wash solution, vortexed briefly and wash discarded. The tissue was cut into small pieces with scalpel then transferred to a tissue grinder, permeablisation buffer was added, then homogenised until an even suspension was obtained (8-10 strokes). The homogenate was transferred to a new tube, incubated for 10 min at 4°C with constant mixing, and then spun at 16,000 × *g or* 13,265 rpm for 15 min at 4°C to pellet permeabilised cells. Solubilisation buffer was added to the pellet (cell or tissue) and resuspended by pipetting up and down. The suspension was incubated at 4°C for 30 min with constant mixing, then spun at 16,000 × *g* or 13,265 rpm for 15 minutes at 4°C. The supernatant containing solubilized membrane and membrane-associated proteins was transferred to a new tube. The membrane fractions were immediately used for BCA for quantification purpose then stored at -80°C.

Table 2.10. Conditions used for membrane protein extraction.

	Cell (approx. 2 x 10^6)	**Tissue (20-40mg)**
Permeabilisation buffer	250μl	500μl
Solubilisation buffer	150μl	150μl

2.7.4 Quantitation of protein by Bicinchoninic Acid Assay (BCA)

The Pierce BCA Protein Assay, a detergent-compatible formulation based on bicinchoninic acid (BCA) was employed for the colorimetric detection and quantitation of total proteins. The purple-colour is produced from the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits

a strong absorbance at 562 nm that corresponds with increasing protein concentrations over a broad working range (20-2,000 μg/ml). The working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B, both supplied in the Pierce® BCA Protein Assay Kit (Thermo Scientific). A series of dilutions were carried out on the 2mg/ml BSA standard (supplied in the kit) to prepare 0 - 2000μg/ml standard solutions. A 0mg/ml standard solution was also prepared to serve as blank standard. An aliquot of 25μl from each standard or unknown sample was added in triplicate into wells of a 96 well plate, followed by the addition of 200μl of working reagent. The plate was mixed thoroughly for 30 s, covered with a tin foil and incubated at 37^{o}C for 1h. Plate was cooled to room temperature and absorbance was measured at 562nm using a Tecan infinite 200 plate reader. The average 562nm absorbance measurement of the Blank standard replicates was subtracted from the 562nm measurements of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average Blank-corrected measurements for each standard versus its concentration in μg/ml (Figure 2.5). Quantitation was carried out by interpolation from the BSA standard curve (0-2000 μg/ml).

2.7.5 Quantitation of protein by High Performance Liquid Chromatography

Size exclusion chromatography (SEC) is a type of HPLC separation techniques for analysing proteins. The SEC column employed is usually packed with highly porous spherical silica beads and operates based on diffusion in and around the beads. The degree of retention time varies and it depends on the size and shape of the dissolved molecules. When protein sample of various sizes is injected into the column, smaller soluble molecules pass slowly through column, interacting and

penetrating into the pores, whereas large soluble molecules are quicker to pass through the column, as they do not enter the pores. As a result, larger proteins elute the column first, then subsequent elution of smaller proteins. As the name goes, size exclusion chromatography sorts the molecules by size.

The mobile phase is held in the solvent bottle and a high-pressure pump meters the mobile phase at a set flow rate, typically in millilitres per minute (ml/min). An auto sampler (injector) injects the sample into the mobile phase stream that is continuously flowing through the HPLC column. Since most compounds do not have colour and cannot be assessed with naked eyes, a detector is required to observe the separated compound peaks as they elute from the HPLC column. Once the mobile phase exits the detector, it can be sent to waste or recycled depending on the mode of separation (i.e. isocratic or gradient). The detector sends an electrical signal to the computer data station, which then translates this into a chromatogram (Figure 2.5). The chromatogram generated is useful in identifying and determining the concentration of the injected sample components. Several types of detection mode are made available because different compounds do possess specific characteristics, with UV and fluorescence detector being the most common. For example, a UV-absorbance detector would be employed for a compound that can absorb ultraviolet light. For a compound that fluoresces, a fluorescence detector is employed (www.waters.com).

All chemicals and reagents were purchased for Sigma Aldrich, Ireland. Agilent HPLC system equipped with a conventional Bio-Sil® SEC 400-5 column (300 × 7.8 mm, 5μm particle size, Bio-Rad) with programmed isocratic and gradient elution and Ultraviolet (UV) detection were employed. The mobile phase (0.2M

Potassium phosphate buffer pH 6.2) is held in the solvent container and the pressure pump was set to meter the mobile phase at a specified flow rate, in 1 ml/min. An auto sampler (injector) was set up to inject 50μl of samples into the mobile phase stream that continuously flowed through the column. A UV detector was required to observe the separated proteins peaks as they elute from the HPLC column (adapted from Diederich et al. 2011). Protein samples were analysed using isocratic parameters. With this separation mode, the mobile phase composition remained constant for the duration of analysis.

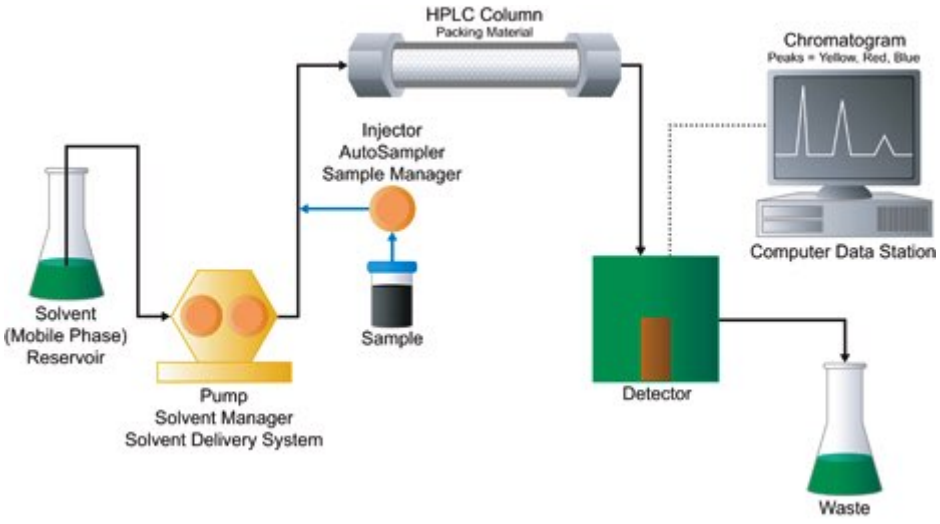

Figure 2.5. Schematic of a High-Performance Liquid Chromatography [HPLC] System. High-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components (www.waters.com)

Wavelength was initially set to 280nm, flow rate 1.0ml/min and injection volume was 20μl of sample (n=2). The chromatograms generated were used to identify and determine the concentration of the injected standards; peak height in mAU and peak

areas obtained from chromatogram was found to correlate with the concentration of standards.

Wavelength was changed from 280nm to 214nm to see the effect this would have on the peak area and absorbance unit. The peak area increased significantly by a fold change of 7 and a major increase in peak height was also observed with wavelength 214nm compared to 280nm. Wavelength 214nm was determined as the optimum wavelength and applied for subsequent analysis. The elution time would differ based on the type of separation used or the type of column, factors such as mobile phase composition, column length, temperature can affect the retention times of proteins.

HPLC parameters

- Column type: Bio-Sil® SEC 400-5 column (300 × 7.8 mm, 5μm particle size)
- Mobile Phase: 0.2M Potassium phosphate buffer pH 6.2, filtered through a 0.2μm membrane filter and degassed for 15min using a sonicator (Branson 5510).
- UV detection: 280nm or 214nm
- Flow rate: Variable
- Injection volume: Variable
- Sample run time: Variable
- Temperature: Ambient

2.7.6 Enzyme linked Immunosorbent assay (ELISA)

The Notch 1 ELISA kit used is an in-vitro enzyme-linked immunosorbent assay for the quantitative measurement of Notch1 in samples. This assay uses an antibody specific for Notch1 coated on a 96-well plate. 0 – 7ng/ml recombinant Notch1 standards and cell lysate generated from MSCs were pipetted into the wells in duplicate. The plate was incubated overnight at $4^{o}C$ with gentle shaking to allow Notch1 present in cell lysate to bind the immobilised antibody. The wells were washed and biotinylated anti-human Notch1 antibody was added. The unbound biotinylated antibody was washed off, followed by the addition of HRP-conjugated streptavidin. The wells were washed again, followed by the addition of TMB substrate solution and colour developed (blue) in proportion to the amount of Notch1 bound. The stop solution (diluted sulphuric acid) was the added to stop the reaction, changing the colour from blue to yellow, and the intensity was measured at 450nm using a Tecan infinite 200 plate reader. A standard curve was prepared by plotting the average measurements for each standard versus its concentration in pg/ml. Quantitation of Notch1 in the generated samples was carried out by interpolation from the standard curve (0-7ng/ml).

2.8 Glyosylated protein detection by Enzyme linked lectin assay (ELLA)

Glycosylated proteins were detected with Lectins (Figure 2.6). Lectins are carbohydrate-binding proteins (CBPs), which have the ability to recognise and bind to specific glycan structures (Hsu, Gildersleeve and Mahal 2008). A large number of lectins particularly from plant and animal origin, with different glycan-binding specificity are now commercially available.

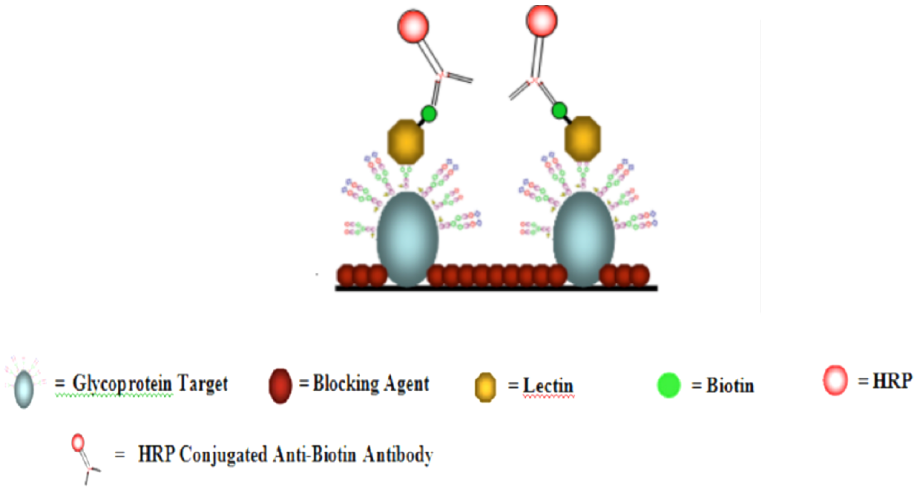

Figure 2.6. Schematic of ELLA. Target glycoproteins are immobilised by non-specific absorption on the surface of an ELISA plate. Plates are blocked, and the target glycoproteins are then probed with biotinylated lectins. Bound lectins are ultimately detected with an anti-biotin antibody (adapted from Thompson R. et al. 2011).

All reagents used were purchased from Sigma Aldrich, Ireland. All biotinylated lectins used were purchased from Vector Laboratories, United Kingdom. The enzyme linked lectin assay was performed on purified glycoprotein solutions as described by Thompson et al. (2011). Although certain assay parameters were varied throughout the series of experiments, here is a description of the basic protocol. Immobilisation of glycoprotein was carried out by adding 50μl of a glycoprotein solution to the wells of an ELISA plate (96well) and incubating overnight at 4 ^{o}C. Glycoprotein solutions used were prepared by dissolving a glycoprotein, typically at a concentration of up to 10μg / ml, in 1 X PBS. For

negative control wells, 50μl of 1 X PBS was added. The glycoprotein solution was removed by inversion. The subsequent steps were performed at room temperature. 200μl of a blocking solution (0.5% PVA in PBS) was added to all wells, and the plate was left to block for 2 h. The wells were washed four times with TBST solution. All biotinylated lectin solutions were prepared in TBST at a concentration of 5μg/ml, 50μl was added to each well, and the plates were incubated for 1 h. Unbound lectins were removed by washing each well four times with TBST solution followed by the addition of 50 μl of 1:10,000 diluted HRP labelled anti-biotin antibody to all wells and incubated for 1 h. Plates were subsequently washed four times with TBST, followed by one final wash with 1 X PBS. Plates were developed by adding 90μl of TMB substrate to each well, and the reaction was stopped with 50μl of 10% sulphuric acid (H_2SO_4) after 10 min. The absorbance was measured at 450 nm using a Tecan infinite 200 plate reader.

2.8.1 Deglycosylation of glycoproteins

Peptide N-glycosidase F (PNGase F) is a 34.8 kDa enzyme produced by the gram-negative bacterium Flavobacterium meningosepticum (supplied by Brennan and Company). It removes most of the common N-linked carbohydrates from glycoproteins. It does this by hydrolyzing the originally glycosylated Asparagine residue to Aspartic acid. Treatment was carried out using standard glycoproteins or generated glycoproteins. 1-20μg of glycoprotein, 1 μl 10X denaturation buffer and distilled water was combined in a tube (10μl total volume). The glycoprotein was denatured by heating at 100°C for 10 min then allowed to cool. The reaction volume was made up to 20μl by adding 2 μl 10X G7 reaction buffer, 2μl 10% NP40, 1 μl PNGase F (500Units) and distilled water. The reaction was then incubated at 37°C

for 3h minimum to overnight. Deglycosylated protein concentration was determined by BCA and stored at -20^{o}C. Analysis of deglycosylated sample was performed using HPLC or ELLA.

2.8.2 Inhibition of N-glycosylation

Glycosylation inhibitors disrupt the post-translational glycosylation process. Tunicamycin from Streptomyces sp, a nucleoside antibiotic, specific inhibitor of N-linked glycosylation blocks the first step of glycoprotein synthesis and 1-Deoxymannojirimycin hydrochloride, a specific α-mannosidase I inhibitor blocks the conversion of high mannose to complex oligosaccharide. Cells in culture were treated with the appropriate dose of the inhibitors or vehicle. For CHO DP-12, supernatant was recovered and purified by protein A affinity chromatography and for stem cells, cell lysate was generated. Purified anti-IL-8 mAb or total protein lysate was quantified using BCA protein assay or HPLC and ELLA analysis to monitor any change in glycosylation.

To establish the modulatory effects of tunicamycin on mesenchymal stem cell growth, 5 x 10^3 cells were seeded per well of a 6 well plate. Quiesced cells were treated with 0-100ng/ml tunicamycin or DMSO (vehicle) in complete maintenance media for 72 h. Spent growth media with dead cells were removed from the wells and the cells were washed twice with PBS. Cells were fixed with 3.7% formaldehyde, then stained with DAPI for nuclear visualisation and analysed by fluorescence microscopy. Image J was used for cell count, taking an average count from 5 images per well. Significant inhibition of cell growth was observed in the wells with tunicamycin concentrations greater than 50ng/ml.

2.9 SDS-PAGE and Western Blot Analysis

Protein concentration in samples was determined by BCA. Equal amounts of samples were then suspended in basic 4 x Laemmli buffer containing 277.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue, 10% v/v β- mercaptoethanol, heated (95^{o}C) for 5 min then immediately placed on ice. The prepared gel (Table 2.11) or commercial pre-cast gel was electrophoresed in running buffer (0.025M Tris pH 8.3; 0.192M Glycine; 0.1% (w/v) SDS) at 15mA per gel using an Atto vertical mini-electrophoresis system until the dye front reached the bottom of the gel. Following electrophoresis, the gel was soaked for 10 min in Pierce ^{TM} 1-step transfer buffer with the Nitrocellulose membrane and 4 sheets of Whatmann filter paper were cut to the same size as the gel and soaked in transfer buffer. Proteins were transferred to the membrane at 25V in a Pierce^{TM} G2 fast blotter semi-dry transfer system. The duration of transfer depends on the molecular weight (MW) of target protein: 5 min for low MW (<25kDa), 7 min for mixed range MW (25-150kDa) and 10 min for high MW (>150kDa). Following transfer, membranes were soaked in Ponceau S solution to confirm transfer of protein to the membrane. Membranes were blocked for a minimum of 1h in blocking solution (5% BSA- PBS-1%Tween) at room temperature.

Membranes were then incubated overnight at 4^{o}C with the appropriate dilution of primary antibody in blocking solution. The blots were then vigorously washed in three changes of PBST and then incubated for 1-2 h at room temperature with a suitable HRP linked secondary antibody diluted in PBST. Following incubation in secondary antibody, the blots were again washed in three changes of PBST.

Antibody-antigen complexes were detected by incubation in TMB at room temperature. Exposure times varied depending on the antibody being used but were typically between 1-5 min. Blots were photographed with a 12-megapixel, five-element lens camera. Bands of interest were identified by use of molecular weight markers (10-250kda).

Table 2.11. SDS-PAGE Gel constituents. The gels were prepared from stock solutions and APS and TEMED were added last to initiate polymerization.

Resolving gel components	Volume (10%)	Volume (12.5%)	Stacking gel Components	Volume (5%)
30% Acrylamide mix	3.3ml	4.125ml	30% Acrylamide mix	670ml
1.5M Tris pH 8.8	2.5ml	2.5ml	1M Tris pH 6.8	500ml
10% SDS	100ml	100ml	10% SDS	40ml
Distilled water	4.09ml	3.265ml	Distilled water	2.4ml
10% APS	50ml	50ml	10% APS	30ml
TEMED	15ml	15ml	TEMED	10ml

2.10 Polymerase Chain Reaction (PCR)

2.10.1 Total RNA preaparation and measurement

Total RNA was isolated from samples using Magcore® automated nucleic acid extractor (Figure 2.8). The kit no. 610 used is a cartridge that contains ready to use buffers and solutions. There are two sets of racks in the instrument where the cartridges slots in. The smaller rack holds the samples and DNAse and the larger rack holds the cartridges. The protocol provided with the instrument for sample preparation was followed. Growth media was removed and cells were washed with

PBS twice. 200μl of reaction buffer containing 1% β-Mercaptoethanol was added to cell pellet in a microcentrifuge tube. The mixture was vortexed briefly and the tube was placed into well 4 on the rack. DNase solution was prepared and placed into appropriate well 3. Program 610 option was selected to run the sample, the total duration for the RNA isolation was 73 min. The purified RNA sample was eluted in 60µl of DEPC treated water into an elution tube in Well 1 (Figure 2.7).

Measurement of the total RNA in the samples (kept on ice) was performed using the Nanodrop® ND-1000 spectrophotometer, blanked with DEPC treated water. Approximately 2.5μl of each sample was measured at wavelengths 260 and 280nm, and the amount of total RNA in each sample was determined automatically. The purity of the isolated RNA was determined from the ratio of absorbance at 260nm : 280nm. A ratio of 1.8 to 2.2 indicated a high purity of RNA. A ratio of approximately 2.0 is generally accepted as pure for RNA, if the ratio is considerably lower than 2.0, it indicates the presence of contaminants that absorb strongly at 280nm. All samples were tested in duplicate and stored at -80^{o}C until further analysis.

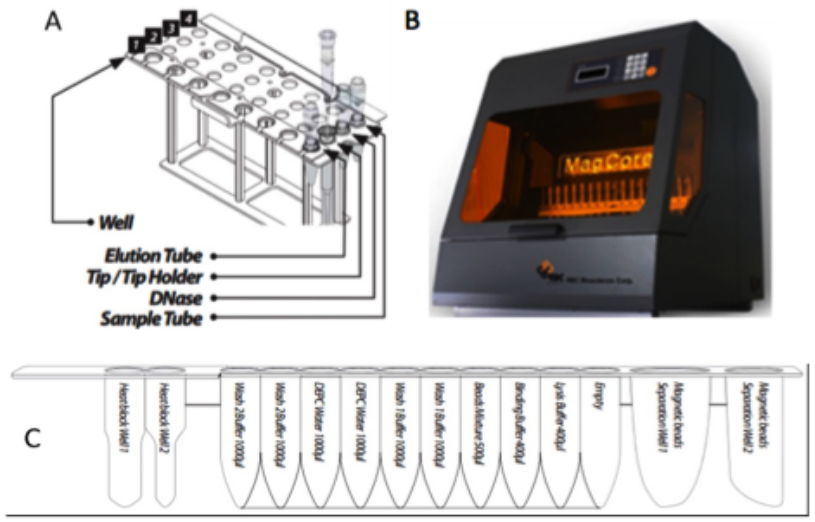

Figure 2.7. Image of Magcore automated nucleic acid extractor. The components are A) T-rack for holding samples and DNase. B) Magcore instrument. C) ready to use cartridge of solutions.

2.10.2 Primer sets and PCR kits

All primers were purchased from Qiagen and IDT (Table 2.5). Bioline Sensifast SYBR no-ROX one step kit and Qiagen QuantiTect ® SYBR Green RT-PCR kit were used with the primers. The SensiFAST™ SYBR® No-ROX One-Step Kit used is formulated for highly reproducible first-strand cDNA synthesis and subsequent real-time PCR in a single tube. The Kit consists of a 2 X SensiFAST SYBR® One-Step mix (the fluorescent component for the detection of the amplicon), as well as separate reverse transcriptase and RiboSafe RNase.

QuantiTect SYBR Green PCR kit consists of a SYBR Green master mix containing a balanced combination of K^+ and $NH4^+$ ions which encourages specific primer annealing and Taq polymerase. The kit also includes reverse transcriptase.

2.10.3 Quantitative Reverse transcription PCR (qRT-PCR)

Quantitative RT-PCR was carried out using a Real-Time Rotor-GeneRG-3000™ lightcycler (Corbett Research) with cycling conditions set up as appropriate for the kits employed (Table 2.13 and 2.15). This method measures fluorescence of the amplified product, which is relative to the amount of target gene present in the sample. It enables the quantification of specific RNA amplifications using the designated primer sets. For each gene, reaction tubes were prepared in triplicate containing all component plus reverse transcriptase and one reaction tube minus reverse transcriptase (Table 2.12 and Table 2.14). The template RNA samples were diluted accordingly with RNase-free water to achieve a suitable concentration, using up to 50ng per reaction. HPRT was used as the reference "house keeping" gene. Data are generated as sigmoidal-shaped amplification curves, in which the number of cycle is plotted against fluorescence (Figure 2.8).

Table 2.12. Sample preparation for qRT-PCR with IDT primers.

Component	Sample + Reverse transcriptase	Sample – Reverse transcriptase
2x Sensifast ™ SYBR® No-ROX One-Step Mix	10μl	10μl
10μM Forward primer	0.8μl	0.8μl
10μM Reverse primer	0.8μl	0.8μl
Reverse Transcriptase (RT)	0.2μl	-
Ribose RNase inhibitor	0.4μl	0.4μl
DEPC water	variable	variable
Template	variable	Variable
Total volume	20μl	20μl

Table 2.13. Bioline Sensifast SYBR no-ROX one step kit and cycling conditions for fast one-step RT-PCR on Rotor Gene cyclers. As per recommendation with the Sensifast kit.

Cycles	Temperature	Time	Step
1	45°C	10 min	Reverse Transcription
1	95°C	2 min	Polymerase activation
40	95°C 60°C 72°C	5s 10 s 5s	Denaturation Annealing Extension

Table 2.14. Reaction setup for fast one-step RT-PCR with Quantitect primers.

Component of tube	Volume (μl)	Final concentration
2 x Rotor-Gene SYBR Green RT-PCR Master Mix	12.5	1 x
10 x QuantiTect Primer Assay	2.5	1x
Rotor-Gene RT Mix	0.25	-
Template RNA	Variable	≤
RNase-free water	Variable	-
Total volume	25	-

Table 2.15. Rotor-Gene cyclers and Cycling conditions for fast one-step RT-PCR on Rotor Gene cyclers (QuantiTect Primer Assay Handbook).

Step	Time	Temperature
Reverse Transcription	10min	55°C
PCR initial activation step	5min	95°C

2-step cycling:

Denaturation	5s	95°C
Combined/annealing extension	10s	60°C
Number of cycles	35-40	

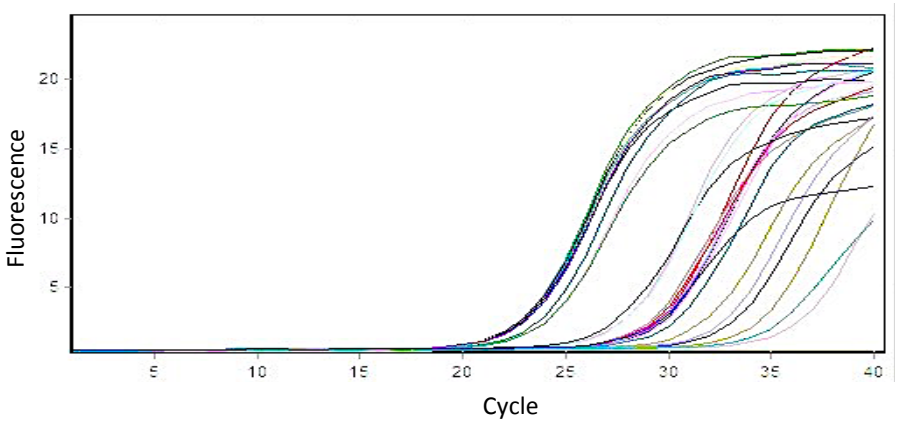

Figure 2.8. Example of qRT-PCR output data generated. The results were analysed using the Comparative CT method (ΔΔCT), described by Livak and Schmittgen, 2001.

2.11 Notch Activation

To stimulate MSCs or MVSCs differentiation towards vascular smooth muscle cells, ligand Jagged1 was employed. All steps were carried out under sterile conditions. To immobilize the Jag-1 Fc ligand on the surface, the appropriate wells of a 6 well plate with coverslips were coated with 50μg/ml protein G overnight at 4^{o}C. The following day, the coated wells were washed with PBS 3 times then blocked with PBS containing 1% BSA for 2 h at room temperature. Jag-1 Fc chimera or control Fc fragment was incubated on the surfaces for 2 h a room temperature. The surfaces were rinsed with PBS 3 times and used immediately or stored at 4^{o}C (procedure adapted from Kurpinski. et al. 2010). In order to monitor the differentiation state of stem cells following Jagged stimulation, smooth muscle markers expression, Calponin1 and Myosin heavy chain (SM-MHC) expressions can be used for early detection of differentiation of MSCs or MVSc towards vSMCs. Changes in Cnn1 and SM-MHC expression before and after differentiation of stem cells were monitored by Immunocytochemistry and qRT-PCR.

For PCR analysis, MSCs or MVSCs were seeded onto Jag-1 Fc or IgG Fc (5µg/ml) coated surface at a density of 2-5 x 10^{5} per well of a 6well plate in complete maintenance media containing 10% FBS and cultured for 12-24 h to allow the cells to adhere. The following day, the cells were supplemented with maintenance media containing 0.5% or 5% or 10% FBS and cultured for a further 72 h. Cells were harvested after treatment and RNA extraction was performed using the Magcore. The RNA samples were analysed by qRT-PCR for Notch activation of Notch target

genes (Hey1) and smooth muscle specific markers (Cnn1 and Myh11). HPRT was used as the reference gene.

For immunocytochemical studies, 10,000 cells were seeded onto Jag-1 Fc or IgG Fc (0.1μg/ml for MSCs and 1μg/ml for MVSCs) coated surface in complete maintenance medium containing 10% FBS and cultured for 12-24 h to allow the cells to adhere. The following day, the cells were supplemented with maintenance media containing 0.5% or 5% or 10% FBS and cultured for a further 5-14 days. The cells were then analysed accordingly.

When monitoring the effect of N-glycosylation inhibition on Notch activation, 200,000 mouse MVSCs were seeded in wells pre-coated with 5 μg/ml Jag-1 or IgG Fc, in complete growth media. 48 h later, spent media was replaced with fresh growth media with 5% serum containing vehicle (DMSO) or 0.5μg/ml tunicamycin. Cells were harvested 24 h after treatment and RNA extraction was performed using the Magcore. The RNA samples were analysed by qRT-PCR for Notch activation of Notch target genes (Hey1) and smooth muscle specific markers (Cnn1 and Myh11). HPRT was used as the reference gene.

2.12 Proteomic analysis by mass spectrometry

2.12.1 Sample preparation for proteomic analysis

Membrane fractions of mouse cell lines from aortic SMC, cultured SMC, cultured MSC, Jag-1 treated MSC and Fc treated MSC were analysed by mass spectrometry to study changes in the proteome of each different cell type. A 50ng/ml human Notch1 (hNotch1) standard was used as a reference standard. The membrane fractions were protein crashed with acetone. The acetone was pre-

chilled to -20 °C and membrane fractions were precipitated by adding four times the sample volume of cold acetone. Each sample was vortexed and incubated for 60 min at -20 °C. Samples were then centrifuged at 13,000 x g or 11,759 rpm for 10 minutes. The supernatant was discarded with care not to dislodge the protein pellet. The resulting protein pellets were resuspended in 6 M urea, 2 M thiourea, 0.1 M Tris-HCL pH 8.0 and quantified again using Bradford protein assay (Bio-rad). 20 μg of each sample was transferred to a fresh tube and was diluted with 50 mM ammonium bicarbonate to a final volume of 50 μl to ensure the final concentration of urea is less than 1.2 M. Each protein sample was reduced with 0.5 M DTT (Sigma) for 20 min at 56 °C and alkylated with 0.55 M iodoacetamide (Sigma) for 20 minutes at room temperate in the dark. 0.5 μL of a 1% Protease Max Surfactant Trypsin Enhancer (Promega) solution was added to each sample. 0.5 μg of sequence grade trypsin (Promega) was added to each sample (1:40 trypsin:protein ratio) and the samples were digested overnight at 37 °C. Digestion was halted by adding Trifluroacetic acid to a final concentration of 0.5%. The peptide samples were desalted using C18 Spin Columns (Pierce, Thermo Fisher Scientific) as per manufacturer's instructions. Eluted peptides from the C18 spin columns were dried using a SpeedVac and stored at -20 °C until they were ready for LC-MS/MS analysis.

2.12.2 LC-MS/MS analysis

Mass spectrometry (MS) facilitates measuring the mass-to-charge ratio (m/z) of gas-phase ions. Within a mass spectrometer, there is an ion source converting analyte molecules into gas-phase ions. There is also a mass analyser that separates the ionized analytes based on m/z ratio, and a detector recording the number of ions at each m/z value. The two soft ionization techniques generally used for ionizing

peptides or proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Han, Aslanian, and Yates 2008).

Dried peptide samples were re-solubilised in 50 μl of LC-MS grade water with 0.1% formic acid (FA) and 2% ACN. Nano LC–MS/MS analysis was carried out using an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) coupled to a hybrid linear ion trap/Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific). SilicaTip™ Standard Coating Tubing OD/ID 360/20μm Tip, ID 10 μm, Length 5cm (New Objective) were used as emitter tips for nano electrospray.

A 2.5 μl injection containing 1 μg of peptide was picked up using the Ultimate 3000 nanoLC system autosampler and loaded onto a C18 trap column (C18 PepMap, 300 μm ID × 5 mm, 5 μm particle size, 100 Å pore size; Thermo Fisher Scientific). The sample was desalted for 3 min using a flow rate of 25 μl/min in 0.1% TFA containing 2% acetonitrile. The trap column was then switched online with the analytical column (PepMap C18, 75 μm ID × 250 mm, 3 μm particle and 100 Å pore size; (Thermo Fisher Scientific)) using a column oven at 40 °C and peptides were eluted with the following binary gradients of: Mobile Phase Buffer A and Mobile phase buffer B: 0–25% solvent B in 280 min and 25–50% solvent B in a further 20 min, where solvent A consisted of 2% ACN and 0.1% FA in water and solvent B consisted of 80% ACN and 0.08% FA in water. Column flow rate was set at 300 nl/min. Data were acquired with Xcalibur software, version 2.0.7 (Thermo Fisher Scientific).

The LTQ Orbitrap XL was operated in data-dependent mode and externally calibrated. Survey MS scans were acquired in the Orbitrap in the 400–1800 m/z

range with the resolution set to a value of 30,000 at m/z 400. Up to three of the most intense ions (1+, 2+ and 3+) per scan were CID fragmented in the linear ion trap. Dynamic exclusion was enabled with a repeat count of 1, repeat duration of 30 seconds, exclusion list size of 500 and exclusion duration of 40 seconds. The minimum signal was set to 500. All tandem mass spectra were collected using a normalised collision energy of 32%, an isolation window of 2 m/z with an activation time of 30.

2.13 Data Analysis

The primary data analysis and calculations were performed with Microsoft Office Excel 2011. Results are expressed as mean ± SEM or STD. Unpaired student t-test was used for comparison of two groups or one-way ANOVA followed by post-hoc Tukey correction for comparison of three or more groups using Graphpad PRISM 6.0 (GraphPad software, La Jalla, California, USA). All statistical tests are described in the figure legends, a value of $p \leq 0.05$ was considered significant.

Mass spectrometry data files obtained were processed for protein identification using Proteome Discoverer 2.1 with Sequest HT (Thermo Scientific) and MASCOT (www.matrixscience.com) against Uniprot-SwissProt fasta database (species; Mus musculus). The following search parameters were used for protein identification: (1) peptide mass tolerance set to 20 ppm, (2) MS/MS mass tolerance set to 0.6 Da, (3) up to two missed cleavages were allowed, (4) carbamidomethylation set as a fixed modification and (5) methionine oxidation as a variable modification. Only high confidence peptides (FDR<1%) with XCorr scores >1.5 for singly charged ions, >2.0 for doubly charged ions and >2.5 for triply charged ions (from SEQUEST) and MASCOT scores of >40 were considered for protein identification.

Chapter 3:

ELLA facilitates detection of N-glycosylation on human IgG standard and a secreted anti-IL-8 mAb glycoprotein using specific lectins

Given that the outer membrane of all cells is decorated with carbohydrates, it is not surprising that glycans play significant roles in several stages of embryonic development and physiology (Varki et al. 2009). Notch receptors are known to be modified by N-linked glycans (Kornfeld et al. 1981; Perdigoto and Bardin 2013). In addition to N-glycosylation, the Epidermal growth factor-like (EGF) repeats of the extracellular domain of the Notch receptor can be modified by different forms of O-linked glycans: O-fucose, O-glucose, O-xylose (Moloney et al. 2000a; Moloney et al. 2000b). Evidence from the literature indicates that glycosylation of Notch receptors is a major regulator of Notch signalling, specifically O-linked glycosylation (Moloney et al. 2000b; Stanley 2007; Matsuura et al. 2008) and the role of N-glycans is unclear (Perdigoto and Bardin 2013).

Identification of the enzymes involved in the construction of sugar chains, using methods for biochemical identification and carbohydrate analysis has allowed several groups to investigate the role of glycosylation in the development of model organisms (Haltiwanger 2002; Haltiwanger and Lowe 2004). Techniques such as mass spectrometry, nuclear magnetic resonance imaging (NMR), high performance liquid chromatography (HPLC) and enzyme linked lectin assay (ELLA) are some of the tools available for glycoanalysis.

N-glycans are highly complex and diverse in structure. It is therefore important to be able to characterise the structure of these glycans in full in order to gain insight into the major mechanisms responsible for glycosylation but also to control activity and stimulate desired cellular responses.

Strategy

The main objective of this thesis is to assess the functional role of N-glycosylation of the Notch1 receptor in controlling stem cell transition and myogenic differentiation to a vascular smooth muscle cell lineage. In order to achieve this, it was necessary to (i) design a system to generate pure N-glycosylated protein (ii), develop tools to detect the N-glycoprotein and (iii) establish techniques to measure changes in N-glycosylation of the protein after treatment with N-glycosylation modifiers. In this context, recombinant CHO DP-12 cells modified to produce anti-IL-8 mAb following methotrexate treatment were used to generate purified N-glycosylated protein that could then be interrogated using various platforms to establish the extent of N-glycosylation and whether pre-treatment of CHO DP-12 cells with N-glycosylation inhibitors modified these N-glycan signatures on this recombinant glycoprotein.

3.1 N-glycosylated anti-IL-8 mAb production and detection.

CHO DP-12 were cultured in serum free media containing methotrexate for 7d before the supernatant was recovered and clarified by centrifugation and then concentrated by ultracentrifugation. The concentrated samples were purified by protein A affinity chromatography and then quantified using HPLC. CHO-DP12 cell line was chosen as the desired system as it is engineered to express N-glycosylated recombinant humanized anti-IL-8 monoclonal antibody following methotrexate selection. HPLC was considered first to measure the N-glycosylated protein. For optimisation purpose, analysis of 0- 1000μg/ml human IgG reference standards was carried out using isocratic mode of separation (Figure 3.1) as described in Materials and Methods section.

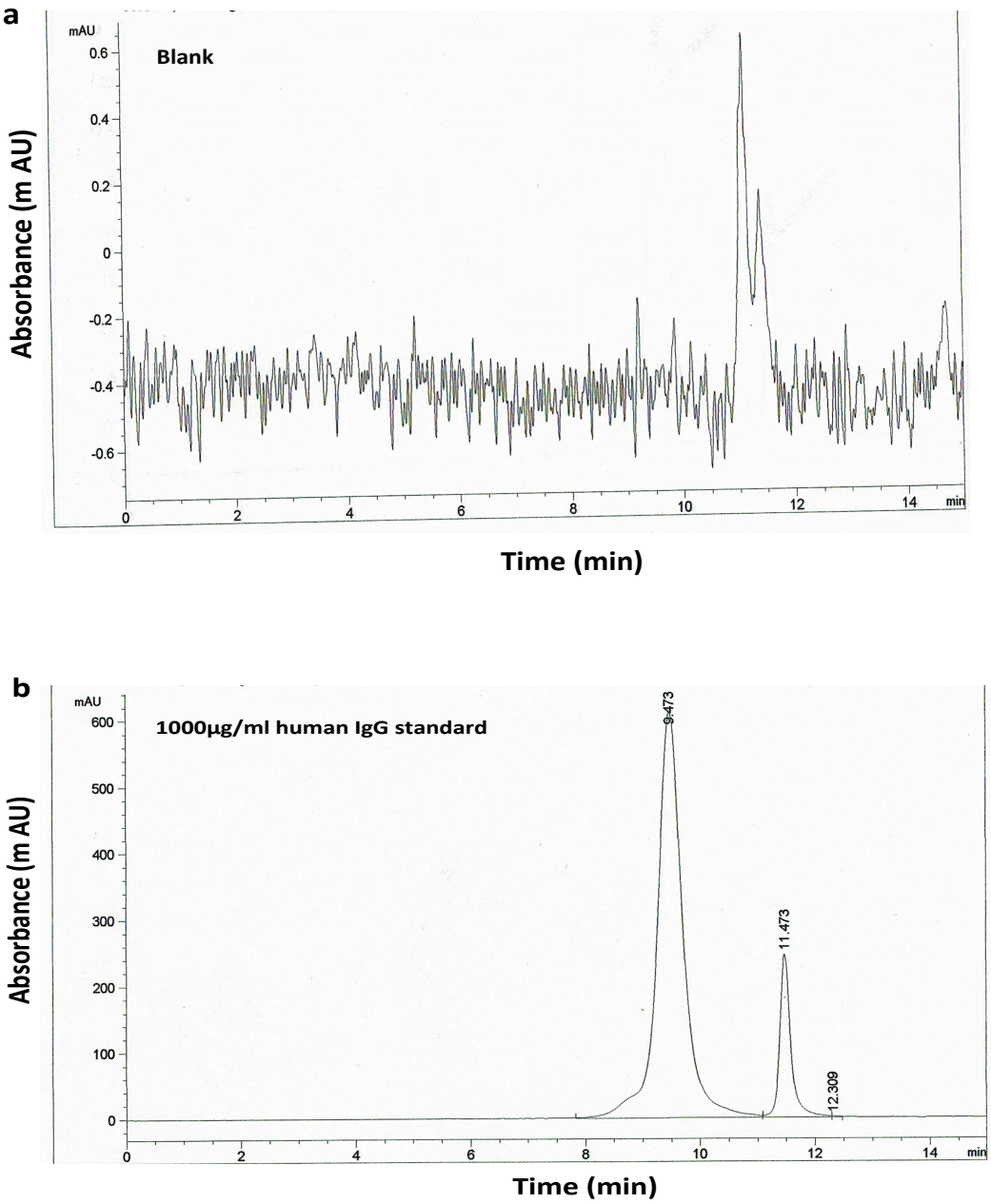

Figure 3.1 HPLC analysis of human IgG standard. (a) Blank sample (mobile phase) and (b) 1000µg/ml human IgG standard were analysed by HPLC SEC on a Biorad 5μm Bio-Sil® SEC column. Sample chromatograms indicating respective peak areas and retention times were obtained; only baseline noise is present in the blank sample. 1000µg/ml human IgG standard peak eluted at 9.473min. Chromatograms are representative of three independent experiments.

A standard curve was generated using the peak area values of 0-1000μg/ml human IgG to check for linearity (Figure 3.2). The linearity of the plot of the peak area against protein concentration indicates that the peak areas change proportionately with the protein concentration. Purified anti-IL-8 mAb generated from CHO DP-12 supernatant was then analysed by HPLC (Figure 3.3) and concentration was determined by extrapolation.

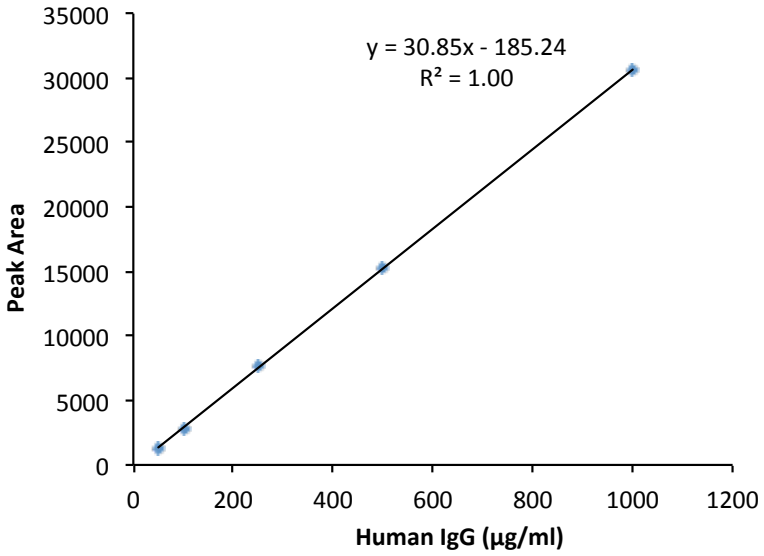

Figure 3.2. Standard curve of human IgG standard. HPLC analysis was carried out on 0-1000μg/ml IgG standard. The peak area of standards was plotted against their respective concentrations. Standard curve is representative of two independent experiments.

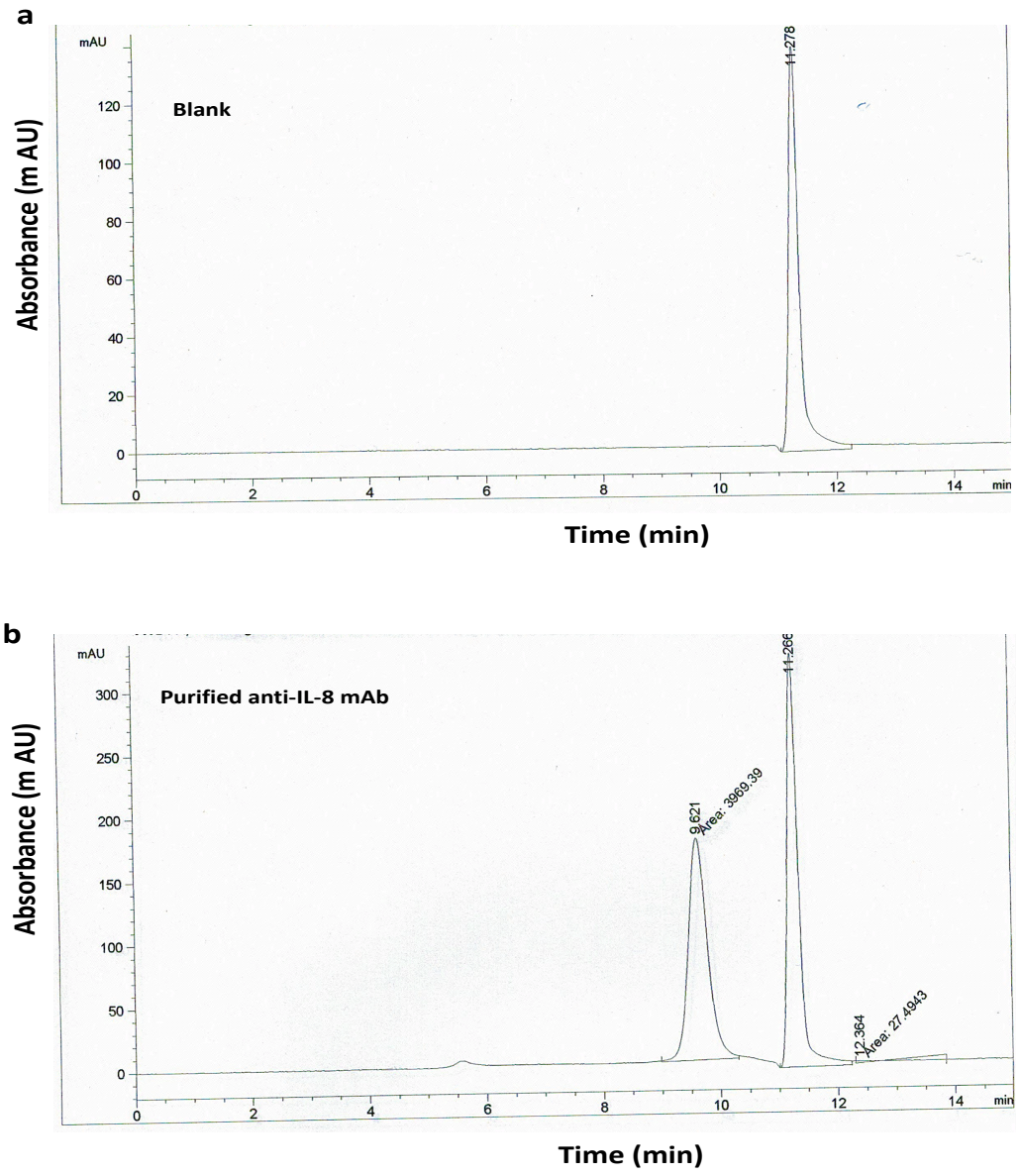

Figure 3.3. HPLC analysis of purified anti-IL-8 mAb. Sample chromatograms of (a) blank sample (elution and neutralization buffer) and (b) purified anti-IL-8 mAb. CHO DP-12 cells were cultured in serum free media in the presence of Methotrexate for 7 days, supernatant was purified by Protein A column. HPLC SEC analysis was carried out on a Biorad 5μm Bio-Sil® SEC column, purified anti-IL-8 mAb eluted from the column at 9.621mins. Chromatograms are representative of three independent experiments.

The amount of secreted anti-IL-8 mAb was determined by BCA protein assay following purification from the conditioned media of CHO DP-12. The amount of anti-IL-8 mAb present in the samples was determined using human serum IgG as reference standard. A standard curve was generated from the absorbance measurement at 562nm (Figure 3.4).

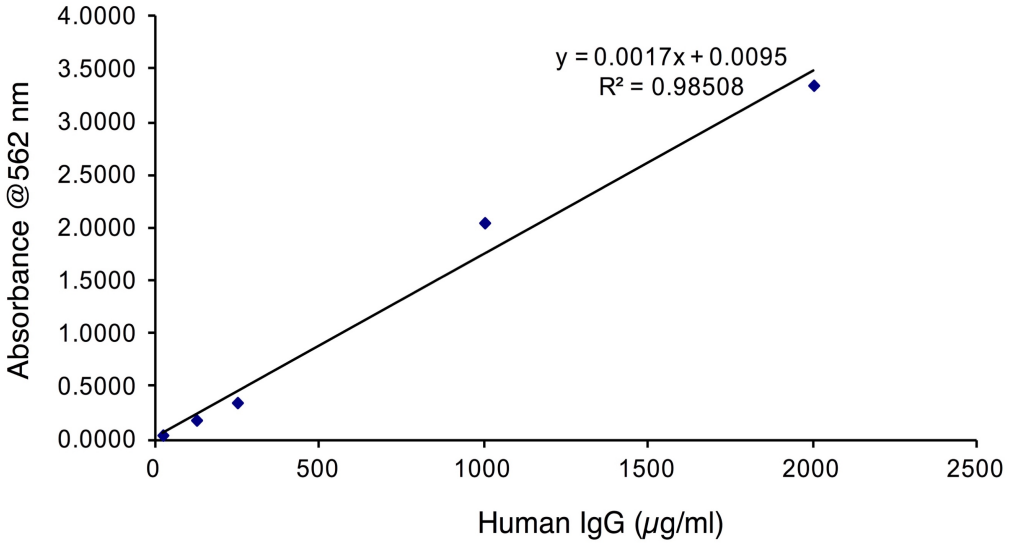

Figure 3.4. Human IgG standard curve. BCA protein assay was used to analyse human IgG standard, a plot of absorbance against concentration (0-2000μg/ml) was generated using Excel. The concentration of unknown sample was determined by extrapolation. Standard curve is representative of three independent experiments.

3.2 Treatment of cells with N-glycosylation inhibitors and the effect on anti-IL-8 mAb N-glycosylation.

Treating cultured cells with glycosylation inhibitors helps to determine N-glycosylation linked carbohydrate structure. Tunicamycin, produced by several bacteria including Streptomyces clavuligerus and Streptomyces lysosuperficus is used as an experimental tool in biology to block N-linked glycosylation. Tunicamycin is a mixture of homologous nucleoside antibiotics that inhibits UDP-HexNAc: polyprenol-P HexNAc-1-P family of enzymes. In eukaryotes, this includes the enzyme GlcNAc phosphotransferase (GPT). Tunicamycin disrupts the first step of glycoprotein synthesis by inhibiting the transfer of N-acetylglucosamine-1-phosphate (GlcNAc-P) from UDP-N-acetlyglucosamine (UDP-GlcNAc) to dolichol phosphate. This disrupts N-glycan processing and causes improperly folded proteins to accumulate within the cells. Tunicamycin also restricts protein transportation and reduces glycoprotein incorporation to the cell membrane (Paszkiewicz-Gadek et al. 2006; Bassik and Kampmann 2011; Heifetz, Keenan and Elbein 1979).

1-Deoxymannojirimycin hydrochloride is employed for studies on golgi-mediated glycoprotein processing and has antiviral activity. 1-Deoxymannojirimycin is a specific α-mannosidase I inhibitor of N-linked glycosylation, blocks the conversion of high mannose to complex oligosaccharide. Deoxymannojirimycin alters cell-surface complex carbohydrate structure as assayed by PHA-L lectin interaction and increases high mannose structures by the increased binding with Con A lectin (Krasnova and Wong 2016; Choi et al. 2006).

CHO DP-12 were cultured in serum free media containing Methotrexate and 0.8 - 4 μg/ml tunicamycin or 1-5 μg/ml Deoxymannojirimycin or vehicle (DMSO) for 7 days. The samples were purified by protein A affinity chromatography and quantified using HPLC or BCA protein assay. Cell viability and growth response (proliferation and apoptosis) of CHO DP-12 cells was determined following treatment with glycosylation inhibitors.

The purified anti-IL-8 mAb from vehicle treated or tunicamycin treated cells were analysed by HPLC to measure and assess any change in glycosylation. No significant change in retention time was observed (Figure 3.5) in the tunicamycin treated sample. Similar result was observed when cells were treated with Deoxymannojirimycin (Figure 3.6). These treatments were expected to alter the N-glycosylation process resulting in decrease in molecular weight of the proteins and as a result should interact longer with the column and elute later than untreated sample.

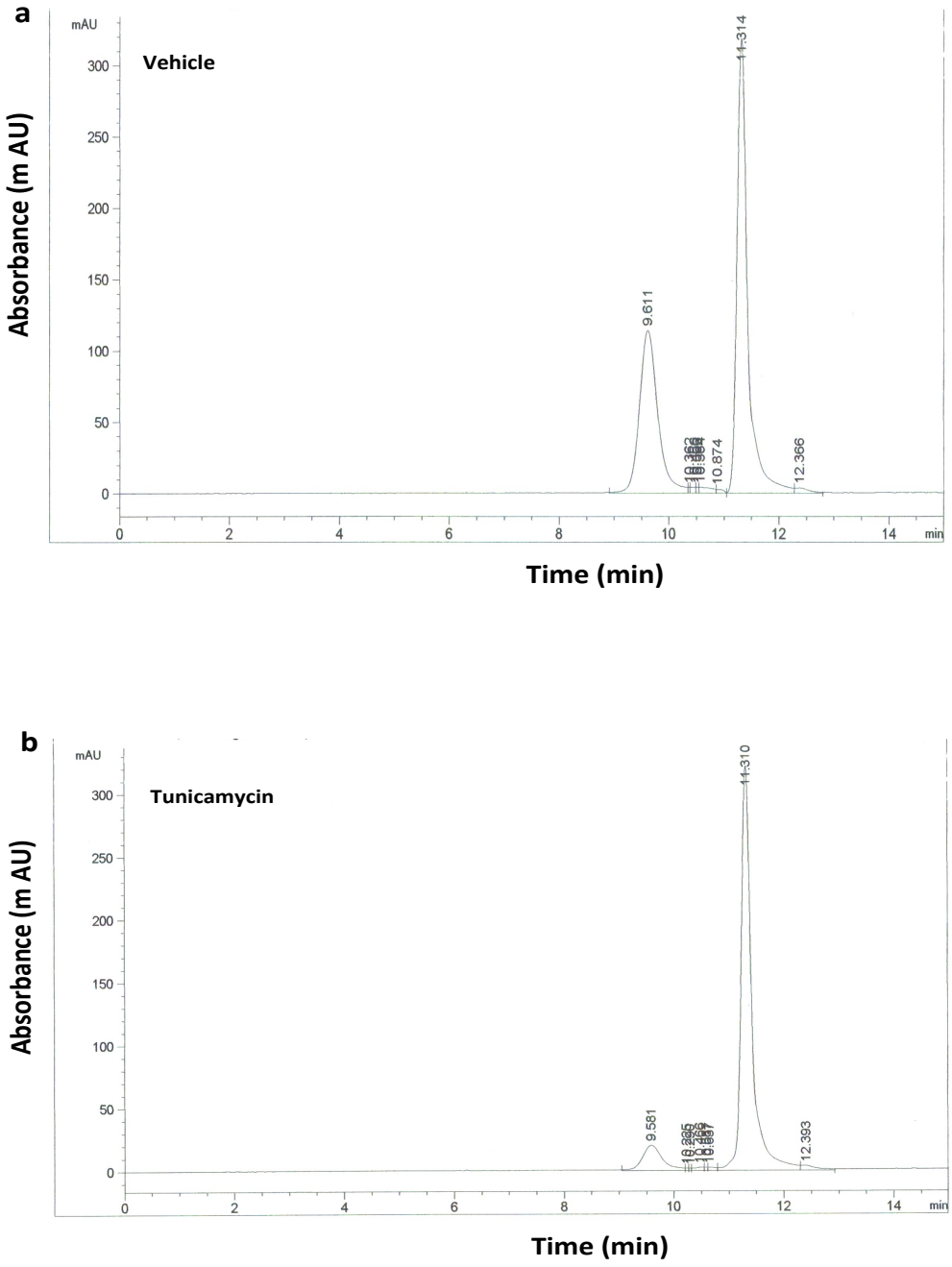

Figure 3.5. The effect of tunicamycin treatment on N-glycosylation of anti-IL-8 mAb. CHO DP-12 was cultured in the presence of (a) DMSO (vehicle) or (b) tunicamycin (4µg/ml), supernatant Protein A purified, separated using SEC analysis on a Biorad 5μm Bio-Sil® SEC column which had been standardized using IgG reference standard. Anti-IL-8 mAb values were calculated for the eluted peaks based on the reference standard profile. Chromatograms are representative of three independent experiments.

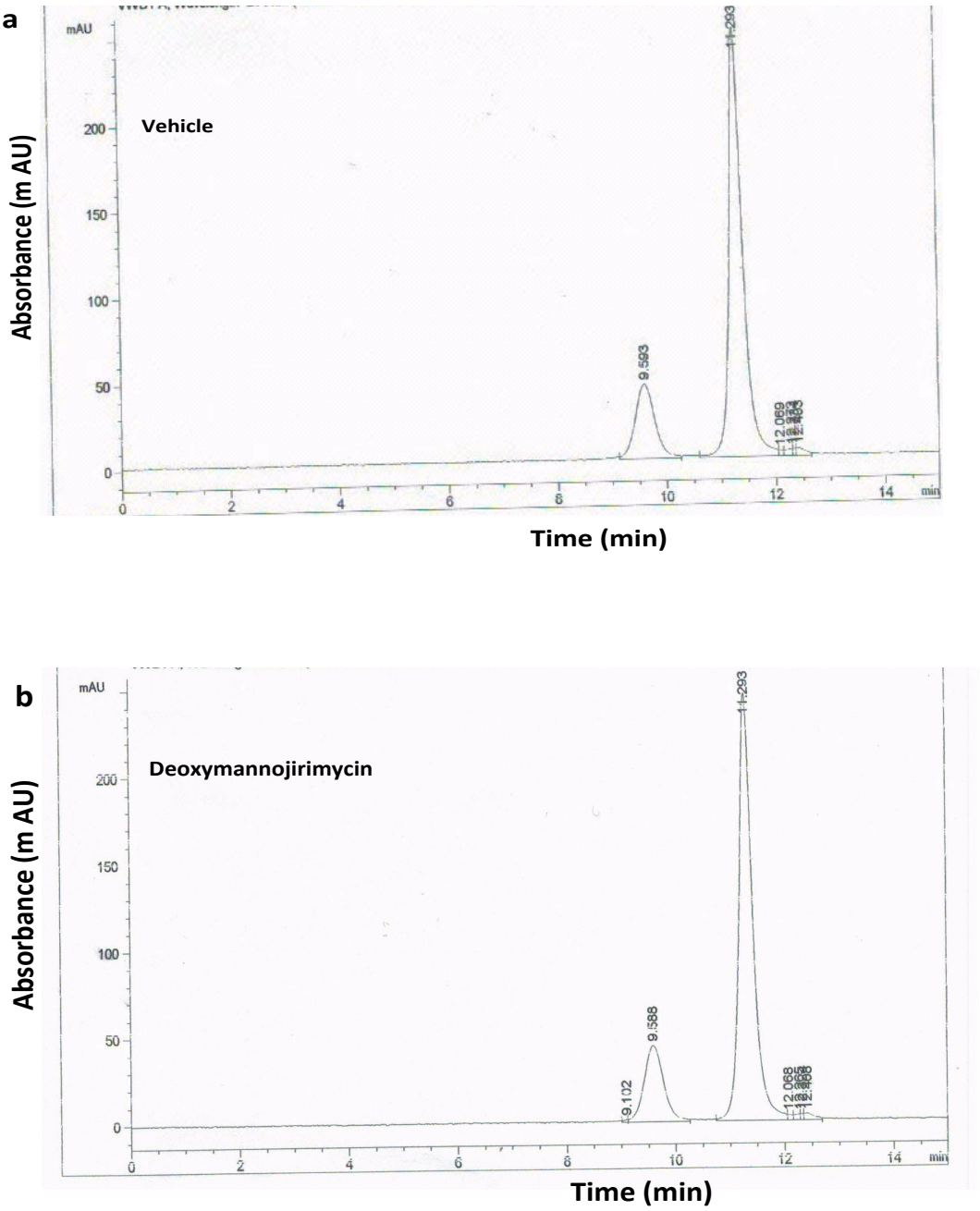

Figure 3.6. The effect of Deoxymannojirimycin treatment on N-glycosylation of anti-IL-8 mAb. CHO DP-12 was cultured in the presence of (a) DMSO (Deoxymannojirimycin vehicle) or (b) Deoxymannojirimycin (5µg/ml), supernatant Protein A purified and separated using SEC analysis on a Biorad 5μm Bio-Sil® SEC column which had been standardized using IgG reference standard. Anti-IL-8 mAb values were calculated for the eluted peaks based on the reference standard profile. Chromatograms are representative of three independent experiments.

3.3 The effect of PNGase F treatment on purified anti-IL-8 mAb.

Purified anti-IL-8 mAb glycoprotein was treated with PNGase F as described in Materials and Methods (section 2.8.1). PNGase F, an asparagine amidase is essential in the study of protein N-glycosylation. It is highly effective in releasing N-glycan from glycoproteins, which enable the successful and accurate characterisation of glycans (Freeze and Kranz 2010; Huhn et al. 2009). The purified deglycosylated anti-IL-8 mAb from vehicle treated or PNGase F treated cells were analysed by HPLC to measure and assess any change in glycosylation. No significant change in retention time was observed (Figure 3.7).

HPLC was useful in measuring the amount of protein of interest following the tunicamycin, Deoxymannojirimycin and PNGase F treatment, however, the determination of the exact glycan structure that may have been removed or modified by the treatment was limited. HPLC was found to be difficult to apply for routine use to measure and determine changes in N-linked glycosylation. An alternative means was sought for easier and faster analysis of glycoproteins.

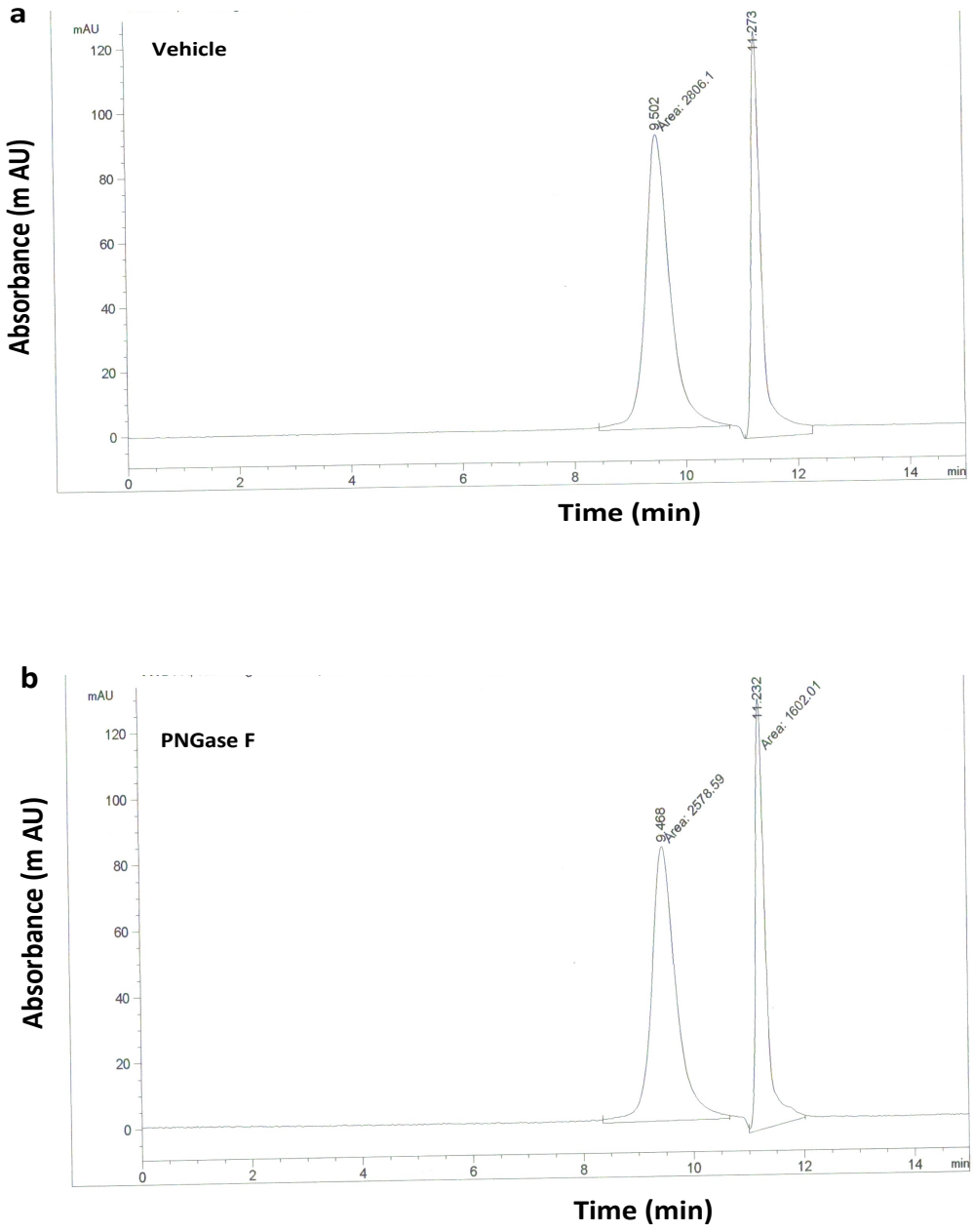

Figure 3.7. HPLC analysis of PNGase F treated anti-IL-8 mAb. Sample chromatograms of (a) anti-IL-8 mAb treated with vehicle and (b) anti-IL-8 mAb treated with PNGase F (500U). The treated samples were Protein A purified and separated using SEC analysis on a Biorad 5μm Bio-Sil® SEC column which had been standardized using IgG reference standard. Anti-IL-8 mAb values were calculated for the eluted peaks based on the reference standard profile. Chromatograms are representative of three independent experiments.

3.4 Anti-IL-8 mAb binds to N-glycan specific lectins.

Lectins are very useful for characterising glycoproteins based on their glycan-binding specificities (Miura and Endo 2016). Lectins are generally divided into five major groups depending on the monosaccharide for which they have the highest binding affinity. Because of the specificity of the interaction between a monosaccharide and lectin, it is possible to determine the glycan components of a glycoprotein (Ramos et al. 2000).

Once the glycoprotein was measured by HPLC or BCA assay, glycan analysis was then carried out using ELLA. In general, ELLA was performed by immobilisation of glycoproteins on the surface of a 96 well plate, followed by blocking of the plate surface with appropriate blocking solution to remove background signals, and then probing with specific biotinylated lectins. This was followed by the addition of TMB substrate solution to develop colour (blue) in proportion to the amount of glycans bound. The stop solution (diluted sulphuric acid) was then added to stop the reaction, changing the colour from blue to yellow, and the intensity was measured at 450nm (Thompson et al. 2011).

Initial studies were carried out on a commercial human serum IgG glycoprotein standard (Figure 3.8a) probed with 7 selected lectins (Table 3.1). The glycosylation profile of anti-IL-8 monoclonal antibody produced in CHO DP-12 cells was also determined and compared to that of human serum IgG standard (Figure 3.8b). In both cases, more than one lectin interacted very strongly with the glycoproteins showing significant binding to Con A, AAL and LCA compared to negative control PBS and therefore confirms the presence of N-glycans. No interaction is seen between the blank (PBS) and lectins as was expected. Con A and LCA have both

been reported to bind strongly to the mannose/glucose core of N-linked glycans and this can be seen from the result with the analysed sample exhibiting high binding to both Con A and LCA. In contrast, AAL, a Fucose specific lectin did not bind as strongly but still significant in comparison to PBS control.

Table 3.1. Commercial lectins used in this study. Lectins were used at 5μg/ml working concentration.

Lectin		**Sugar specificity**
Con A	Concanavalia ensiformis	Mannose / Glucose
LCA	Lens culinaris	Mannose / Glucose
PNA	Peanut agglutinin	Galactose
SBA	Soybean agglutinin	Galactose / N-acetylgalactosamine
AAL	Aleuria aurantia	Fucose
WGA	Wheatgerm agglutinin	Sialic acid / N-acetyl glucosamine
MAL 1	Maackia amurensis	Lactose / Galactose

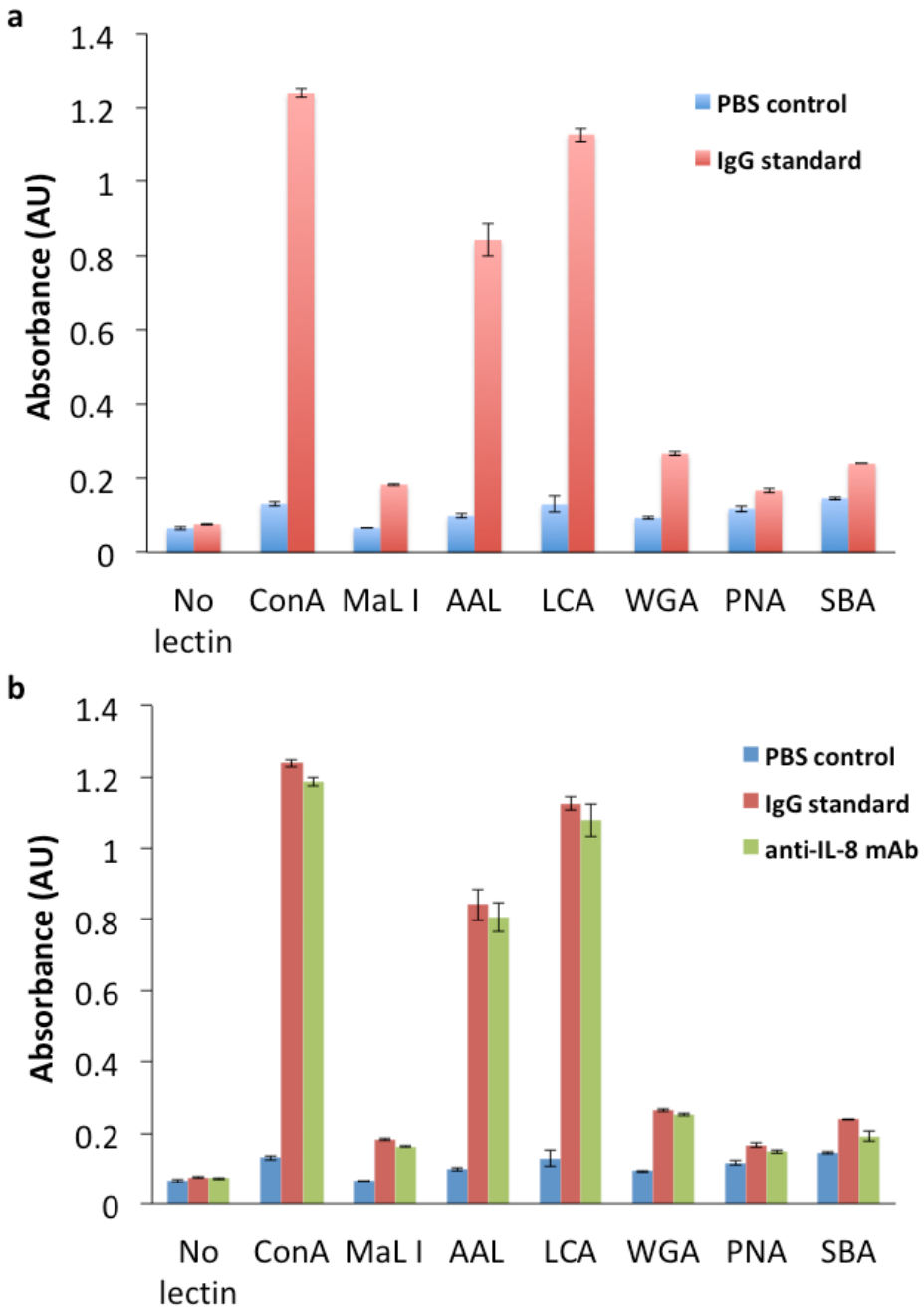

Figure 3.8. Glycosylation profile of human IgG standard. (a) ELLA was used to analyse human serum IgG standard (10μg/ml) to verify its glycosylation and presence of N-glycans. (b) Purified anti-IL-8 mAb was analysed by ELLA to verify glycosylation and presence of N-glycans on the glycoprotein. Profile of IgG standard was compared to that of anti-IL-8 mAb. The experiments were performed in triplicate with the graph representative of three independent experiments. Data are mean ± SD.

3.5 N-glycosylation inhibition affects anti-IL-8 mAb binding to N-glycan lectins.

To determine the effect of tunicamycin on the glycoprotein anti-IL-8 mAb, CHO DP-12 cells were treated with 0.8μg/ml tunicamycin glycosylation inhibitor as described in Material and Methods (section 2.8.2) and ELLA was used to monitor the effect on the anti-IL-8 mAb glycosylation. The ELLA profile shows reduced binding with the Con A, AAL and LCA compared to the untreated sample as expected (Figure 3.9a).

To determine the effect of the 1-Deoxymannojirimycin on anti-IL-8 mAb N-glycosylation, CHO DP-12 cells were treated with 5μg/ml 1-Deoxymannojirimycin hydrochloride as described in Material and Methods (section 2.8.2) and ELLA was carried following affinity purification of secreted anti-IL-8 mAb. As Con A and LCA lectins are mannose specific, no effect was observed on the Con A binding but reduced binding was seen with LCA and more significant reduction was observed with AAL, a Fucose specific lectin. WGA, a GlcNAc specific lectin demonstrated a reduced binding with anti-IL-8 mAb (Figure 3.9b).

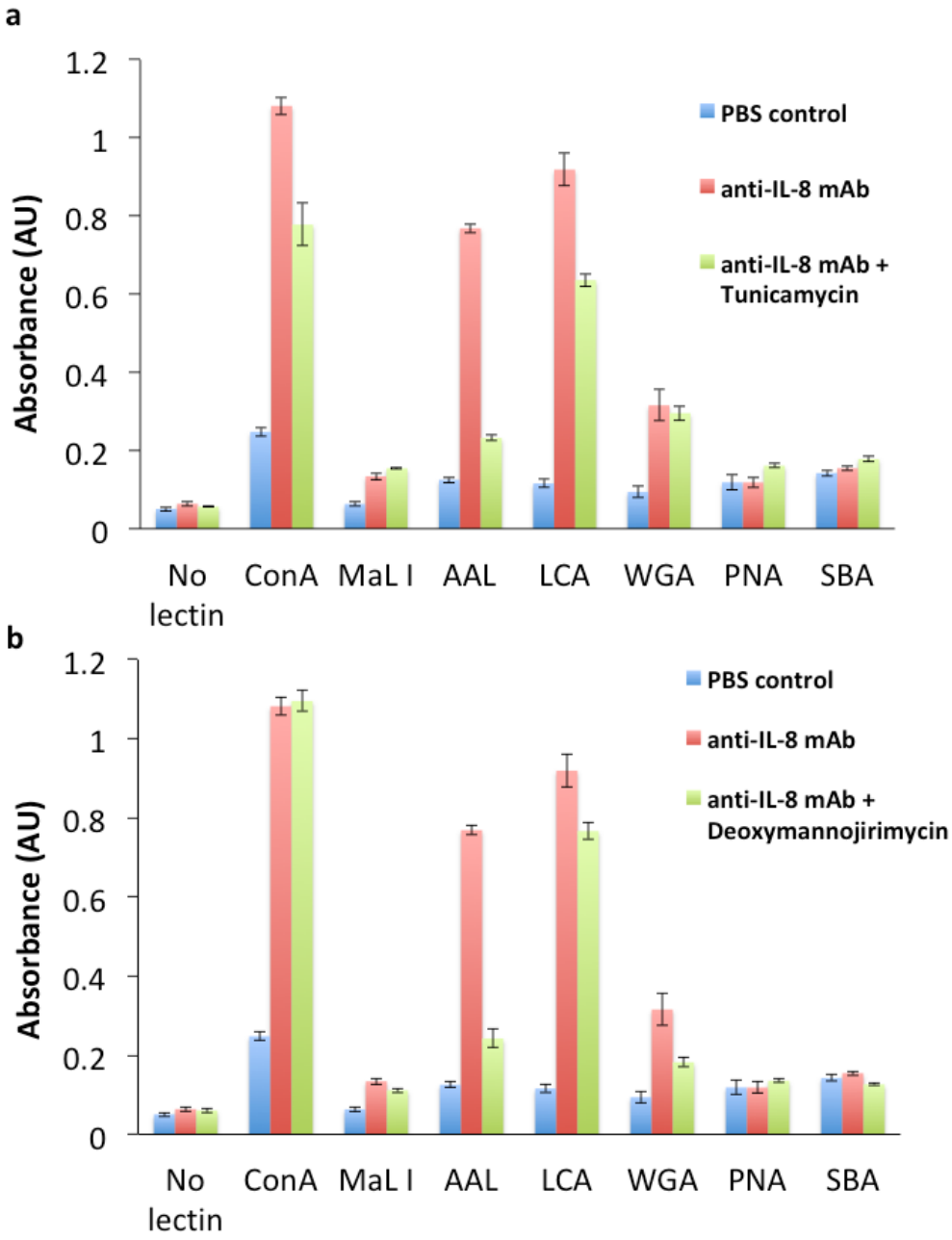

Figure 3.9. The effect of N-glycosylation inhibitors on anti-IL-8 mAb and lectin binding. CHO DP-12 cells were treated with (a) tunicamycin (0.8μg/ml) or Deoxymannojirimycin (5μg/ml) for 48 h, ELLA analysis was done on the expressed anti-IL-8 mAb. The experiments were performed in triplicate with the graph representative of three independent experiments. Data are mean ± SD.

3.6 PNGase F treatment inhibits N-glycan lectin binding.

The model IgG standard was treated with peptide N- glycosidase F (PNGase F). PNGase F cleaves all types of N-glycans from glycosylated proteins, including mannose, complex and hybrid types. It operates by removing most of the common N-linked carbohydrates from proteins by hydrolyzing the originally glycosylated asparagine residue to Aspartic acid. The digestion of IgG with PNGase F greatly reduced the binding to Con A, AAL and LCA in comparison to that without enzyme (Figure 3.10a), suggesting that most or all of the N-glycans had been removed. In order to increase deglycosylation efficiency, denaturation of glycoproteins is usually done prior to PNGase F digestion (Szabo, Guttman and Karger 2010). To prove that PNGase F treatment is responsible for the deglycosylation and not the denaturation of the glycoproteins, a denatured IgG was also analysed by ELLA and no major difference was observed when compared to the non-denatured glycosylated IgG (Figure 3.10a).

To further ascertain the effectiveness of the N-glycan removal procedure, Invertase, another standard N-glycoprotein was treated with PNGase F. Binding of treated sample with Con A and LCA lectins, both specific for N-glycans reduced greatly compared to untreated control, confirming that the treatment removed the N-glycans present (Figure 3.10b).

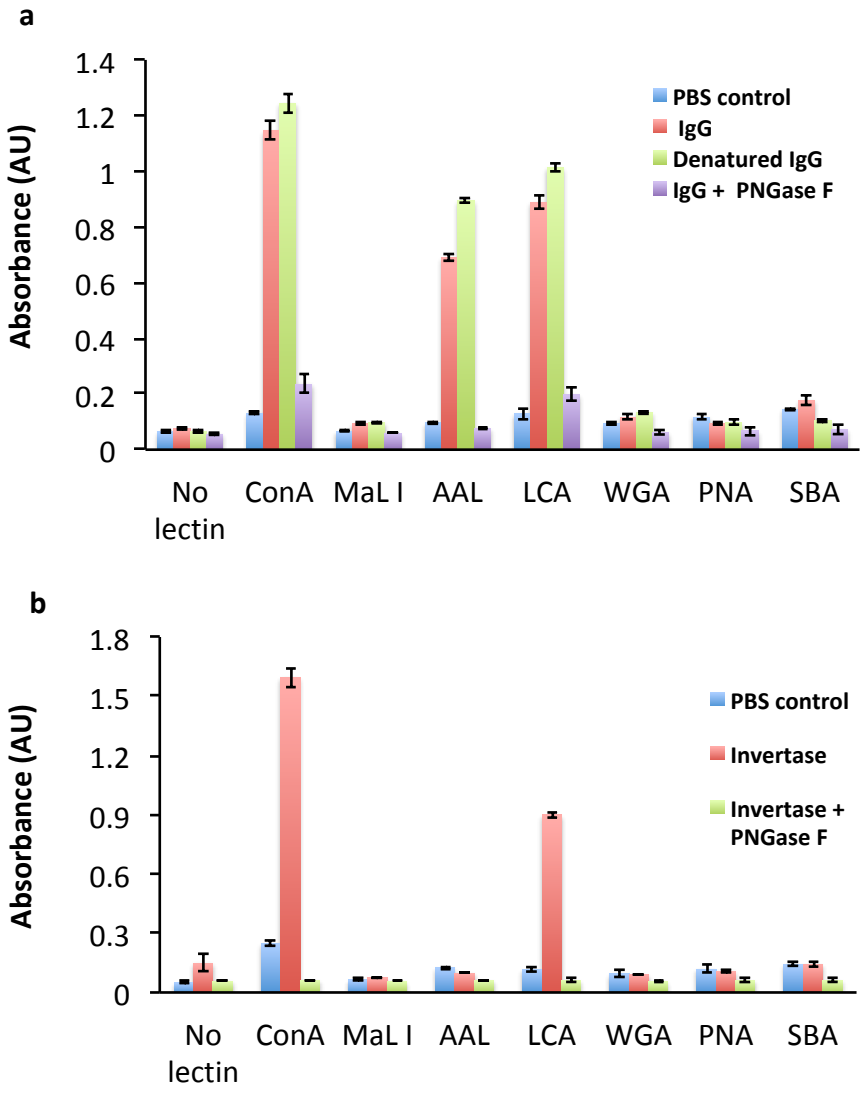

Figure 3.10. The effect of PNGase F treatment on N-glycan lectin binding. (a) anti-IL-8 mAb (10μg/ml) was treated with PNGase F for 3h at 37°C, followed by ELLA analysis**. (b)** N-glycoprotein Invertase (10μg/ml) was analysed by ELLA following treatment with PNGase F. The experiments were performed in triplicate with the graph representative of three independent experiments. Data are mean ± SD.

Summary

- ELLAs facilitated detection of N-glycans on human IgG standard and a secreted anti-IL-8 mAb glycoprotein using specific lectins.
- PNGase F treatment attenuated binding to Con A, AAL and LCA lectins suggesting human IgG and anti-IL-8 mAb glycoproteins have high levels of mannose and fucose.
- Tunicamycin treatment of cells attenuated binding to Con A, AAL and LCA further suggesting the anti-IL-8 mAb has high levels of mannose and fucose.
- Deoxymannojirimycin treatment of cells attenuated binding to AAL, LCA and WGA but Con A lectin suggesting the presence of high mannose N-glycans and complex N-glycans.

Discussion

Glycosylation profiling of proteins is based on the expression of various glycosyltransferase / sialyltransferase enzymes that are situated in the Golgi and Endoplasmic Reticulum of the cell. Several factors can influence the level of expression of these enzymes within a cell; these variations can account for the obvious differences in structure and activity of glycosylated proteins. There can be variation in the proportion of glucose, mannose, terminal galactose, core fucose and bisecting GlcNAc (N-Acetyl glucosamine). It is also possible to have variation in the structure of sialic acid Neu5Ac (Butler 2005).

The aim of this study was to establish platforms for detecting and measuring N-glycosylated proteins. To achieve this, a system was designed to generate pure N-glycosylated protein, anti-IL-8 monoclonal antibody, an IgG1 produced in CHO DP-12 cells. The model N-glycosylated protein was isolated from the supernatant of cells in culture using a protein affinity chromatography purification method. This system offers easy rapid small-scale antibody purification.

Analytical techniques were then developed to detect the glycoprotein. A High Performance Liquid Chromatography (HPLC) method was successfully employed to detect and quantify the expressed anti-IL-8 mAb glycosylated protein. The BCA protein assay was employed alternatively for measuring purified anti-IL-8 mAb, though not as accurate, it is a much faster and easier technique compared to HPLC.

A set of glycosyltransferase or glycosidase inhibitors were then used to modify the N-glycans of anti-IL-8 mAb and the effect was monitored using a variety of techniques. Initially, a range of concentrations of the inhibitors was used and the

optimum doses were selected and used as appropriate. The glycoprotein processing inhibitors were added to the growth media of CHO DP-12 cells expressing anti-IL-8 mAb and changes in N-glycosylation patterns were studied using a combination of biochemical techniques.

At first, the effect of the glycosylation inhibition treatment on the secreted glycoprotein was examined by SEC analysis. This method sorts protein by size, larger proteins elute the column first, then subsequent elution of smaller proteins. Differences in their retention times can be used as a means of identification. There were no noticeable differences in the retention times of the treated glycoprotein samples and the control when cells were treated with tunicamycin or Deoxymannojirimycin. There is a possibility that the treatment might not have worked or the glycosylation modifications did not impact hugely the size of the glycoprotein. Although the HPLC technique was successfully applied to measure the glycoprotein following the glycosylation inhibition treatment, it was not possible to determine the exact glycan structure that may have been modified by the treatment.

Another approach was taken to release N-glycans from the glycoprotein. Different enzymes have been utilised for N-glycan release, including PNGase F, PNGase A and endoglycosidase H. PNGase F releases all N-linked sugars and is capable of deglycosylating intact glycoprotein while PNGase A is only efficient at glycopeptide level and Endo H is only specific for high-mannose like N-glycans (Huhn et al. 2009). PNGase F was selected as the most suitable to cleave N-glycans from the glycoprotein. Purified anti-IL-8 mAb was treated with PNGase F and the resulting glycoprotein was analysed by SEC and compared to untreated

glycoprotein. Again, no noticeable difference was observed in the retention times of the treated glycoprotein, similar to findings from the glycosylation inhibition studies. This suggests that the size of glycoprotein is not significantly modified following treatment, although individual differences in glycan composition may be too subtle to detect. Glycoproteomic studies using analytical techniques such as mass spectrometry and high performance liquid chromatography have been successfully applied to characterise N-glycans from recombinant IgG antibodies. It may be possible to use a reversed-phase high performance liquid chromatography/mass spectrometry method for profiling and characterising N-glycans from glycoproteins (Chen and Flynn; Flynn et al. 2010).

Release of N-Glycans from glycoprotein by enzymatic cleavage is necessary followed by derivatisation with fluorescently tagged 2-aminobenzamide and then separation on reversed phase HPLC with fluorescence detection coupled to a mass spectrometer. This method enables the segregation of all major glycan types: high mannose, hybrid and complex, allowing accurate quantification of N-glycans based on the fluorescence (Chen et al. 2007; Flynn et al. 2010).

The difficulties encountered with SEC application in determining changes in N-linked glycosylation made it impossible to continuously use it for glycan analysis. It was concluded that an alternative means was required for easier and faster analysis of glycoproteins.

Enzyme-linked lectin assay (ELLA) was then selected, given that it's been reported to be the simplest model for glycoanalysis and does not have a complex set up. It's basically set up in a similar format to that of a standard enzyme-linked immunosorbent assay (ELISA) supporting the analysis of glycoproteins and lectin

interactions in a standard microtiter plate arrangement. A major benefit associated with the use of lectins is that analysis does not require prior glycan removal from glycoprotein and derivatisation procedure as would be required for HPLC analysis, hence making the analysis easy to perform (Pilobello et al. 2005; Chen et al. 2007).

To validate the ELLA method for the analysis of N-glycosylated proteins, groups of lectins of known glycan-binding specificities were analysed with anti-IL-8 mAb glycoprotein and human serum IgG standard. With this, more information was gained about the types of sugar moieties that are present on the glycoprotein.

Of the 7 lectins tested, N-glycan specific lectins Con A, LCA and AAL bound strongly to the glycoprotein indicating that anti-IL-8 mAb glycosylation has high levels of mannose and fucose, the finding was similar for IgG standard. Addition of glycosylation inhibitor tunicamycin to CHO DP-12 produced anti-IL-8 mAb with reduction in mannose and fucose according to diminished binding with Con A, AAL and LCA by 30%, 50% and 30 % respectively. Con A lectin has affinity for branched α-mannose structures, high-mannose type, hybrid type and complex type N-glycan, LCA lectin is specific for fucosylated core region of complex type N-glycans (Roth,Yehezkel and Khalaila 2012). AAL binds Fucose region of complex N-glycans (Kaji et al. 2006).

N-glycosylation analysis of anti-IL-8 mAb expressed in the presence of Deoxymannojirimycin whose function is to block the conversion of high mannose N-glycans to complex N-glycans. ELLA demonstrates reduced binding in AAL, LCA and WGA by 53% 15% and 13% respectively, the binding affinity of Con A was not affected following treatment with the specific mannosidase I inhibitor.

WGA binds GlcNAc (N-acetyl glucosamine) of complex N-glycans (Kaji et al. 2006).

Glycosidase enzyme (PNGase F) treatment was used to further characterize IgG glycoprotein. The resulting glycoprotein was then tested to evaluate the ability of ELLA to detect removal of N-glycans following PNGase F treatment. Lectins were probed with intact IgG and deglycosylated IgG and changes in the interactions between the carbohydrate and the lectins were assessed.

PNGase F treatment decreased the affinity of IgG to bind to lectins AAL and LCA by 60 and 70% respectively while treatment had a much stronger effect on Con A binding with 90% reduced binding. The decrease in affinity can be explained by the decrease in level of mannose and fucose compared to the untreated samples.

The main focus of this project is to study the functional role of the N-glycans in Notch signalling activity. N-glycosylation inhibitors and PNGase F treatment have both been shown by ELLA to affect the N-glycosylation of anti-IL-8 mAb produced in CHO DP-12. One of the earliest studies on Notch protein suggests that it is decorated with N-glycans (Kornfeld et al. 1981) due to interaction with Lentil lectin (Johansen, Fehonand Artavanistsakonas 1989). The enzyme linked lectin assay offers a simple, more affordable approach to glycoanalysis. This study demonstrated the potential use of ELLA technique for N-glycosylation analysis of protein of interest Notch1.

Chapter 4 :

A co-operative effect of PNGase F, Tunicamycin and Lunatic Fringe siRNA on Notch1 receptor N-glycosylation

Post-translational modifications of Notch receptors can influence the relative strength of receptor-ligand interactions in stem cells. The extracellular domain of Notch is composed of several epidermal growth factor (EGF)- like repeats. There are at least four different glycans located in specific regions of these EGF-like repeats; N-linked glycan and three O-linked glycans (O- fucose, O-glucose and O-GlcNAc) (Matsumoto et al. 2016; Andersson, Sandberg and Lendahl 2011). Protein O-fucosyltransferase adds O-fucose to Notch receptors, though this modification alone isn't necessary for Notch activity (Okajima et al. 2008), it is required for the following glycosylation step by Fringe proteins (such as lunatic Fringe, manic Fringe and radical Fringe in mammals). O-fucose glycan is then subjected to further modification by the addition of N-acetyl glucosamine (GlcNAc) by Fringe proteins. This glycosylation regulates the interaction of Notch receptors with ligands by promoting a response with delta like ligand 1 (DLL-1) and inhibiting responsiveness to Jag-1 (Andersson, Sandberg and Lendahl 2011). Notch EGF repeats can also be glycosylated by Rumi (protein O-glucosyltransferase, Poglut), an enzyme involved in the addition of O-glucose (Takeuchi and Haltiwanger 2010). The mechanisms involved in glycan modification of Notch receptors have been investigated by several groups to establish their specific roles and attain a better understanding for how glycosylation controls receptor activity (Andersson, Sandberg and Lendahl 2011; Takeuchi and Haltiwanger 2014; Matsumoto et al. 2016). The mechanism of the specific enzyme(s) involved in the N-glycosylation of the Notch receptor is not fully understood. In order to establish the role of Notch receptor N-glycosylation in this process, it is therefore essential to be able to detect Notch1 and monitor its glycosylation state.

Strategy

In order to determine the N-glycosylation of native Notch1 receptor in MSC, the following strategy was adopted. Cultured MSCs were used as resident vascular stem cells (MVSCs) are MSC-like as they transition down a myogenic lineage.

The major objectives of this chapter were to: (i) Quantify native Notch1 receptor in target mesenchymal stem cell lysates (ii) Verify N-glycosylation of Notch1 receptor extracellular domain (ECD) using the ELLA technique developed in Chapter 3 (iii) Ectopically express Notch1 and immunoprecipitation to generate pure Notch1 (iv) Assess cell viability and growth response of stem cells and determine optimum dose following treatment with the N glycosylation inhibitor (v) Verify the N-glycosylation state of immunoprecipitated Notch1 and monitor changes following N-glycosylation modification (vi) Establish N-glycosylation profile for total glycoproteins in stem cell lysate and monitor differences following N-glycosylation modification.

4.1 Endogenous Notch1 is detected in cell lysate.

ELISA was used to measure the level of endogenous Notch1 receptor in MSC lysate. Quantitation was carried out by interpolation from the standard curve (0-7 ng/ml) (Figure 4.1). The yield of endogenous Notch1 in MSCs was found to be 3ng/ml, 0.3 nanogram per 9.6 million cells in 100μl lysate or 0.3 attogram per cell.

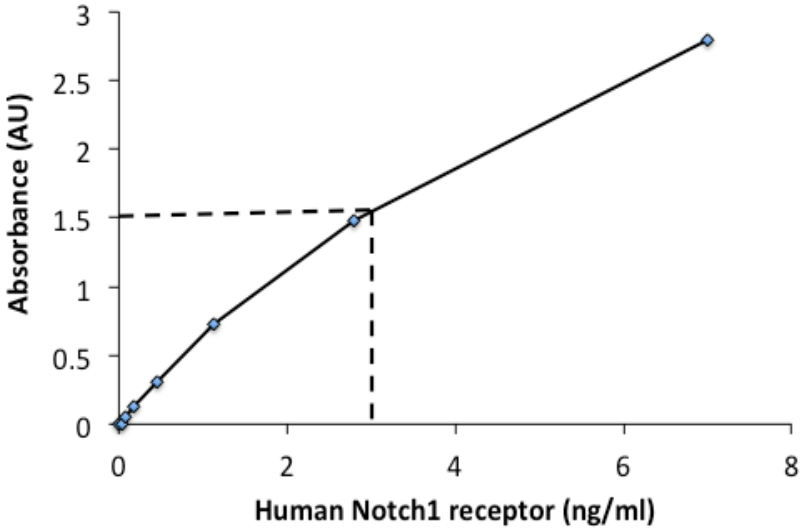

Figure 4.1. ELISA standard curve for Human Notch1 standard. ELISA was performed 0-7ng/ml Notch1 standard. The average absorbance of each standard was determined and plotted against their respective concentrations. Standard curve is representative of three independent experiments.

4.2 Notch1 Fc binds to N-glycan specific lectins.

ELLA analysis was initially performed on human Notch1 standard (50ng/ml) using Con A lectin. No signal was observed for the lectin used. This indicated that the concentration of the standard was too low for detection by ELLA (Figure 4.2).

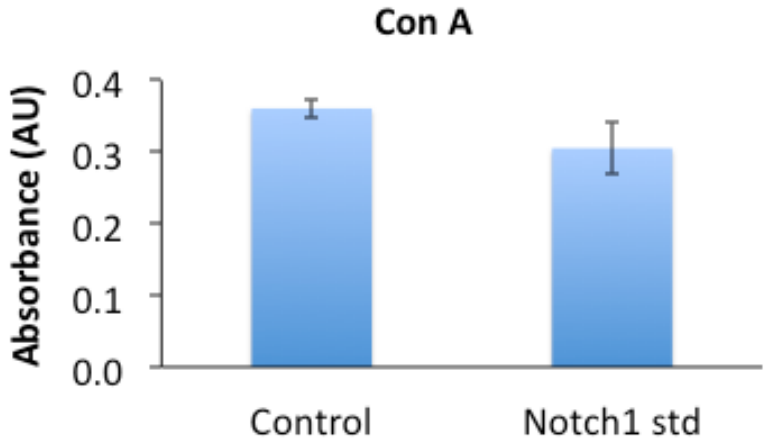

Figure 4.2. Glycosylation analysis of the human Notch1 standard. A 50ng/ml Notch1 standard sample was analysed by ELLA using Con A. Bar chart is representation of triplicate experiments. Data are mean ± SD.

Next, ELLA was performed on recombinant Notch1 Fc as it was not possible to obtain purified Notch1 standard with a high enough concentration. IgG1 Fc serves as the non-glycosylated (negative) control. A sample of 5μg/ml Notch1 Fc or IgG Fc was probed with a panel of five lectins, four of which are specific for N-glycan structures, namely Con A, Mal 1, AAL, LCA and WGA. These four lectins are useful in detecting N-glycosylation changes in glycoproteins. In particular, Con A lectin has affinity for branched α-mannose structures, high-mannose type, hybrid type and complex type N-glycan.

The LCA lectin is specific for fucosylated core region of complex type N-glycans (Roth,Yehezkel and Khalaila 2012). AAL binds Fucose region of complex N-glycans and WGA binds GlcNAc N-acetyl glucosamine of complex N-glycans (Kaji et al. 2006). Significant binding of the Notch1 Fc was observed across the N-glycans specific lectins in comparison to its non-glycosylated control. No significant binding was detected with the Mal 1. This profile indicates that there are N-glycans present in 5μg/ml Notch1 Fc.

Of the 5 lectins tested, 4 exhibited strong signals against Notch1 Fc, which could be grouped according to carbohydrate-binding specificities. Both Con A and LCA binds to the core mannose structure of N-linked glycans, AAL binds fucose and WGA binds GlcNAc. Mal 1 binds to sialic acid. ELLA analysis demonstrated low signals across all lectins in negative control wells containing no glycoprotein (PBS) or recombinant human IgG1 Fc (Figure 4.3).

To establish the detection limit for the Notch1 Fc, 0-5μg/ml solutions of Notch1 Fc were probed with four N-glycan specific lectins. Con A, AAL and LCA bound very strongly across the varying concentration of Notch1 Fc, even at the lowest

concentration of 1μg/ml (Figure 4.4). The concentration was then further reduced to 0.01μg/ml, only Con A and AAL bound at this concentration. The detection limit for both LCA and WGA lectins was noted to be above 0.5 μg/ml (Figure 4.5) as no binding was observed with either of the lectins below this concentration.

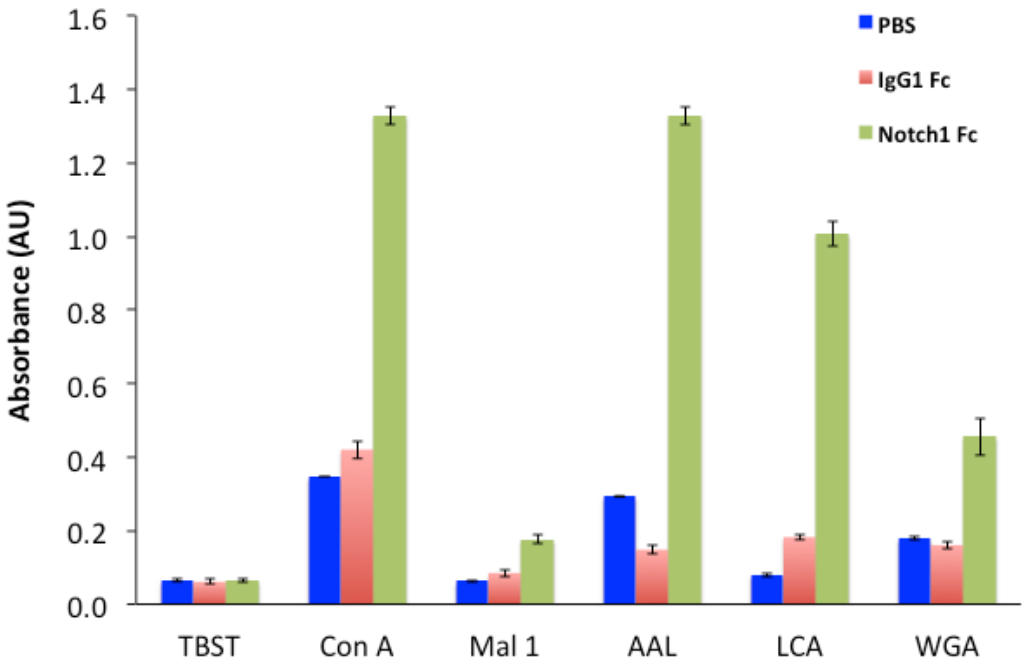

Figure 4.3. Detection of N-glycans on Notch1 Fc. Notch1 Fc and IgG1 Fc (5 μg/ml) were probed with 5 lectins. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

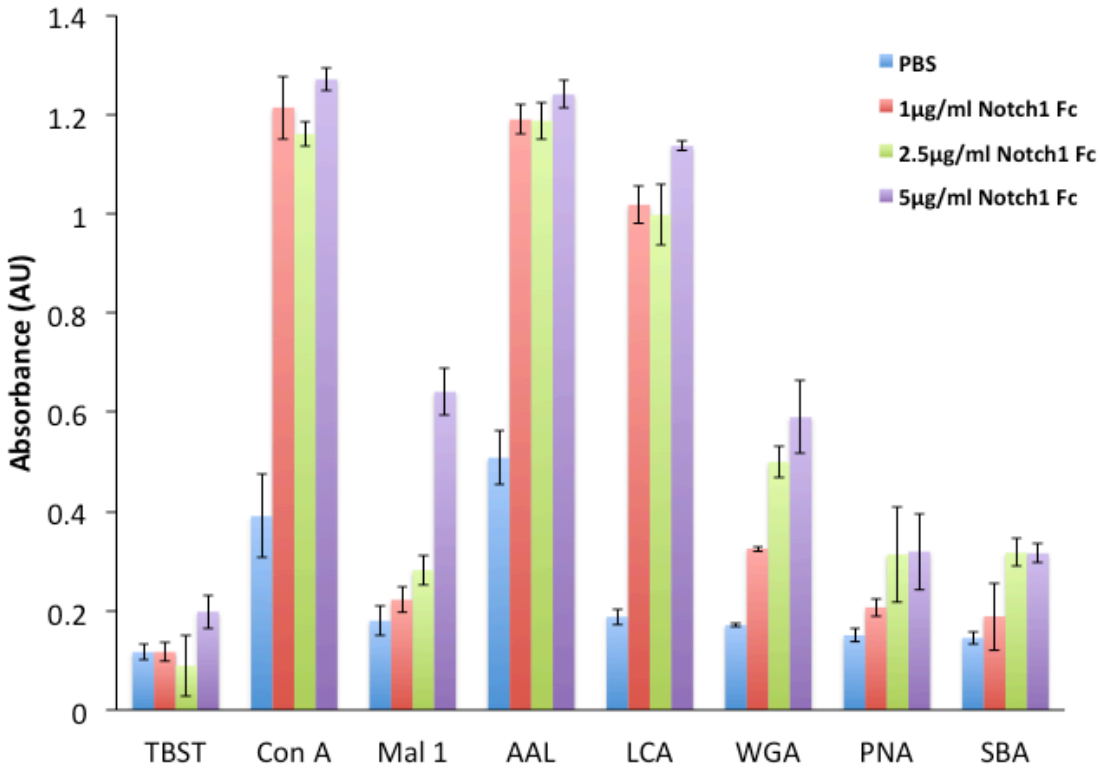

Figure 4.4. The detection limit of different lectins. ELLA was used to analyse different concentration of Notch1 Fc (0-5μg/ml) to determine the limit of detection for specific lectins. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

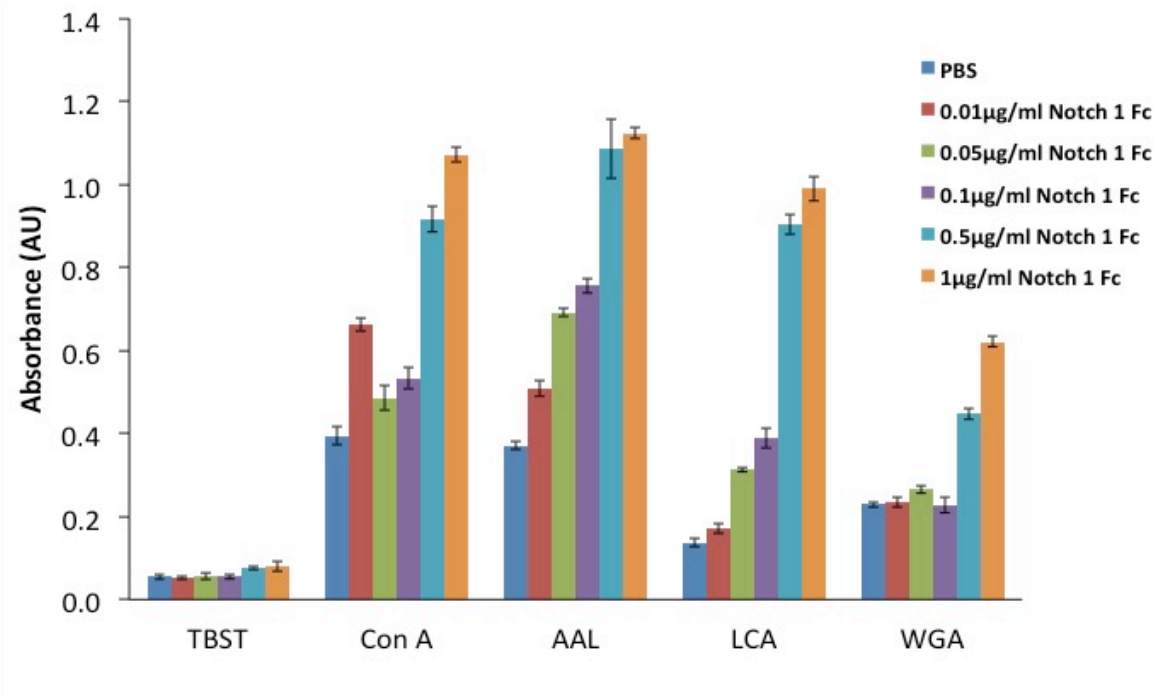

Figure 4.5. ELLA detection limit for Notch1 Fc. Notch1 Fc (0 - 1 μg/ml) was probed with four N-glycan specific lectins. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

4.3 Notch1 Fc and Notch1 overexpression lysate have similar N-glycan profile.

A comparative study was performed using ELLA under similar conditions to compare the N-glycan profile of Notch1 Fc and commercial Notch1 overexpression lysate (NBL1-13725, NOVUSBIO) generated in HEK293T cells. This was done to investigate if there is similarity between the N-glycan profile of pure Notch1 Fc and crude over expression lysate containing Notch1. Similar binding was observed with Con A and WGA (Figure 4.6). Notch1 Fc and overexpression Notch1 lysate were then treated with PNGase F to remove N-glycans. In both cases, a decrease in binding was observed across the N-glycan specific lectins (Figure 4.7) when compared to the non-treated. The result confirmed the removal of N-glycans.

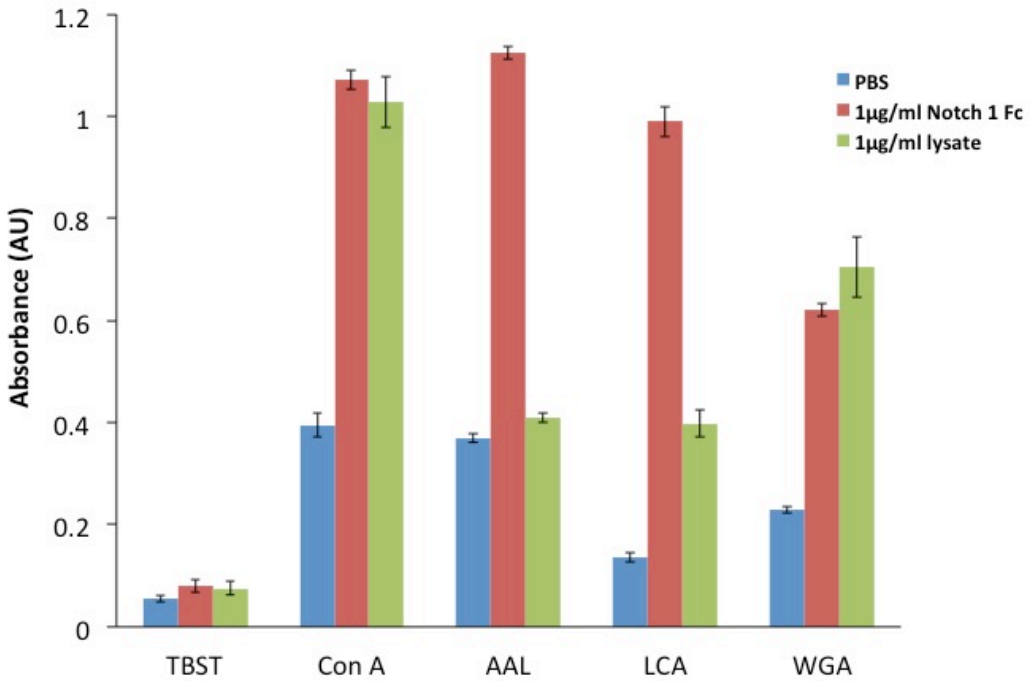

Figure 4.6. N-glycosylation profile of Notch1 Fc and overexpression lysate. Commercial Notch1 Fc (1 μg/ml) and Notch1 overexpression lysate (1 μg/ml) generated in HEK293T cells were analysed by ELLA to compare their N-glycosylation profile. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

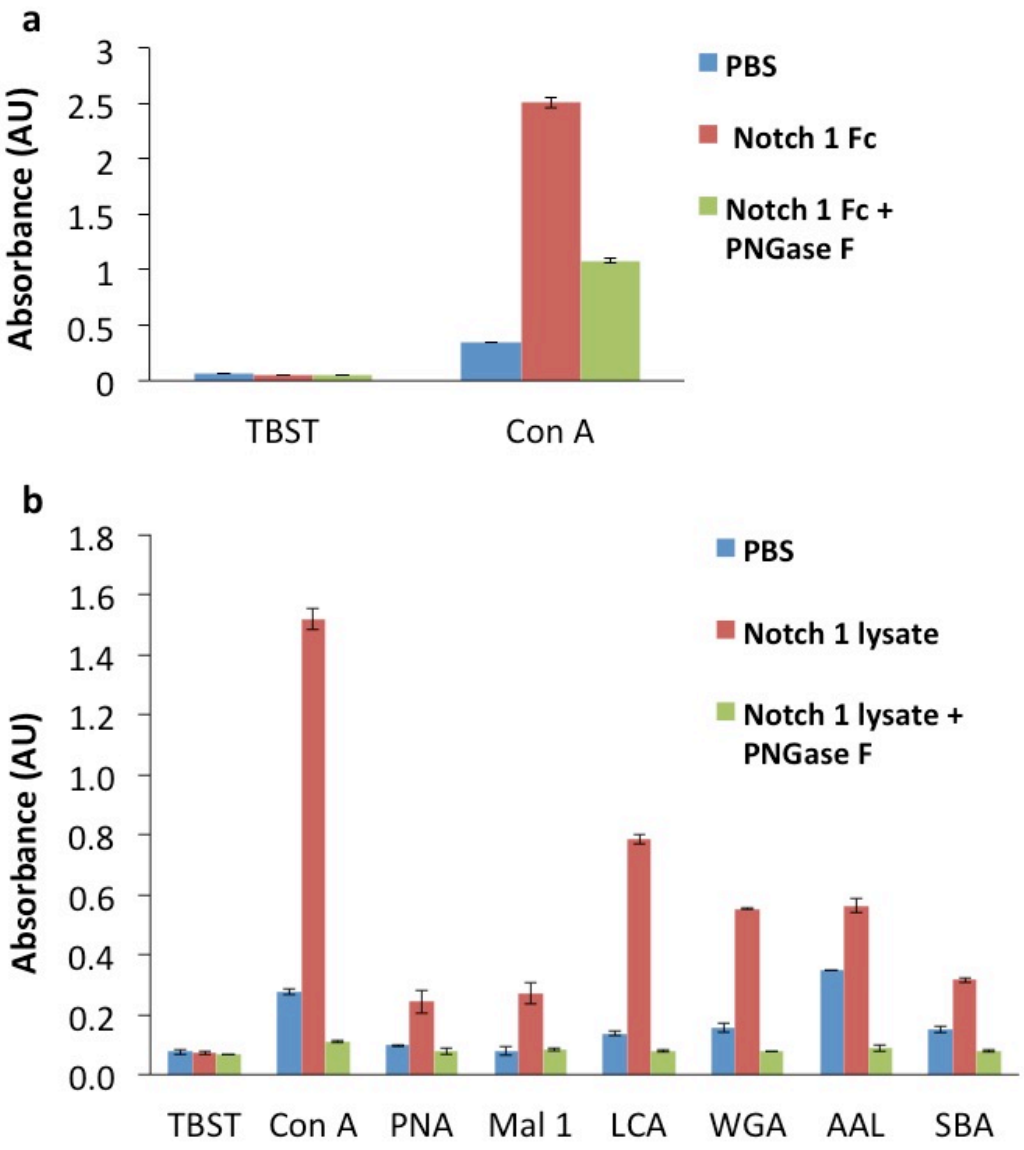

Figure 4.7. PNGase F treatment diminishes N-glycan binding. (a) Notch1 Fc was treated with PNGase F (1000U) to remove N-glycans, ELLA confirms the removal of N-glycans on the deglycosylated form (2.5 μg/ml) with significant reduction in Con A binding. (b) Notch1 overexpression lysate generated in HEK293T cells was treated with PNGase F (1000U), the deglycosylated sample (2.5 μg/ml) was then analysed by ELLA. Bar charts representative of triplicate experiments. Data are mean ± SD.

4.4 Glycoproteins of stem cells and smooth muscle cells interact with lectins.

In order to determine the changes in N-linked glycosylation during stem cell differentiation, it was decided to first compare the N-glycan profile of murine bone marrow derived MSC with murine vascular smooth muscle cells.

ELLA was used to establish the glycosylation profile of total glycoproteins present in lysates of cultured SMC and MSC. Different level of glycoproteins was detected in cultured MSC and SMC lysate. Although it can be assumed that any lectin interaction observed is not from the glycoprotein of interest, Notch1, still it was interesting to see that more N-linked glycoproteins are present in the MSC lysate as stronger binding was observed with Con A and AAL (Figure 4.8).

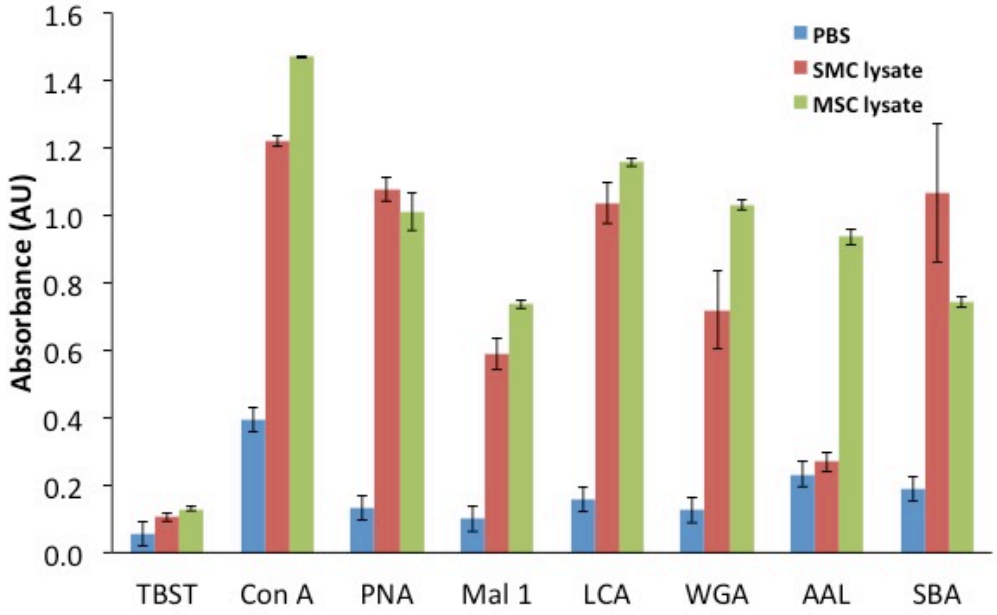

Figure 4.8. N-glycoprofiling of lysates generated from MSC and SMC lysates. Lysates (5 μg/ml) from cultured SMC and MSC were tested with 7 lectins to monitor the level of glycosylation in the different cell lines. Bar chart representative of triplicate experiments. Data are mean ± SD.

4.5 Ectopic over expression and glycosylation profiling of Notch1 in MSC.

The detection of Notch1 in MSCs lysate by ELISA indicated that the abundance was very low. Therefore, it would be a challenge to investigate its glycosylation state by ELLA. A technique for over-expressing Notch1 and easy isolation was decided upon to enable easier glycosylation profiling. Transfecting cells with full length Notch1 cDNA with lentiviral particles was initially considered for this purpose but it was not possible to obtain these commercially. The alternative was to ectopically express the Notch1 by transfecting the cells with c-Myc tagged full length Notch1 cDNA. To achieve this, it was required to optimise specific transfection conditions to achieve the desired transfection efficiencies for the chosen cell type. The optimisation was performed by transfecting MSCs with Green Fluorescent Protein (GFP) plasmid. Important parameters that were considered are the type of transfection reagent, ratio of transfection reagent to DNA, amount of transfected nucleic acid and length of time cells are exposed to the transfection reagent. Optimal transfection efficiency of approximately 70% was determined by fluorescence imaging, with low levels of cytotoxicity (Figure 4.9a).

MSCs or SMCs were then transfected with Notch1 plasmid tagged with a c-Myc epitope (peptide sequence EQKLISEEDL), the levels of myc tag expression in transfected and non-transfected MSCs was analysed by immunochemistry (Figure 4.9b and 4.9c) and by western blot (Figure 4.9d). Non-transfected cells were used as control rather an empty vector transfected cells, as the empty vector could not be obtained commercially.

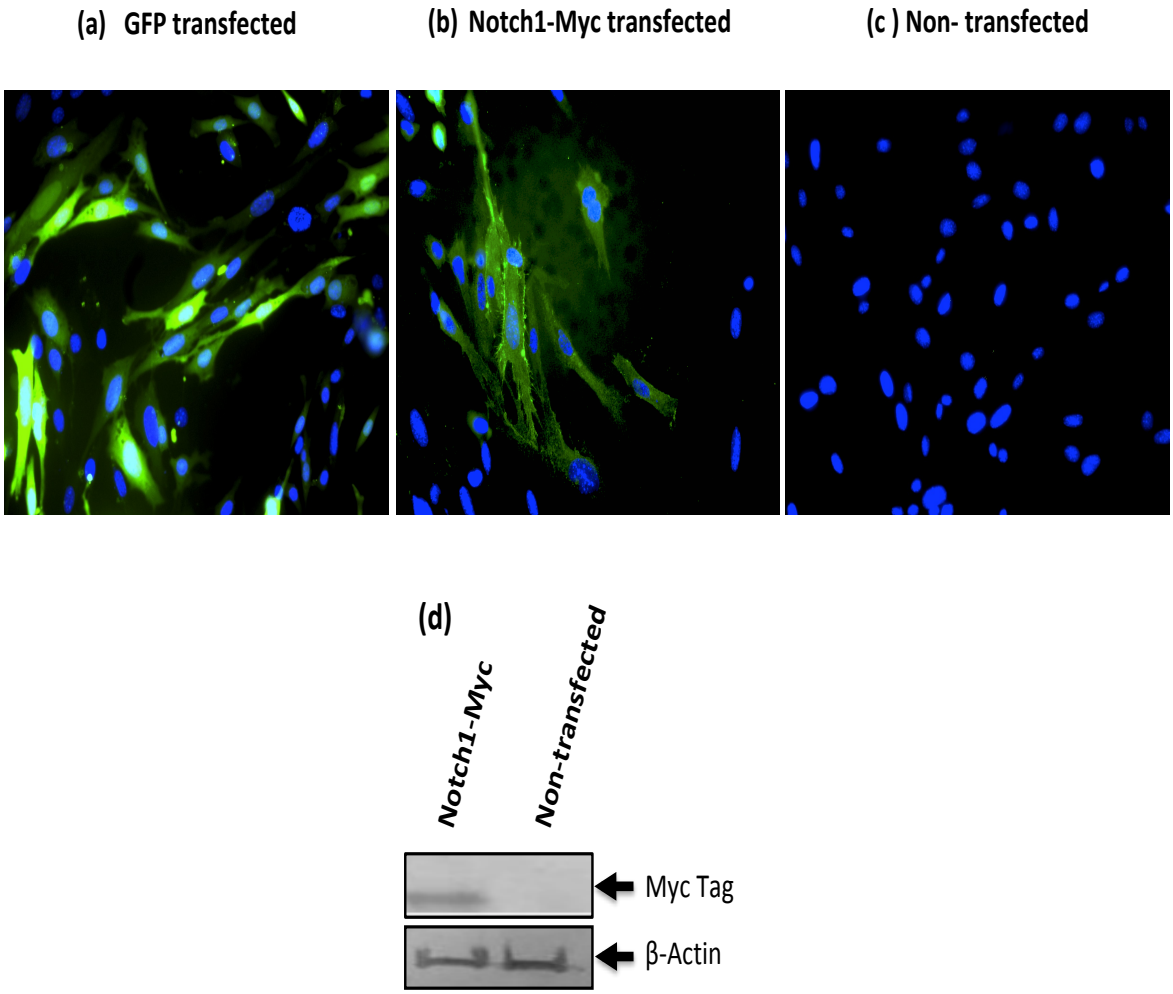

Figure 4.9. Ectopic Expression of the Notch1 receptor in MSC. (a) GFP expression following transient transfection of a GFP-expression plasmid, a positive control in murine MSCs. (b) Myc tag expression following transient transfection of a Notch1- Myc tag plasmid in murine MSCs. (c) Non-transfected cells were used as negative control. (d)Western blot of Myc tag expression in MSC lysates following transient transfection of MSCs with full length Notch1. MSCs were co-transfected with the puromycin resistance plasmid, pGKpuro and cells pooled following treatment with puromycin (2μg/ml, 72h). Cell lysates were precipitated with an anti-Myc antibody and probed with an anti-Myc and an anti-Beta actin antibody. Representative of 6 images. Gels depict representative of two independent experiments.

4.6 Notch1 is glycosylated with both O and N-glycans.

An ELLA glycosylation profile for immunoprecipitated (IP) Notch1 was determined using the purified Notch1 obtained from two different cell lines. The experiment was carried out using same concentration under similar conditions. Since full length Notch1 has both N and O glycans, binding across all of the 7 lectins was expected. The IP Notch1 generated from transfected SMCs and MSCs shows similar binding across the lectins (Figure 4.10). Immunoprecipitation was also carried out on the non-transfected control to establish the efficiency of the procedure. The control generated from non-transfected cells was also analysed and showed no binding to the lectins.

In order to establish any similarities in the glycosylation profile of total protein generated from MSCs lysate (containing Notch1 receptor in very low concentration and possibly other glycoproteins) and IP Notch1 generated from transfected MSCs, ELLA was carried out using same concentration under similar conditions. It is clear that the total proteins in the crude MSC lysates bound more strongly to 6 of the 7 lectins in comparison to the IP Notch1 (Figure 4.11). Results from ELISA and ELLA suggests that the concentration of endogenous Notch1 was too low to be detected by the lectins, therefore any interaction observed with the lectins and the proteins in the crude MSC lysate was considered to be from other glycoproteins present in the lysate and not from the glycoprotein of interest, Notch1. The interaction of the lectins and the purified Notch1 from transfected cells was observed to be significant and of true relevance. The IP Notch1 shows binding to Con A, which has affinity for core mannose of biantennary complex N-glycan and a

much stronger binding to LCA which is specific for fucosylated core mannose of complex N-glycan. The ELLA confirms the presence of N- glycans on Notch1.

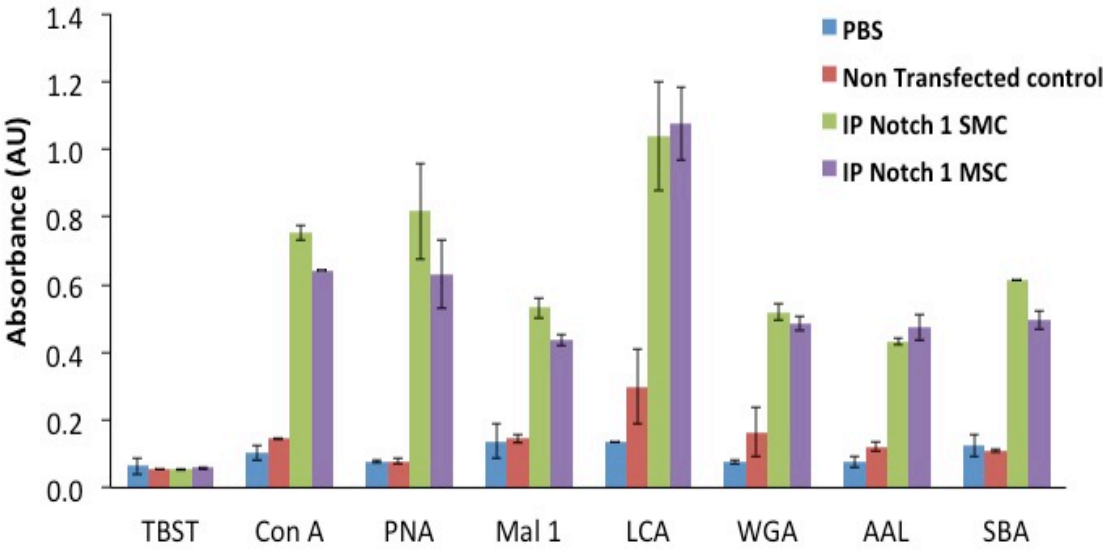

Figure 4.10. Glycosylation profile of IP Notch1 in different cell lines. Following transient transfection of SMCs or MSCs, purified Notch1 was generated by Immunoprecipitation (IP) procedure using anti-Myc (9E10) magnetic beads and quantification was performed by BCA. The IP Notch1 (1 μg/ml) was then analysed by ELLA. Pulled down fraction from non-transfected cells was used as negative control. The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

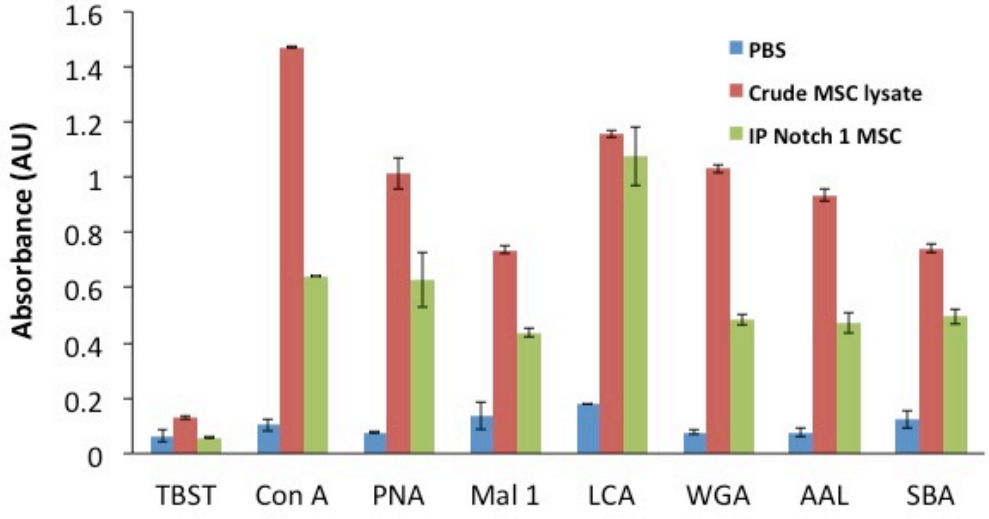

Figure 4.11. Glycosylation profile of crude lysate and IP-Notch1 derived from MSCs. Immunoprecipitated Notch1 (IP Notch1, (1 μg/ml) generated from transfected MSC and crude MSC lysate (1 μg/ml) were probed with lectins using the ELLA assay. The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

The glycosylation state of c-Myc tag was also investigated to verify that any lectin binding observed with the IP Notch is true and not from the tag. An experiment was performed with a c-Myc peptide (Sigma Aldrich) as a representative, the result indicates that the tag is not glycosylated (Figure 4.12).

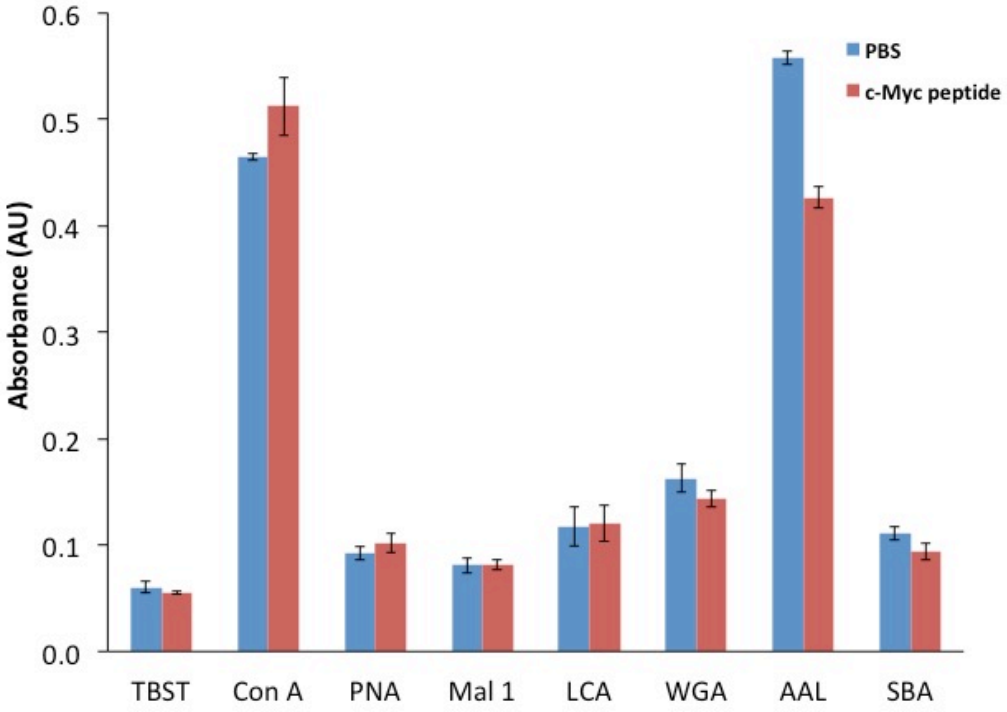

Figure 4.12. c-Myc peptide is not glycosylated. Glycosylation analysis was done on c-Myc peptide (5 μg/ml) by probing with 7 lectins. Bar chart is a representative of triplicate experiments. Data are mean ± SD.

4.7 PNGase F treatments remove N-glycans on IP Notch1 and MSC lysate glycoproteins.

The ELLA was then employed to study the de-glycosylated form of IP Notch1. Immunoprecipitated Notch1 from transfected MSCs was treated with PNGase F and the removal of N-glycans was monitored. Con A is known to bind strongly to the mannose/glucose core of N-linked glycans. The treatment of Notch1 with PNGase F greatly reduced the binding to Con A in comparison to that without treatment, suggesting that most or all of the N-glycans had been removed (Figure 4.13a). To release the N-linked glycans from total glycoproteins in MSC lysate, lysate was treated with PNGase F. The N-deglycosylated lysate was analysed by ELLA and the result showed removal of N-glycans (Figure 4.13b).

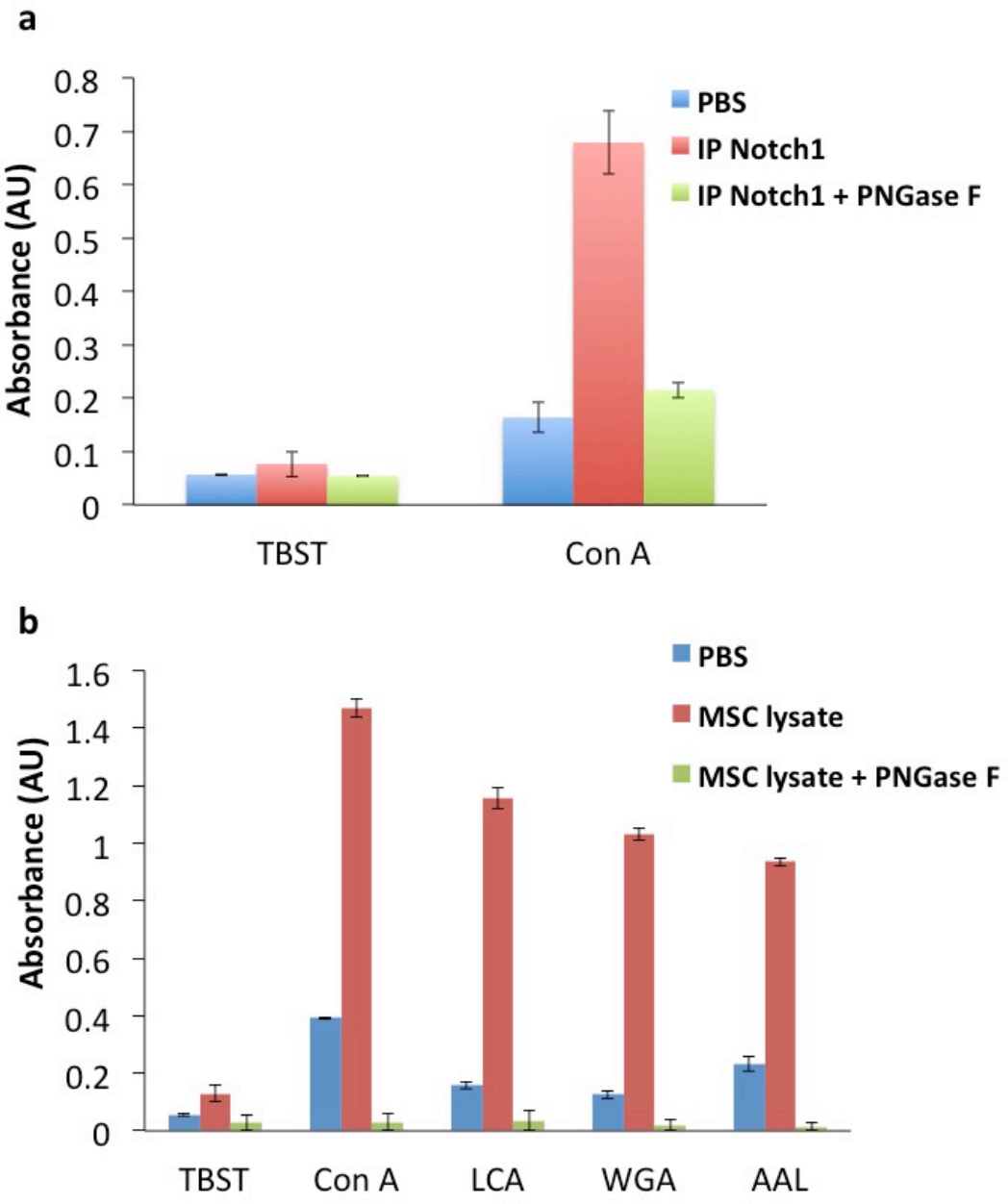

Figure 4.13. The effect of PNGase F on IP Notch1 and MSCs N-glycome. Immunoprecipitated Notch1 and MSC lysate were treated with PNGase F (500U) for 24 h followed by ELLA analysis of (a) deglycosylated IP Notch 1 (1 μg/ml) and deglycosylated MSC lysate (1 μg/ml). The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

4.8 The effect of PNGase F or Tunicamycin treatments on MSC N-glycome.

To target the N-glycans on the cell membrane, MSCs in culture were treated with 0 or 1000U PNGase F in media for 24 h in complete growth media. Following treatment, whole cell lysate (Figure 4.14a) and membrane fraction lysate (Figure 4.14b) were analysed by ELLA to monitor changes in glycosylation profile with and without PNGase F treatment. In both cases, the result showed no difference in lectin binding between the treated and untreated samples. It seems the treatment targeting the surface N-glycans had little to no impact on the overall N-glycosylation.

In order to determine the effect of tunicamycin on the total N-glycoproteins expressed in MSCs, it was first necessary to determine the MSC survival profile. Cells were treated with 0-100ng/ml Tunicamycin or vehicle (DMSO) for 72 h, 25ng/ml tunicamycin was found to be the safest concentration for cell survival (Figure 4.15a). Cells were then treated 25ng/ml tunicamycin for 72 h, total protein lysate was obtained followed by ELLA analysis to see if there's a reduction in N-glycan specific lectin binding following tunicamycin treatment. The ELLA result indicated no difference in the N-glycosylation between lysates from treated and non-treated group (Figure 4.15b).

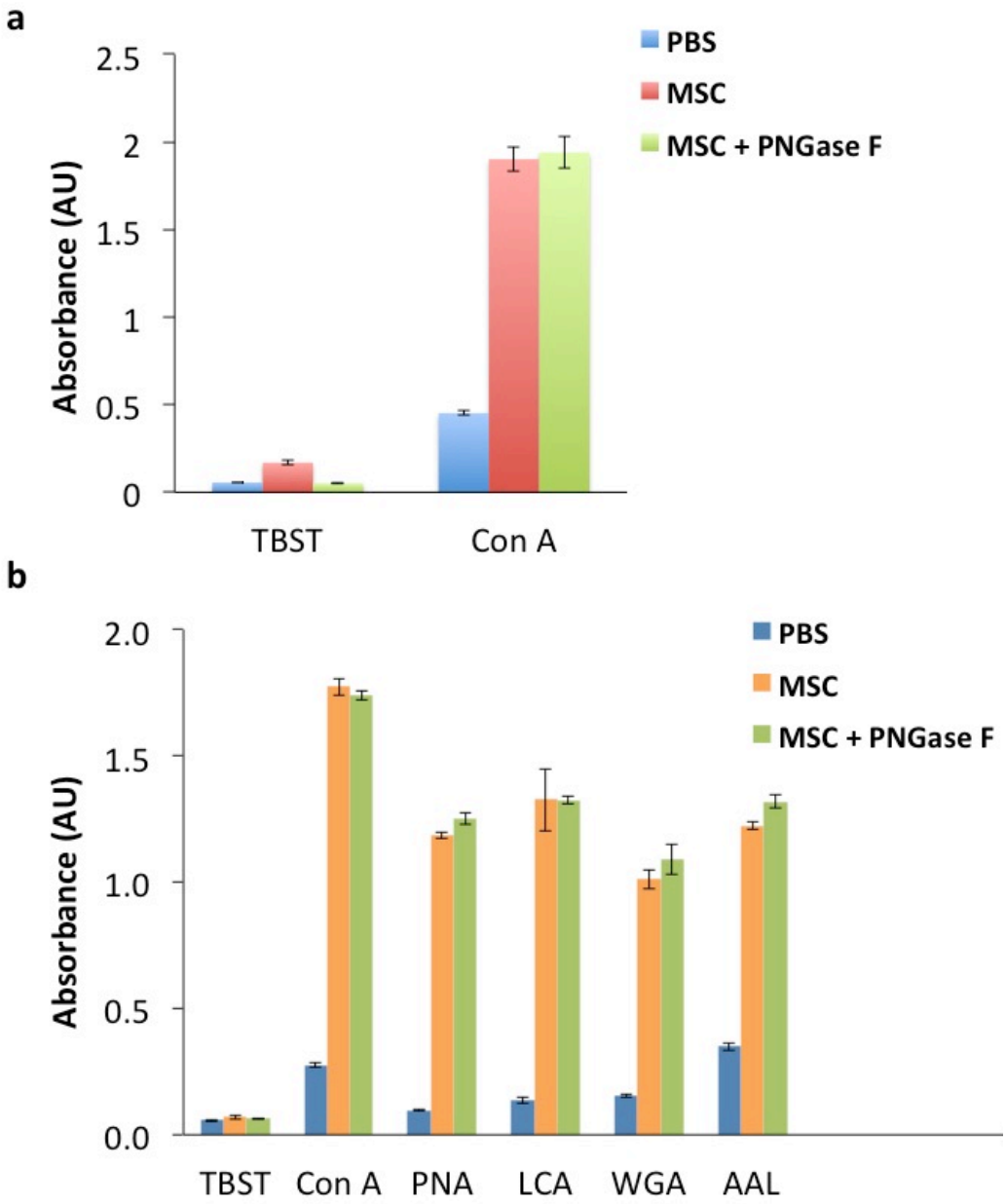

Figure 4.14. The effect of PNGase F on MSC membrane N-Glycans. MSCs in culture were treated with PNGase F for 24h. a) Total protein lysate (5 μg/ml) from treated and non-treated cells were analysed by ELLA b) Membrane protein lysate (5 μg/ml) from treated and non-treated cells were analysed by ELLA. Chart is a representative of triplicate experiments. Data are mean ± SD.

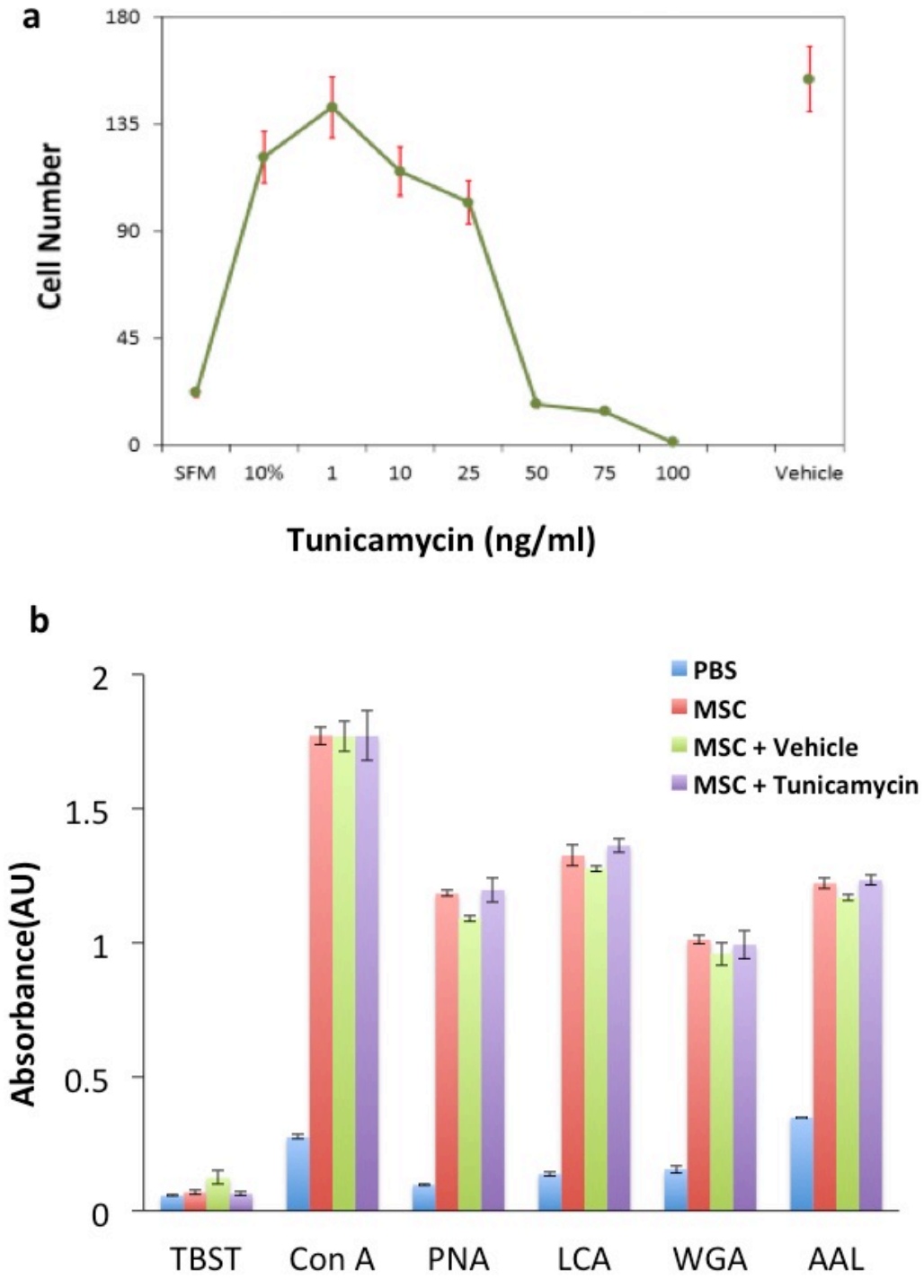

Figure 4.15. The effect of tunicamycin on MSCs N-glycome. (a) Cells were treated with varying doses of tunicamycin (0-100ng/ml) for 72 hours to determine the safe dose to inhibit N-Glycosylation without significant inhibition of growth. A plot of concentration against average cell count was generated. Data are the mean ±SEM of triplicate wells. (b) MSCs were treated with tunicamycin (25ng/ml) for 72h before protein lysate (5 μg/ml) was analysed by ELLA. The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

4.9 The effect of PNGase F on MVSC membrane N-Glycans.

Further glycosylation studies were carried out on MVSC with the aim of achieving a better glycosylation profile for Notch1 / stem cell N-glycoproteins following tunicamycin or PNGase F treatment. Live cells were incubated with PNGase F in PBS for 6 h. Membrane protein extraction was carried out on one set of cells and total protein extraction was carried out on the other set. ELLA was used with N-glycan specific lectins to confirm the effect of PNGase F on proteins in the whole lysate (Figure 4.16a) and the membrane fractions (Figure 4.16b). Between these two groups, no significant binding intensity changes were detected in the expression of cell surface N glycans. This demonstrates that the total amount of cell surface glycans is not significantly modified after PNGase F treatment, although individual changes to protein of interest may be masked.

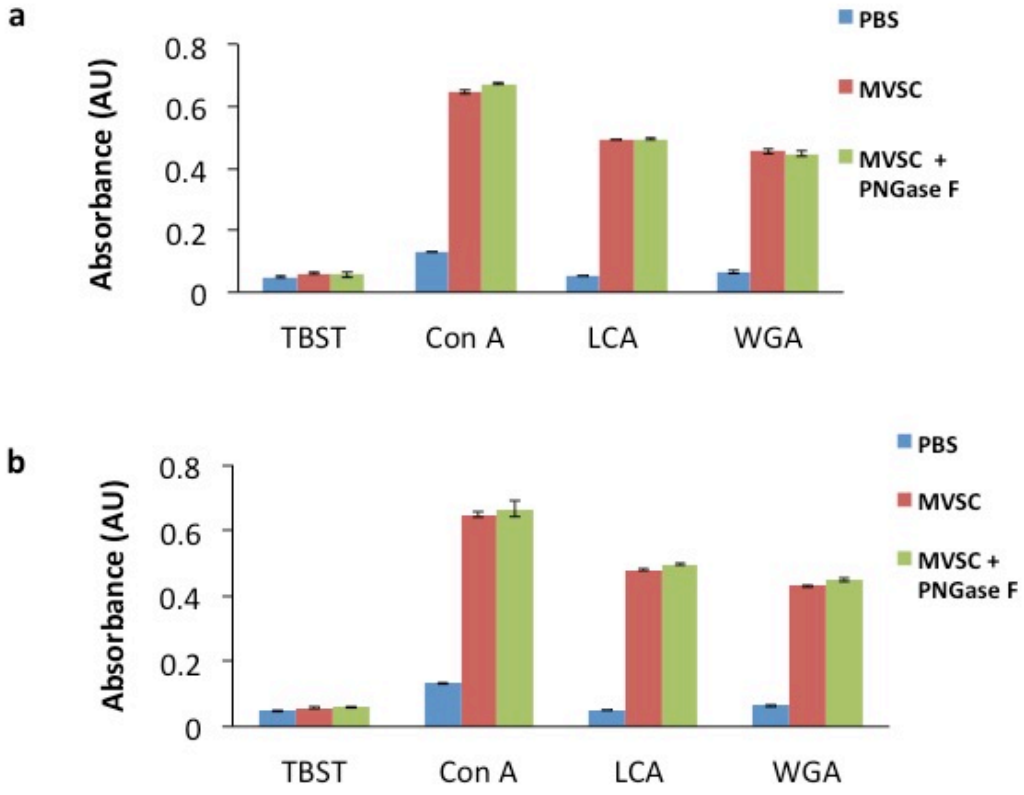

Figure 4.16. The effect of PNGase F treatment on MVSC membrane N-Glycans. MVSCs in culture were treated with 0 or 500U PNGase F in PBS and incubated for 6 h at 37^{o}C before either (a) whole cell lysate or (b) membrane fraction lysate was prepared and analysed for lectin binding by ELLA. BCA protein assay was carried out on both sample groups and all samples were diluted to 7.5 μg/ml with PBS. The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

MVSC lysate was treated with PNGase F to remove N-glycans of present glycoproteins and verify the effectiveness of the treatment. ELLA analysis indicated that PNGase F was effective in removing the N-glycans of glycoproteins in lysate (Figure 4.17) whereas no effect was observed in the glycoproteins from PNGase F treated cells (Figure 4.16a). It can be inferred that PNGase F has greater access to the asparagine bond of glycoproteins in the cell lysate.

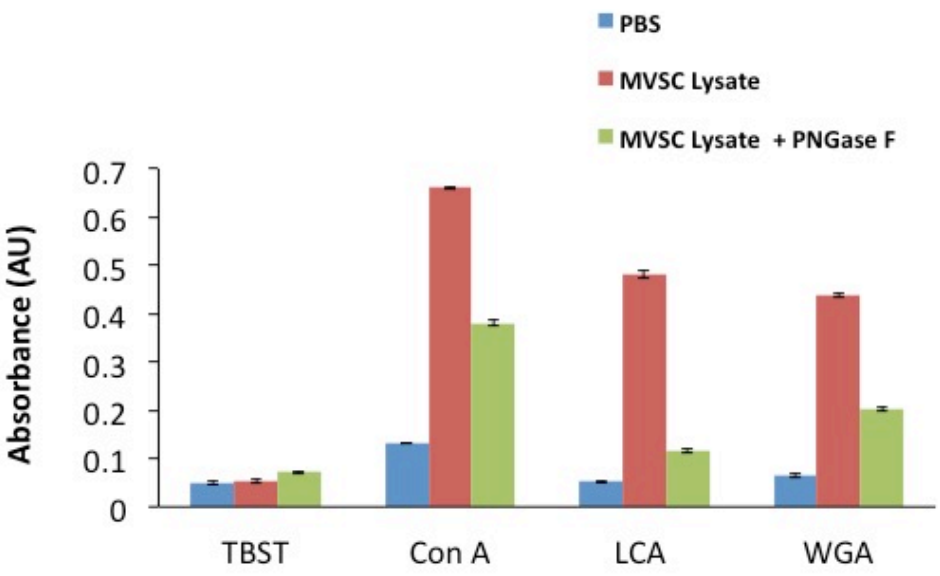

Figure 4.17. PNGase F treatment is effective in removing N-glycans of MVSC glycome. Cells were grown for 5 days in complete growth media until confluent. The cells were harvested and lysed using RIPA buffer and BCA assay was used to determine the concentration of total protein. Deglycosylation was carried out on 20µg protein using PNGase F (500U), ELLA analysis was carried out on PNGase F treated total protein lysate. The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

4.10 Tunicamycin treatment affects N-glycoproteins of MVSC.

In order to determine the effect of tunicamycin treatment on MVSC membrane N-glycans, it was first necessary to establish the modulatory effects of tunicamycin on multipotent vascular stem cell growth and determine the effective lower dose to modify N-glycosylation. Cells were treated with 0 - 4μg/ml tunicamycin or DMSO (vehicle) in complete maintenance media for 24 h. A significant reduction in cell number was observed with all tunicamycin concentrations (Figure 4.18a).

Next, the effect of tunicamycin on the total N-glycoproteins expressed in MVSCs was determined. Cells in culture were treated with 0.5μg/ml tunicamycin for 24h. Lysate containing total protein was prepared from the cells and analysed by ELLA to investigate the state of N-glycosylation with N-glycan specific lectin binding. The ELLA result indicated slight differences in the N-glycosylation of glycoproteins from treated and non-treated group. There was reduced interaction with Con A, LCA, Mal 1 and WGA in comparison to the untreated control (Figure 4.18b).

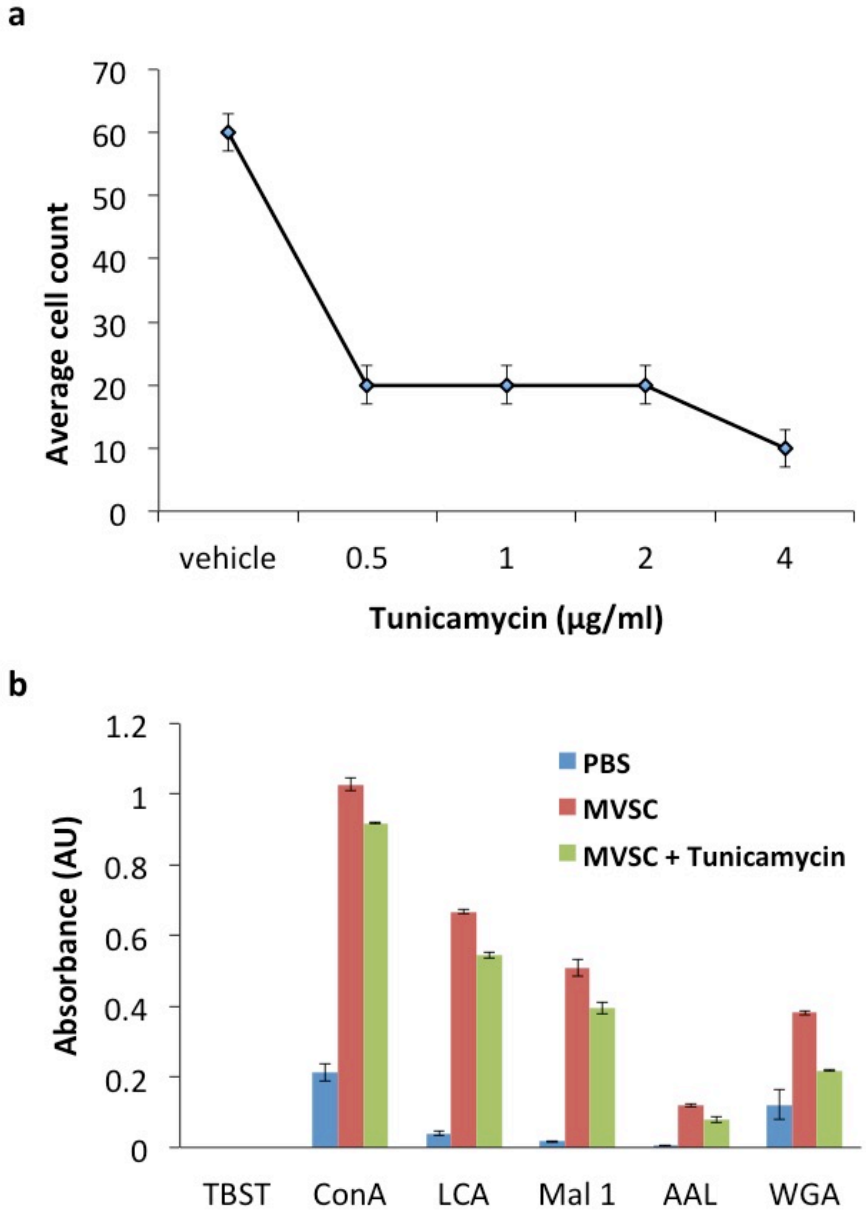

Figure 4.18. Tunicamycin treatment affects N-glycoproteins of MVSC. (a) Cells were treated with 0 - 4µg/ml tunicamycin or DMSO (vehicle) in complete maintenance media for 24 h. Cells were washed with PBS and fixed with 3.7% formaldehyde, before staining of cells with DAPI for nuclear visualisation and analysed by fluorescence microscopy, taking an average count from 5 images per well. Image J was used for cell count, a plot of concentration against average cell count was generated. Data are the mean ± SD of six wells/concentration. (b) MVSCs were treated with tunicamycin (0.5µg/ml) for 24 h and total lysate (7.5 µg/ml) was analysed by ELLA. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

4.11 Ectopic over-expression and glycosylation analysis of Notch1 in MVSC.

MVSC were transfected with Notch1 plasmid tagged with a c-Myc epitope (peptide sequence EQKLISEEDL) or GFP plasmid. Optimal transfection efficiency of approximately 70% was determined by Fluorescence imaging of GFP transfected cells (Figure 4.19a). The levels of Myc tag expression in transfected and non-transfected MVSCs was analysed by immunocytochemistry (Figure 4.19b and Figure 4.19c) and by western blot (Figure 4.19d). The level of Notch1 expression by western blot (Figure 4.19d) confirmed Notch1 ectopic expression.

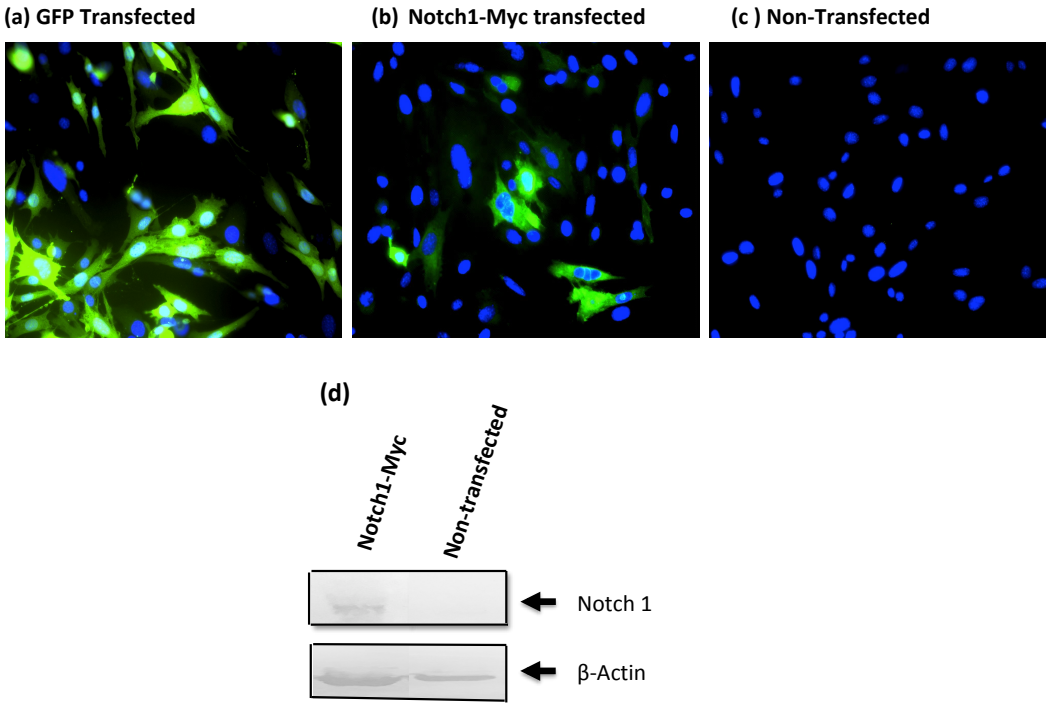

Figure 4.19. Ectopic Expression of the Notch1 receptor in MVSC. (a) GFP expression following transient transfection of a GFP-expression plasmid, a positive control in MVSCs. (b) Myc tag expression following transient transfection of a Notch1- Myc tag plasmid in MVSCs. (c) Non-transfected cells were used as negative control. (d)Western blot of Myc tag expression in MVSC lysates following transient transfection of MVSCs with full length Notch1. Cell lysates were precipitated with an anti-Myc antibody and probed with an anti-Notch1 and an anti-Beta actin antibody. Representative of 6 images. Gels depict representative of two independent experiments.

4.12 Tunicamycin treatment modifies N-glycosylation of Notch1 overexpression lysate.

To establish the effect of tunicamycin on over-expressed Notch1 N-glycosylation, MVSCs were transfected with Notch1 plasmid and incubated for 48 h followed by 24h tunicamycin treatment. Following immunoprecipitation, it was discovered that the level of antigen for the Myc-antibody was so low that no protein was detected in the pulled-down sample by BCA, possibly lower than the level of detection, therefore only the overexpressed Notch1 lysate was analysed by ELLA. The over expressed Notch1 lysate demonstrated reduced binding to Con A, LCA, Mal1 and WGA as a consequence of the tunicamycin treatment (Figure 4.20).

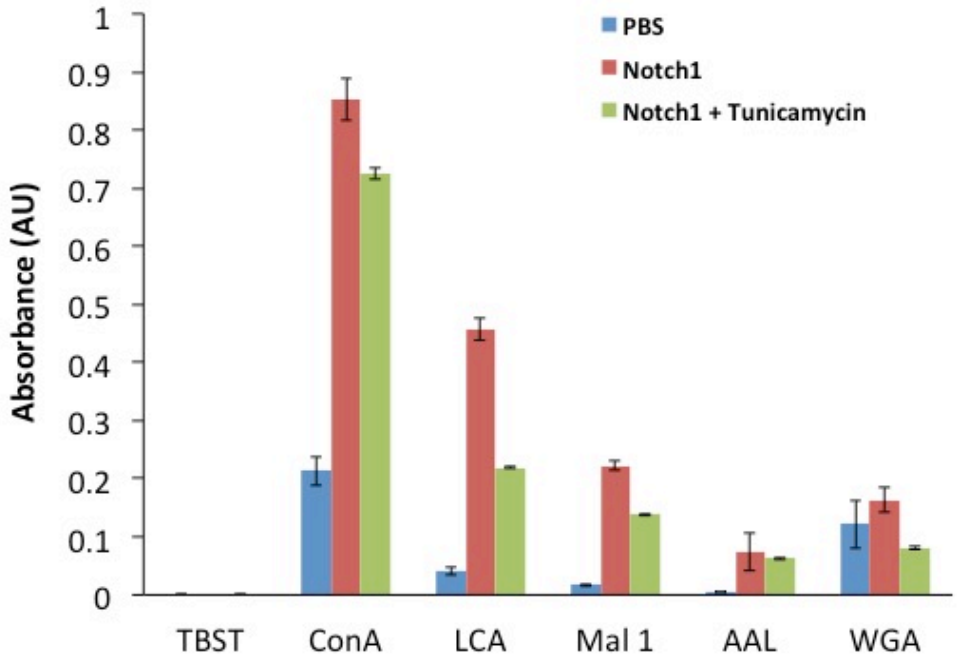

Figure 4.20. Tunicamycin treatment modifies N-glycosylation in Notch1 overexpression lysate. MVSCs were transfected with Notch1 plasmid before treatment with tunicamycin (0.5μg/ml for 24h). Quantification of protein was performed using the BCA assay and glycosylation analysis of over-expressed Notch1 lysate (7.5 μg/ml) by ELLA. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

4.13 Transient knockdown of Lunatic Fringe (Lfng) gene expression modifies N-glycosylation of Notch1 overexpression lysate.

Lunatic fringe, a N-acetylglucosamine sugar transferase, was identified in Drosophila and controls which ligand types activate Notch in many developmental contexts. There are three fringe genes in mammals; Lunatic fringe (Lfng), Manic fringe (Mfng), and Radical fringe (Rfng). Many genetic studies have discovered a large number of tissues where Delta like or Jagged ligand induced Notch signalling and fringe genes are expressed. Despite this, very little is known about how fringes control Notch-dependent development. During somitogenesis, Lfng regulates molecular clock by restricting Notch activation downstream of Delta like ligands. Similarly, Lfng facilitates Notch activation by Delta like ligands during lymphocyte, blood vessel, and neuronal development. Importantly, Lfng potentiates Notch signalling cell-autonomously. In vivo overexpression experiments with Notch ligands suggest that Lfng strongly augments Notch signalling mediated by Delta-like 1 but not Jagged 1 (Kato et al., 2010; Evrard et al., 1998; Cohen et al., 1997).

Short, interfering RNAs (siRNAs) can be used in mammalian cells to gain information about gene function through inactivation of specific gene expression (Wall 2003). In order to investigate if Lunatic Fringe (Lfng) is essential for Notch1 N-glycosylation, the Lfng was transiently knocked down in MVSCs using siRNA specific to Lfng. Optimal transfection was established by transfecting MVSCs with an IDT fluorescent-labelled siRNA using Trans IT-X2™ Dynamic Delivery system, as described in Material and Methods, section 2.6. Greater than 90% of cells took

up dye when analysed by fluorescence microscopy 24h post-transfection (Figure 4.21a).

Transfection of MVSCs was performed with a positive control siRNA (HPRT) to verify the effect of RNA interference. A significant knockdown of HPRT mRNA level was achieved (0.31 ± 0.05 fold, n=3) (Figure 4.21b).

MVSCs were transfected with a commercially available Qiagen Gene solution siRNA targeted against Lfng and a knockdown of Lfng mRNA level was observed (0.47 ± 0.09 fold, n=3) (Figure 4.21c).

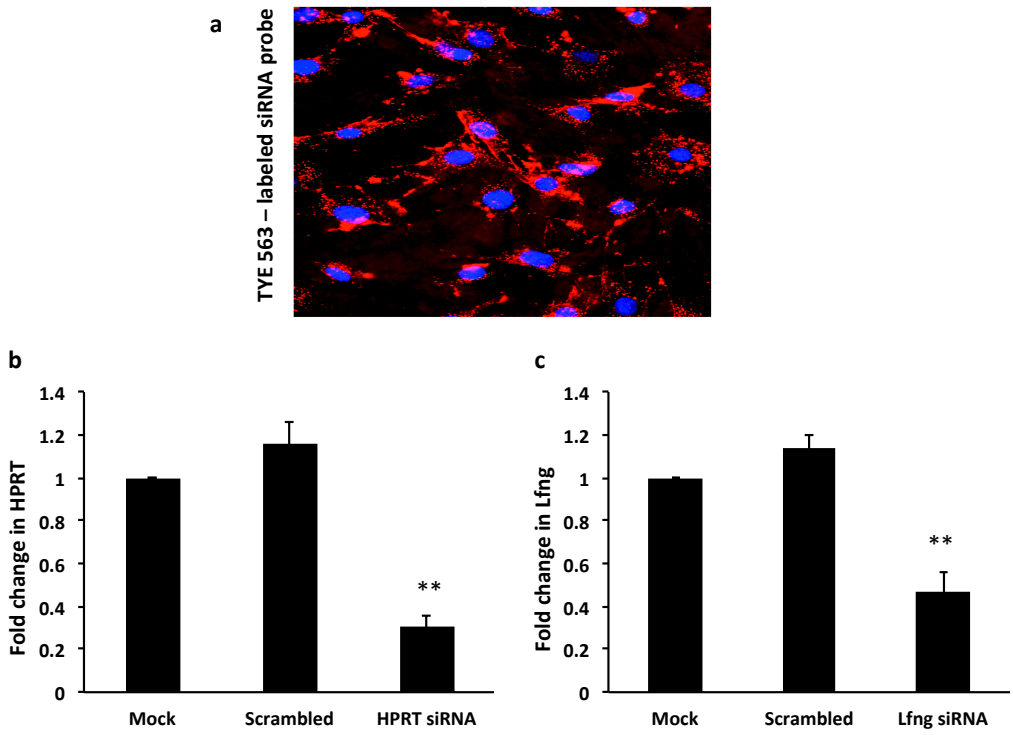

Figure 4.21. Lfng knockdown by siRNA. (a) Transfection efficiency visualised 24h post-transfection using a TYE 563 siRNA probe. MVSCs were transfected at a concentration of 10nM with the TYE ^{TM} 563 DS Transfection control duplex using Trans IT-X2^{TM} Dynamic Delivery system. Cells were imaged 24h post-transfection. MVSCs were seeded at a density of 200,000 cells /well in a 6well plate. 18-24h later, the cells were transfected using (b) 10nM HPRT positive control duplex or (c) Lfng siRNA. 72h post-transfection, RNA was isolated using Magcore instrument, and HPRT and Lfng levels were measured using qRT-PCR. Relative expression was normalized to internal control mRNA using scrambled control as baseline (100%). **$p<0.01$ as compared to scrambled siRNA treated control (Students t test). The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.

MVSCs were co-transfected with Lfng or scrambled siRNA and Notch1 cDNA. Seventy-two hours post transfection, an attempt was made to immunoprecipitate the Myc-tagged Notch1 in order to fully characterise the glycosylation of purified Notch1. The level of antigen for the Myc-antibody was so low that no protein was detected in the pulled-down sample by BCA, possibly lower than the level of detection, therefore only the overexpressed Notch1 lysate was analysed by ELLA (Figure 4.22). Lfng knockdown did not affect the binding of LCA, Mal 1 or AAL to overexpressed Notch1 lysate. Only N-acetyl glucosamine binding lectin WGA demonstrated significant reduction in binding by 50%, indicating the modification of complex N-glycan.

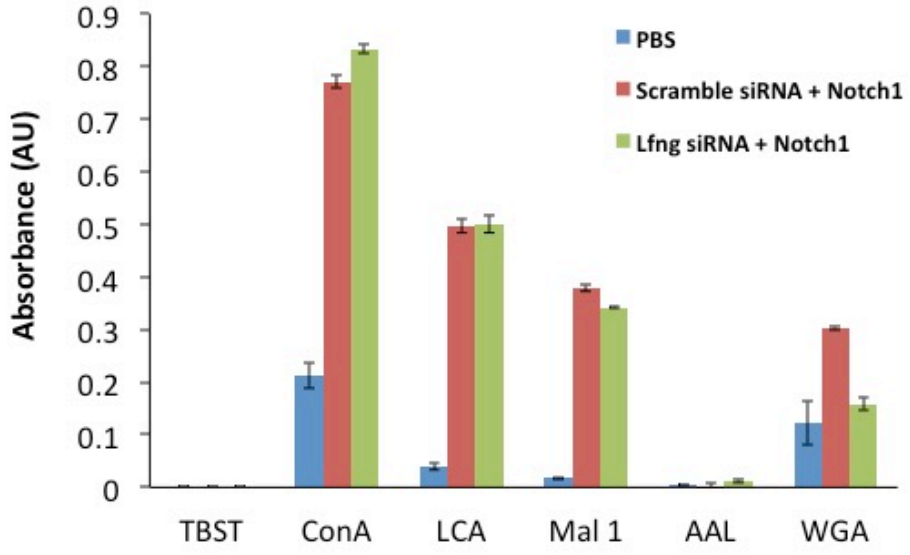

Figure 4.22. Transient knockdown of Lunatic Fringe (Lfng) gene expression modifies N-glycosylation in Notch1 overexpression lysate. MVSCs were transfected with Lfng siRNA or scrambled siRNA, protein extracts were obtained 72 h post transfection. Quantification of protein was performed using the BCA assay and glycosylation analysis of over-expressed Notch1 lysates (5 μg/ml) by ELLA. The experiments were performed in triplicate with the chart representative of two independent experiments. Data are mean ± SD.

4.14 Notch Ligand Jagged1 is glycosylated.

Ligand Jagged1 is essential for activating Notch and stimulating mesenchymal stem cell differentiation to smooth muscle cells (Kurpinski et al. 2010). Jag 1 Fc was analysed by ELLA (Figure 4.23), of the 7 lectins tested, 5 bound very strongly to Jag-1 in comparison to the negative control IgG Fc. This indicated that Jagged1 is glycosylated.

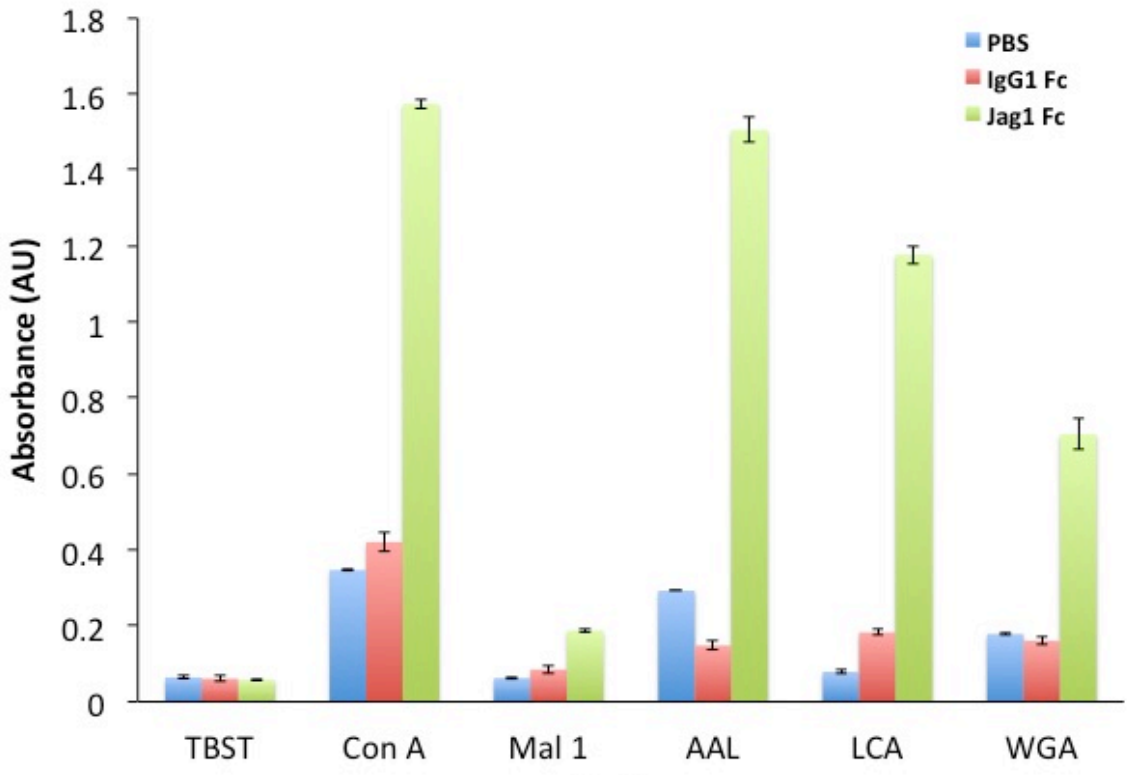

Figure 4.23. Notch Ligand, Jagged1 is glycosylated. Jag-1 Fc (5 μg/ml) was analysed by ELLA and compared to the IgG Fc negative control. Chart is a representative of triplicate experiments. Data are mean ± SD.

Summary

- ELLAs facilitated detection of N-glycan binding to specific lectins using purified recombinant hNotch1 Fc, hNotch1 overexpression lysate and immunoprecipitated mNotch1 following ectopic expression in MSC and MVSC.
- Increasing concentrations of purified Notch1 preferentially bound to Con A and AAL while higher concentrations bound to LCA and WGA suggesting high levels of mannose and fucose and complex N-glycans are present. PNGase F treatment attenuated binding to these lectins.
- PNGase F treatment of Notch1 lysates and IP Notch1 from MSC and MVSCs attenuated binding to Con A confirming mNotch1 has high levels of mannose.
- Lunatic Fringe (Lfng) knockdown attenuated Notch1 N-glycan binding to WGA and Mal 1 lectins.

Discussion

This study investigated the N-glycosylation profile of Notch1 receptor expressed on both resident like murine MSCs and MVSCs. To achieve this, it was necessary to first determine the level of endogenous Notch1 receptor protein that can be obtained from a given number of cultured MSCs. ELISA was employed for this purpose given its specificity to determine the concentration of target protein rather than total protein. It was discovered that only 3ng/ml of endogenous Notch1 was present in the MSC lysate. Although the ELISA method was useful in determining the level of endogenous Notch1 present in MSC lysate, the technique was not used routinely, as there was no means of easy isolation of Notch1 from the total protein in the lysate to study its glycosylation.

Next, ELLA was used to analyse the N-glycosylation of human Notch1 standard (50ng/ml), there was no interaction with Con A lectin tested. This indicates that concentration of the Notch1 tested was too low for lectin binding. A decision was then made to use commercially available recombinant human Notch1 Fc chimera, a representative of Notch extracellular domain (ECD) as it was not possible to obtain purified Notch1 standard in higher concentration. Human Notch ECD 1 (Notch1 amino acids Ala19 - Gln526, including first 13 EGF repeats) was shown to have 91% aa identity with corresponding regions of mouse and rat (R&D systems).

The main lectins tested are Con A, LCA, AAL, WGA and Mal 1, these five lectins cover the majority of N-glycan types. Con A lectin has affinity for branched α-mannose structures of high-mannose type, hybrid type and complex type N-glycan, LCA lectin is specific for fucosylated core region of complex type N-glycans (Roth, Yehezkel and Khalaila, 2012). AAL binds the Fucose region of complex N-

glycans (fucose linked to N-acetyl glucosamine) and WGA recognises terminal β-GlcNAc (N-acetyl glucosamine) of complex N-glycans, Mal 1 detects glycans composed of NeuAc-Gal-GlcNAc with sialic acid at the 3-position of galactose (Kaji 2006; Murakami et al. 2014).

Each of the lectins interacted with hNotch1 Fc in an expected manner based on the glycan structures known to be present on this glycoprotein. Notch receptor is known to be modified by O-fucose, O- glucose glycans and complex N-glycan. Structural analysis performed on Notch1 expressed as ECD fragments in mammalian cells suggests that the composition of O-fucose glycan observed to date is sialic acid α-(2,3)Galβ(1,4)GlcNAcβ(1,3)Fuc-O –Ser/Thr (Stanley 2007) or (2,6)Galβ(1,4)GlcNAcβ(1,3)Fuc-O –Ser/Thr (Harvey et al. 2016). ELLA was then employed to determine the detection limit for specific lectin binding with a series of concentration of hNotch1 Fc. The glycoprofile obtained indicated that a minimum of 50ng/ml is required to observe any significant lectin interaction and enable monitor of Notch1 receptor glycosylation.

A comparison study on hNotch1 Fc and commercially available hNotch1 overexpression lysate was undertaken to see if there is any similarity in their glycosylation profile. The lysate was created following transient transfection of HEK293T cells with plasmid ID RC211365, C-terminal DDK tag (NOVUSBIO). It was thought that this lysate would mimic the lysate generated from cultured MSCs. Con A and WGA interacted with both at the same intensity, suggesting that they have similar levels of mannose and N-acetyl glucosamine, possibly due to high amount of Notch1 also present in the overexpression lysate. It was interesting to

find that AAL and LCA, which has strong affinity for fucose region of complex N-glycans, demonstrated stronger binding to hNotch1 Fc.

To verify that N-glycans are present on hNotch1 Fc and hNotch1 overexpression lysate, they were treated with PNGase F and resulting glycoproteins were analysed by ELLA. In both cases, a significant reduction in binding to Con A was observed suggesting that N-glycans had been modified.

Having established that N-glycans are present on hNotch1 Fc and hNotch1 overexpression lysate, an ELLA profile was obtained for glycoproteins in SMC and MSC lysate. The seven lectins tested were Con A, AAL, PNA, Mal 1, LCA, WGA and SBA. Of the 7 lectins probed to detect both N and O glycans, 6 interacted with SMC lysate glycoproteins with no signal detected with AAL similar to that of no glycoprotein control. MSC lysate glycoproteins interacted with all lectins except MAL 1, with slightly more binding observed with Con A and LCA in comparison to SMC lysate. The interaction of MSC lysate glycoproteins was much stronger for AAL and WGA and lesser with SBA. PNA binds β-Galactose and SBA binds α N-acetylgalactosamine (GalNAc). Glycosylation profiling of glycoproteins in stem cells have been reported by several groups. Dai and co-workers presented a differential profiling study to detect change in glycosylation pattern following treatment of stem cells with Gamma secretase inhibitor. Profiling was done on N-linked glycoproteins from the soluble fraction of cell lysates by coupling multi-lectin (Con A and WGA) chromatography with a label–free quantitative mass spectrometry technique (Dai et al. 2011).

Given that the level of endogenous Notch1 detected in the cultured MSC lysate was only 3ng/ml and the glycoprofile of hNotch1 Fc by ELLA indicated that a minimum of 50ng/ml is required for any significant lectin binding, it was not possible to conclude that any lectin binding observed from the lysates is from Notch1 receptor but possibly from interaction with other glycosylated proteins expressed within the cell.

It was then necessary to over-express full length mNotch1 receptor and find means to separate it for ease of glycosylation profiling by ELLA. Initially, a lentiviral method was considered but it was not possible to obtain one with full length Notch1. It was thought that the NECD portion might not be a true representative of glycosylation of Notch1. A puromycin selection based method of transfection was then employed and the optimisation for this procedure was carried out using Green Fluorescent Protein (GFP) plasmid DNA and mouse MSC; the transfection efficiency was determined by fluorescence microscopy. MSCs or SMCs were co-transfected with c-Myc tagged full-length Notch1 plasmid and pPGK-puromycin plasmid to allow for exclusion of non-transfected cells. This greatly improved the transfection efficiency. The Notch1 is tagged with a c-Myc epitope (peptide sequence EQKLISEEDL) to which anti-Myc antibody has high affinity which enabled easy isolation (pull down) of Notch1 by Immunoprecipitation. The procedure required a lot of optimisation to ensure maximum recovery of purified Notch1. A major concern with working with tagged proteins is that the Myc tag on the Notch1 may interfere with protein function hence its glycosylation. Another concern is that only the overexpressed tagged glycoprotein Notch1 is immunoprecipitated and not the endogenous one. Stem cells were employed as the host for transfection purpose since they naturally express Notch receptor protein so

it is assumed that the endogenous and IP Notch1 protein should be processed in similar format regarding structure and activity, however, there is still a possibility of differences in post-translational modification i.e., glycosylation.

ELLA was performed on IP Notch1 following transient transfection of SMC or MSC to investigate if there are similarities or differences in the glycosylation profile. No significant difference was observed in the N-glycosylation of IP Notch1 from the two different cells, subsequent transfections were carried out in MSCs. However, there was also a concern that the c-Myc tag on Notch1 could interfere with the lectin binding. This was resolved by testing a c-Myc peptide against several lectins. All of the lectins showed low signals similar to that of no glycoprotein control. IP Notch1 from SMC and MSC displayed similar N-glycosylation pattern, all the lectins interacted with Notch1 in an expected manner. When MSC lysate glycoproteins were compared to the IP Notch1, all lectins interacted strongly with IP Notch1, but lesser intensity than MSC lysate. PNGase F treatment of MSC and IP Notch1 demonstrates successful removal of N-glycans present on the glycoproteins with binding to N-glycans specific lectins greatly reduced following treatment. PNGase F cleaves between the asparagine residues and innermost GlcNAc of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins (O'Neill 1996).

To determine the modulatory effects of N-glycosylation inhibitors on mesenchymal stem cell growth, MSCs were treated with a concentration range of 0.5-10μg/ml tunicamycin for 48 h. Total loss of cellular viability was observed at all concentrations of tunicamycin. Thus, no data was generated as there were no viable cells to analyse. The experiment was repeated with a lower concentration range of

0.5-5μg/ml tunicamycin and a reduced exposure time of 24 h. There was still a significant decrease in cell numbers with increasing concentrations of tunicamycin.

Due to issues encountered during these experiments it was necessary to decrease the concentration of this inhibitor in order to attempt to reduce the cytotoxicity of this treatment. The concentration of tunicamycin was further reduced to 0-100ng/ml and MSCs were treated for 72h, 25ng/ml was found to be the dose that did not impact the health of cells. Proteins from cells treated with this dose were compared for lectin binding against untreated cells in order to determine whether N-glycosylation was affected at this lower concentration of tunicamcyin. ELLA profile indicated that this dose of tunicamycin did not affect the N-glycosylation of proteins from treated cells.

Further N-glycosylation studies were performed with MVSCs. It was observed that the tunicamycin treatment did affect cell health negatively at low density but not at high density. Having established the modulatory effect of tunicamyicn on cell growth, MVSC were treated with 0.5 μg/ml for 24h, proteins from treated and untreated cells were probed with lectins to assess the effect of this dose on N-glycosylation. Reduced binding was observed with Con A, LCA, MAL 1 and more profound with WGA in the treated sample, confirming modifications of GlcNAc residues of complex N-glycans. MVSC were transiently transfected with Notch1 cDNA to generate a Notch1 over expression lysate followed by tunicamycin treatment (0.5 μg/ml). The purpose of this experiment was to be able to isolate Notch1 by Immunoprecipitation and study its glycosylation after tunicamycin treatment. The procedure was unsuccessful even though it had been applied successfully in previous studies with MSC. The non-IP protein lysate from treated

and non-treated cells were assessed by ELLA and interestingly, the glycosylation pattern was similar to that of non-transfected cells following tunicamycin treatment.

Tunicamycin is an inhibitor of UDP-GlcNAc, known to cause endoplasmic reticulum stress, improperly folded proteins, loss of cellular function and induction of apoptosis, and as a result, tunicamycin treatment was found to significantly affect cell health even at very low dose. Inhibiting N-glycosylation with tunicamycin does not remove N-glycans already processed but blocks the enzyme responsible for the first step in N-glycoproteins synthesis. N-glycosylation is important in the correct folding of proteins, treatment with tunicamycin may impact the correct processing of Notch receptor and trafficking to cell surface. This global inhibition would impact not only protein of interest but also all other N-glycoproteins that may be responsible for many cellular functions.

The use peptide N-glycosidase F was then considered based on report from previous studies whereby treatment of cultured cells with PNGase F has been shown to reduce N-glycans at the cell surface (Noma et al. 2009; Hamanoue and Okano 2011; Hall et al. 2014). With this approach, endoplasmic reticulum function should not be impacted, however, prolonged exposure to treatment could not be accomplished to avoid issues of cell detachment. Stem cells (MSC or MVSC) in culture were treated with PNGase F or PBS for a specified time, total protein lysate or membrane fractions of the treated and untreated cells were prepared and analysed by ELLA to confirm the removal of N-glycans. Following treatment, no reduction was observed in the binding of Con A with either of the group tested. PNGase F cleaves between the asparagine residues and innermost GlcNAc of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins

(O'Neill 1996). The treatment of cells may not have altered the surface N-glycoproteins significantly enough to be detected by the ELLA method.

Identified glycosyltransferases involved in the glycosylation of Notch in the literature are protein O-fucosyltransferase 1 (Pofut1), protein O-glucosyltransferase 1 (Rumi/Poglut1) and Fringe β 1,3-N- acetylglucosaminyl transferase, UDP Gal β-1,4-galactosyltransferase (β4GalT-1). The mechanism of the specific enzyme(s) involved in the N-glycosylation of the Notch1 receptor is not fully understood. It is known that N-glycans are located on Notch1 receptor and Fringe proteins modify the already O-fucosylated Notch receptor by adding N-acetyl glucosamine. This modification has been shown to influence Notch activity by altering Notch ligand interaction (Panin et al. 2002; Chen, Moloney and Stanley 2001; Takeuchi and Haltiwanger 2014; Harvey et al. 2016). There are three different Fringes in mammals with four different receptors and five different ligands. How Fringe regulates Notch in the mammalian system is not fully known despite all the effort of researchers to date (Bruckner 2000; Moloney et al. 2000b; Taylor et al. 2014; Harvey et al. 2016; Takeuchi and Haltiwanger 2014).

A more targeted approach to study the N-glycosylation of Notch receptor as opposed to inhibiting N-glycosylation using tunicamycin or PNGase F to remove cell surface N-glycans is the depletion of the specific glycosyltransferases responsible for addition of N-glycans. MVSC were transiently co-transfected with c-Myc tagged-Notch cDNA and Lunatic Fringe siRNA to diminish the ability of Fringe in adding GlcNAc to the O-fucose of Notch receptor protein. Notch1 overexpression lysates were extracted from the Lfng siRNA transfected and scrambled siRNA transfected samples and probed with N-glycan specific lectins

Con A, LCA, MAL 1 and WGA. The glycoprofile of Lfng depleted Notch1 overexpression lysate signifies the modification of complex N-glycan with significant reduction in binding observed with WGA lectin. It was not possible to isolate Notch1 following transfection; therefore the consequences of Lfng depletion on Notch1 N-glycosylation could not be assessed.

The overall aim of this project is to investigate to role of N-glycosylation on Notch receptor activity and myogenic differentiation in stem cells. The experiments were therefore designed to employ Jagged1 to induce Notch activation; this requires incubation of ligand Jag-1 with stem cells. A preliminary experiment was carried out to verify the glycosylation state of Jag-1 Fc by ELLA analysis. The glycoprofile indicates the presence of both O and N-glycans on the glycoprotein suggesting that Jag-1 is glycosylated as expected.

The consequence of inhibiting N-glycosylation of the Notch1 receptor using tunicamycin or depletion following Lunatic Fringe knockdown on Notch activity in multipotent vascular stem cells is investigated in the next chapter.

Chapter 5:

A role for Notch1 receptor N-glycosylation in Jagged1-induced myogenic differentiation of stem cells and its regulation by Tunicamycin and Lfng

Stem cells are thought to be involved in the pathogenesis of intimal medial thickening (IMT) typical of atherosclerosis, arteriosclerosis, in-stent restenosis and transplant arteriosclerosis, although the process is not yet fully understood (review by Wang et al. 2015; Tang et al. 2012; Xu 2008).

Lineage tracing analysis has provided substantial evidence for the involvement of stem cell-derived progeny, in addition to 're-programmed' differentiated SMC, as well as SMC derived from endothelial-mesenchymal transition (EndMT) in progressing IMT. These cells may become activated/re-programmed, differentiate towards myogenic and myeloid lineages and subsequently dictate, in part, vessel remodelling (Medici et al. 2010; Yuan et al. 2017).

It is evident that stem cells have the ability to differentiate into vascular smooth muscle cells and play a significant role in vascular development and IMT. Several biochemical factors, including transforming growth factor-beta (TGF-b), the Notch pathway (Kurpinski et al. 2010), and hedgehog signalling (Erbilgin et al. 2013) has been shown to influence this vascular myogenic differentiation.

Our group hypothesised that vascular SMC accumulation following vascular injury or during in-stent restenosis is due in part to differentiation and transition of resident vascular stem cells to SMC under the control of a Notch/Hedgehog pathway (Redmond et al. 2013a, Redmond et al. 2013b; Morrow et al. 2007) contrary to the original hypothesis that de-differentiated re-programmed SMCs were exclusively responsible. However, the distinct mechanism involved in this signalling pathway in the differentiation of stem cells is yet to be fully determined (Kurpinski et al. 2010).

Several factors regulate the activity of Notch receptor activity and signalling. Evidence from literature has indicated the significance of glycosylation of the Notch receptors in regulating Notch signalling (Stanley 2007; Okajima, Matsuura and Matsuda 2008; Luther and Haltiwanger 2009), although the mechanism is yet to be fully understood. Earliest studies of the Notch protein indicate the presence of N-glycans (Kornfeld, Reitman and Kornfeld 1981) as a result of interaction observed with Lentil lectin (Johansen, Fehon and Artavanistsakonas 1989). In addition to *N*-glycosylation, three types of *O*-linked glycosylation have been defined on the EGF-like repeats of the Notch proteins: *O*-fucosylation, *O*-glucosylation and *O*-GlcNAcylation (Moloney et al. 2000; Matsuura et al. 2008). A lot of research has been done to determine the role of O-glycosylation modification on Notch signalling pathway (Sala et al. 2012). Elongation of O-fucose on Notch receptor by Fringe is known to modify Notch –ligand interactions (Rampal et al. 2005; Luther and Haltiwanger 2009). However, to date a defined role for N-glycosylation of Notch receptors in controlling Notch function has yet to be demonstrated.

Strategy

In order to demonstrate that N-glycosylation of Notch1 receptor has a role in stem cell transition and myogenic differentiation, it was proposed to: (i) Verify Notch activity is involved in Jagged-induced stem cell differentiation by treating cultured MSC and resident vascular MVSC with recombinant Notch ligand, Jag-1 before myogenic differentiation is confirmed by immunocytochemistry and qRT-PCR of discrete SMC differentiation markers, Cnn1 and Myh11. (ii) Investigate the effect of inhibiting N-glycosylation of the Notch1 receptor using tunicamycin or Lunatic Fringe knockdown to remove the glycans before assessing whether Notch's ability to promote myogenic differentiation is compromised.

5.1 Overexpression of NICD increases the expression of Notch target genes in MSC

The effect of over expression of the constitutively activated form of Notch (NICD) on Notch target genes expression was investigated. Following overexpression of NICD, a significant increase in the levels of Notch target genes, Hey1 and Hey2 was detected by PCR analysis (48.13 ± 0.84 fold and 7.90 ± 1.11 fold respectively, n = 3) (Figure 5.1).

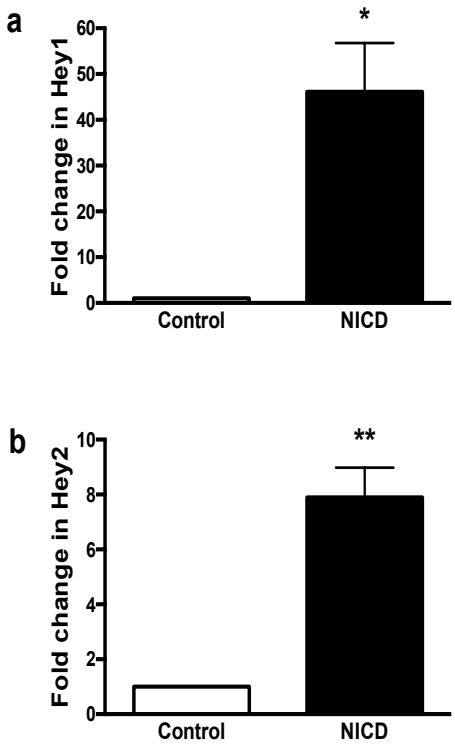

Figure 5.1. NICD overexpression increases Notch target genes mRNA. RT-PCR analysis of (a) Hey1 and (b) Hey2 following transfection of MSCs with NICD plasmid (1μg). Expression was normalised to non-transfected control. The experiments were performed in triplicate with the chart representative of three independent experiments. Data are mean ± SD.*P < 0.05, **p < 0.01 as compared to control (students t-test).

5.2 Notch signalling is involved in MSC transition to SMC lineage

Analysis by qRT-PCR found Jag-1 ligand to be effective in activating Notch signalling in MSCs, significant up regulation of Notch target genes Hey1 and Hey2 was observed. Hey1 and Hey2 mRNA expression increased by 30.76 ± 3.30 fold change and 23.01 ± 2.09 respectively (Figure 5.2).

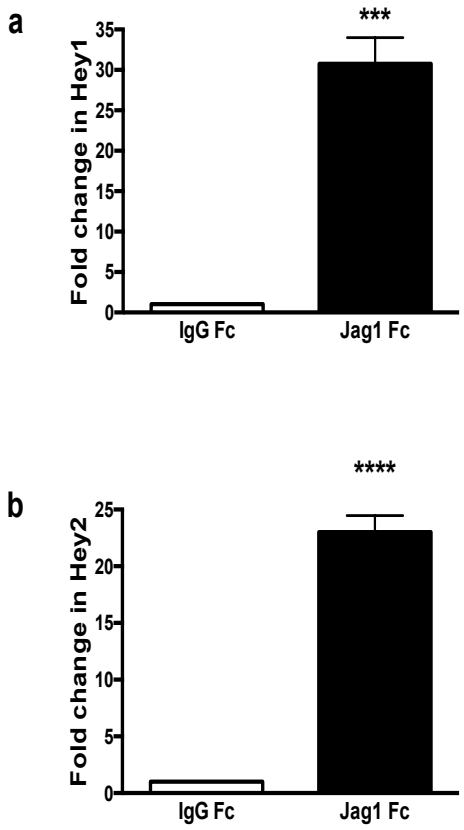

Figure 5.2. Jag-1 induced Notch signalling increases Notch target genes mRNA. RT-PCR analysis of a) Hey1 and b) Hey2 mRNA following treatment of MSCs with Jag-1 (5µg/ml) for 72h. Chart is a representative of triplicate experiments. ***P < 0.001, ****p < 0.0001 as compared to control (students t test).

A parallel study by immunocytochemistry demonstrated an up regulation of smooth muscle markers alpha actin, Cnn1 and myosin heavy chain (SM-MHC, Myh11) (Figure 5.3). The induction of SM-MHC was the most profound in this experiment with clearly visible myosin filaments. These findings suggest that Jagged alone is sufficient in stimulating activity of Notch and causing myogenic differentiation (MSC differentiation to SMC) and therefore suggests that Notch signalling pathway is directly involved in the process.

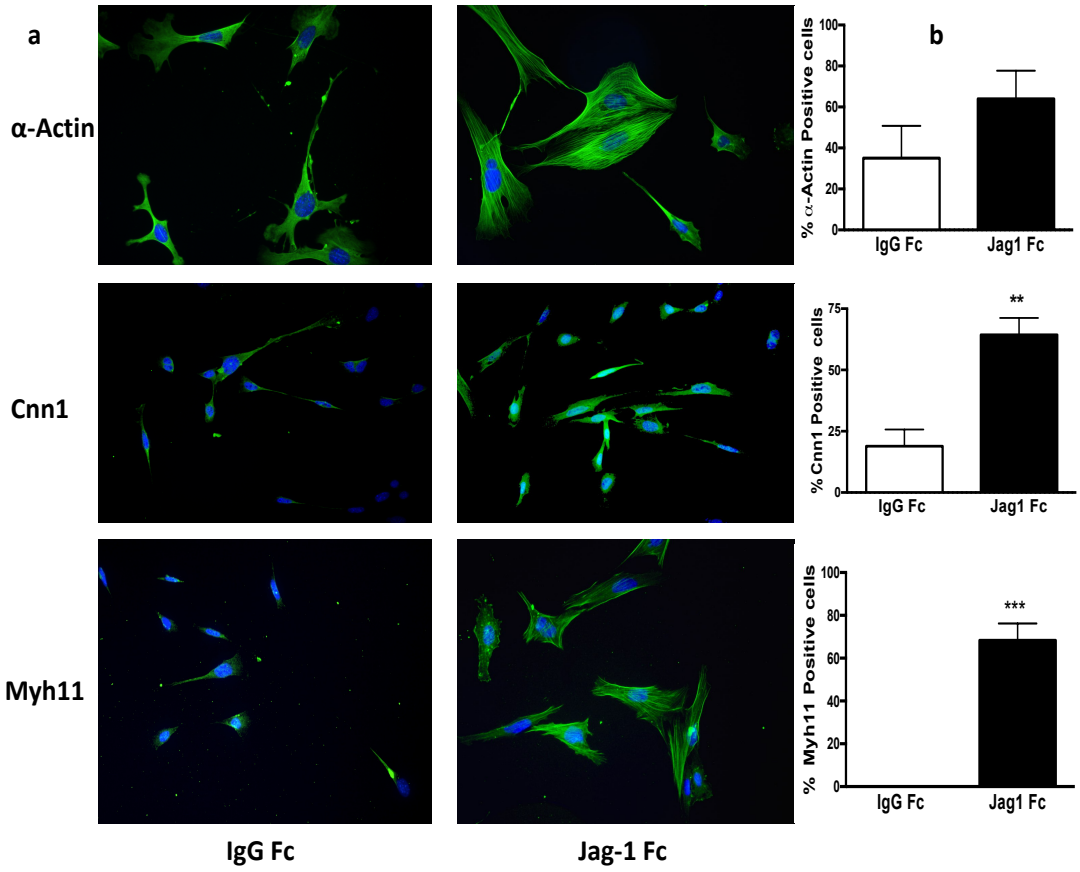

Figure 5.3. The effects of Notch1 activation on MSCs myogenic differentiation through immobilised Jag-1. MSCs were cultured on Jag-1-Fc or Fc-coated (as control) surfaces for 5 days in 0.5% serum growth media. (a) Cells were fixed with formaldehyde and stained for smooth muscle cells markers (a-actin (SMA), Cnn1 and Myh11) using anti-a-actin, anti-Cnn1 and anti-Myh11 primary antibodies. Cells were probed with Alexafluor 488 Goat anti-mouse IgG, Alexafluor 488 Goat anti-rabbit IgG and Alexafluor 488 Goat anti-mouse IgG secondary antibody respectively. The nucleus was counterstained with DAPI and examined by fluorescence microscopy, 20x magnification. Representative of 6 images. (b) Representative percentage of a-actin (SMA), Cnn1 and Myh11 positive MSCs from (a). Data are the mean ± SEM. **p<0.01 vs. IgG Fc control, ***p < 0.001 vs. IgG Fc control.

5.3 Proteomic analysis by mass spectrometry

A qualitative study was performed by mass spectrometry on aortic SMC, cultured SMC, cultured MSC, Jag-1 treated MSC and Fc treated MSC to check what proteins are present or absent. Several proteins were identified from the preliminary proteomic data, with the few selected ones listed in Table 5.1. Cnn1, Transgelin (SM 22α) and Myh11 are markers for SMC with Myh11 being the most distinct signature for identifying smooth muscle cells. Non-related Myosin isoforms were identified in the cultured SMC and MSC. Actin, an aortic smooth muscle protein was detected in freshly isolated SMCs, Fc treated MSCs, Jag-1 treated MSCs and untreated MSCs. Actin, alpha cardiac muscle protein was identified in cultured SMC only. Cnn1 and Myh11 were found in Aortic SMC only but not in the cultured SMC or in Jag-1 treated MSC. Transgelin 1 (SM22 Alpha) was detected in Aortic SMC, Fc treated MSCs and untreated MSCs. Transgelin 2, an homolog of SM22 alpha, was identified in SMC, MSC, Jag-1-treated MSC, Fc treated MSC and aortic SMC. Transgelin, an actin-binding protein of Cnn1 family is a novel marker used to detect smooth muscle differentiation (Robin et al. 2013).

A control sample (50ng/ml Notch 1 standard) was also analysed, Notch 1 could not be detected in this or in any of the samples possibly due to low abundance. However, some Notch glycosylation related glycosyltransferases were identified across the samples, listed on Table 5.3 and their respective functions in Table 5.4.

Table 5.1. List of cell types and total proteins identified.

Cell type	Total proteins identified
Cultured mouse MSC	708
Cultured mouse SMC	898
Jagged treated mouse MSC	790
IgG Fc treated mouse MSC Jagged	813
Freshly isolated aortic mouse SMC	287

Table 5.2. List of SMC markers identified. **http://www.uniprot.org/**

Accession	Description	Function
P62737	Actin, aortic smooth muscle	Highly conserved protein that is involved in cell motility
P68033	Actin, alpha cardiac muscle 1	Highly conserved protein that is involved in cell motility
Q08091	Calponin 1	Thin filament-associated protein that controls smooth muscle contraction
O08638	Myosin Heavy Chain 11	Involved in smooth muscle contraction
P37804	Transgelin (SM22 α)	Responsible for Actin cross-linking /gelling

Table 5.3. List of glycosyltransferases and glycosylation related proteins identified

Description	Cell type
Alpha-(1-3)-fucosyltransferase	MSC + Jag-1
Dolichol-phosphate mannosyltransferase	MSC + Jag-1, SMC
GDP-fucose protein O-fucosyltransferase 1 (pofut1)	MSC + Fc
UDP-glucose: glycoprotein glucosyltransferase	MSC + Jag-1, MSC + Fc MSC
Alpha mannosidase 2	MSC, MSC + Fc
Alpha-N-acetylgalactosamidase	MSC + Jag-1, MSC + Fc
Neutral alpha-glucosidase AB	MSC, MSC + Jag-1, MSC + Fc, SMC
N-acetylglucosamine-6-sulfatase	MSC + Jag-1, MSC + Fc SMC

Table 5.4. Function of identified glycosyltransferases (**http://www.uniprot.org/**)

Accession	Description	Function
Q8BHC9	Alpha-(1-3)-fucosyltransferase 11	This enzyme is involved in the glycosylation of protein Transfers a fucose onto a GlcNAc in a 1,3 linkage (Baboval and Smith 2002).
Q9D1Q4	Dolichol-phosphate mannosyltransferase subunit 3	Synthesis of dolichyl-phosphate mannose, involved in protein N-glycosylation
Q91ZW2	GDP-fucose protein O-fucosyltransferase 1(Pofut 1)	Catalyses the process of fucose addition to the EGF repeats use GDP-fucose as donor substrate
Q6P5E4	UDP-glucose: glycoprotein glucosyltransferase 1	Provides quality control for protein assembly in the ER by recognizing glycoproteins with minor folding defects Reglucosylates individual N—glycans located near the misfolded area of the protein
P27046	Alpha mannosidase 2	Initiates the processing of complex N-glycans, controls conversion of high mannose to complex N-glycans.
Q9QWR8	Alpha-N-acetylgalactosamidase	Cleaves terminal alpha-n-acetylgalactosamine residues from glycolipids and glycoproteins
Q8BHN3	Neutral alpha-glucosidase AB	Involved in N-glycan metabolism pathway.
Q8BFR4	N-acetylglucosamine-6-sulfatase	Involved in the breakdown of sulfated glycosaminoglycans that are located on cell surface or the extracellular matrix Glycosaminoglycans are made up of a repeating disaccharide units of N-acetylgalactosamine or N-acetyl glucosamine and a uronic acid (http://themedicalbiochemistrypage.org/glycans.php).

5.4 MVSCs characterisation by Immunocytochemistry

Resident MVSC were derived from the thoracic region of a mouse aorta and characterised to confirm a pure culture. These cells were generated to serve as a myogenic differentiation model of resident vascular stem cells transitioning to vascular smooth muscle cells. Therefore, it was necessary to initially characterise these cells in order to establish their stemness. The cells were cultured for 2 weeks in growth media and then stained for neuroectodermal stem cells markers, Nestin and Sox 10 (Figure 5.4).

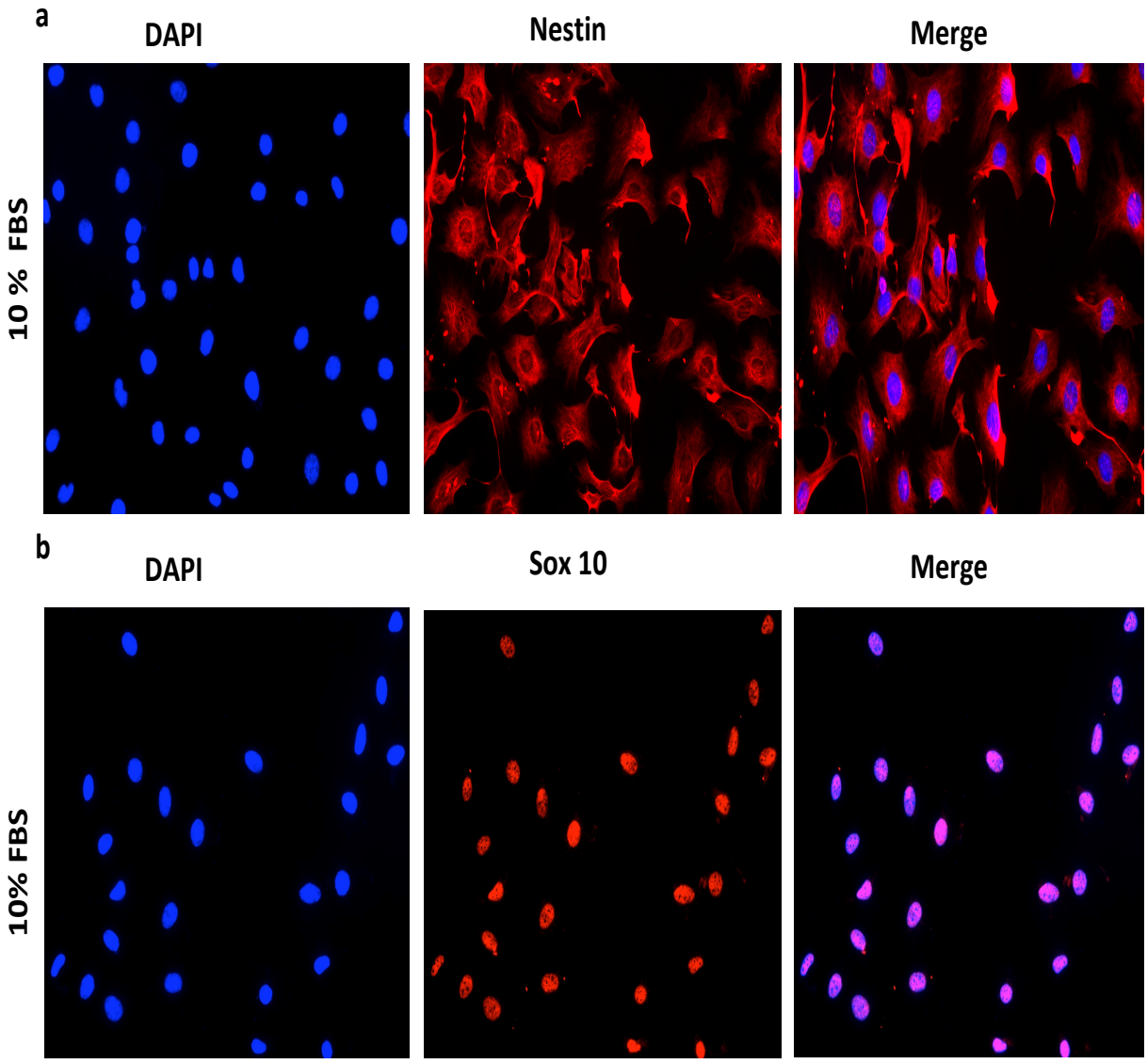

Figure 5.4. Nestin and Sox 10 expression in MVSCs. Cells were cultured in maintenance growth media for 2 weeks, then fixed with formaldehyde and stained for (a) Nestin or (b) Sox 10 using anti-nestin or anti-sox10 primary antibody. Cells were probed with an alexafluor 546 anti-mouse secondary antibody and fluorescent DAPI was used for nuclear staining, 20 X magnification. The images are representative of 12 images from two independent experiments.

5.5 Activation of Notch1 promotes myogenic differentiation of MVSCs

Having observed the positive effect of Jag-1 activation of Notch signalling on myogenic differentiation in MSC, the effect of Notch activation on myogenic differentiation was investigated in MVSCs. MVSCs were grown in complete maintenance media before cells were seeded on glass coverslips immobilised with Jag-1 Fc ligand or the control Fc fragment (1μg/ml) in media supplemented with 0.5%, 5% and 10% FBS, respectively. Varying the serum concentration provides other inductive factors that enhance the Jag-1 stimulated myogenic differentiation response. In order to monitor myogenic differentiation of MVSCs following Jag-1 stimulation, smooth muscle markers expression was used as an early indication of myogenic differentiation. Following treatment of MVSCs with Jag-1, there was an upregulation of smooth muscle specific markers, Myh11 (Figure 5.5 and 5.6) and Cnn1 (Figure 5.7 and 5.8). The most pronounced increase in SMC differentiation marker expression was observed for Cnn1 in 10% FBS (Figure 5.9) and thereby confirmed that Notch activation with Jag-1 promoted myogenic differentiation of MVSCs.

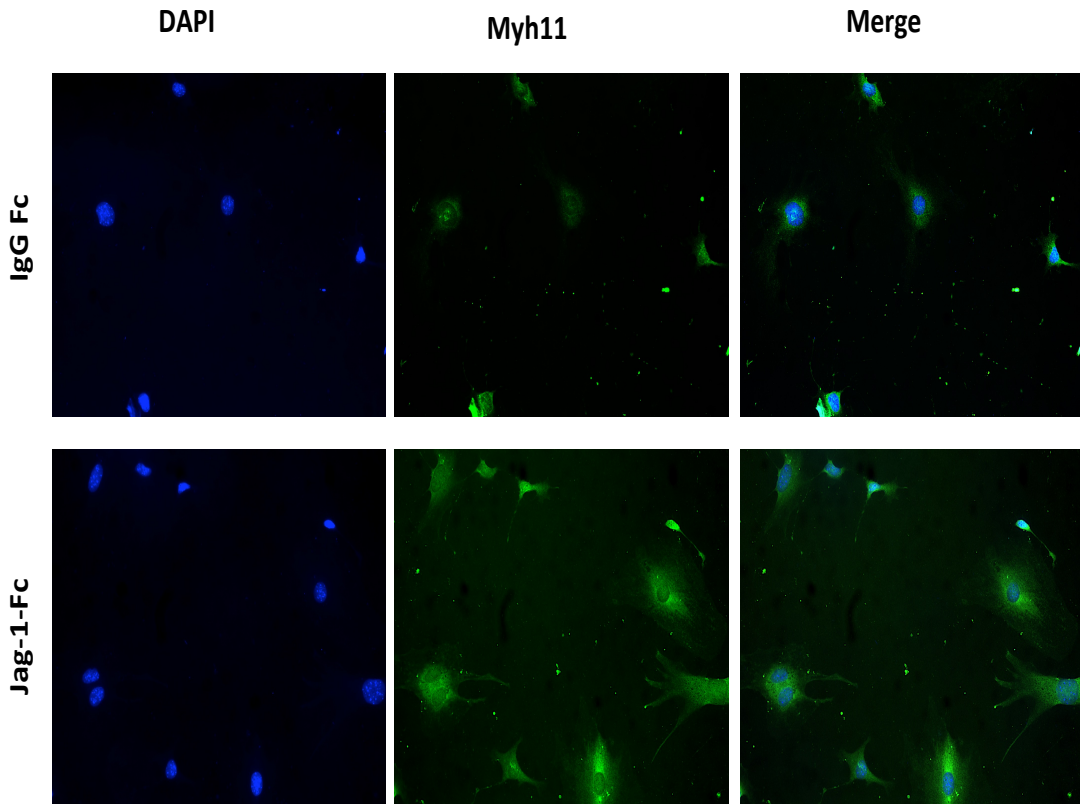

Figure 5.5. Myh11 expression in MVSCs exposed to Jag-1. Mouse MVSCs were treated with Jag-1-Fc or control IgG-Fc in growth media containing 0.5% FBS. Cell were fixed in formaldehyde and stained with antibody against smooth Myh11 and probed with Alexafluor 488 anti-mouse IgG secondary antibody. DAPI was used for nuclear visualisation, 20 X magnification. The images are representative of 12 images from two independent experiments.

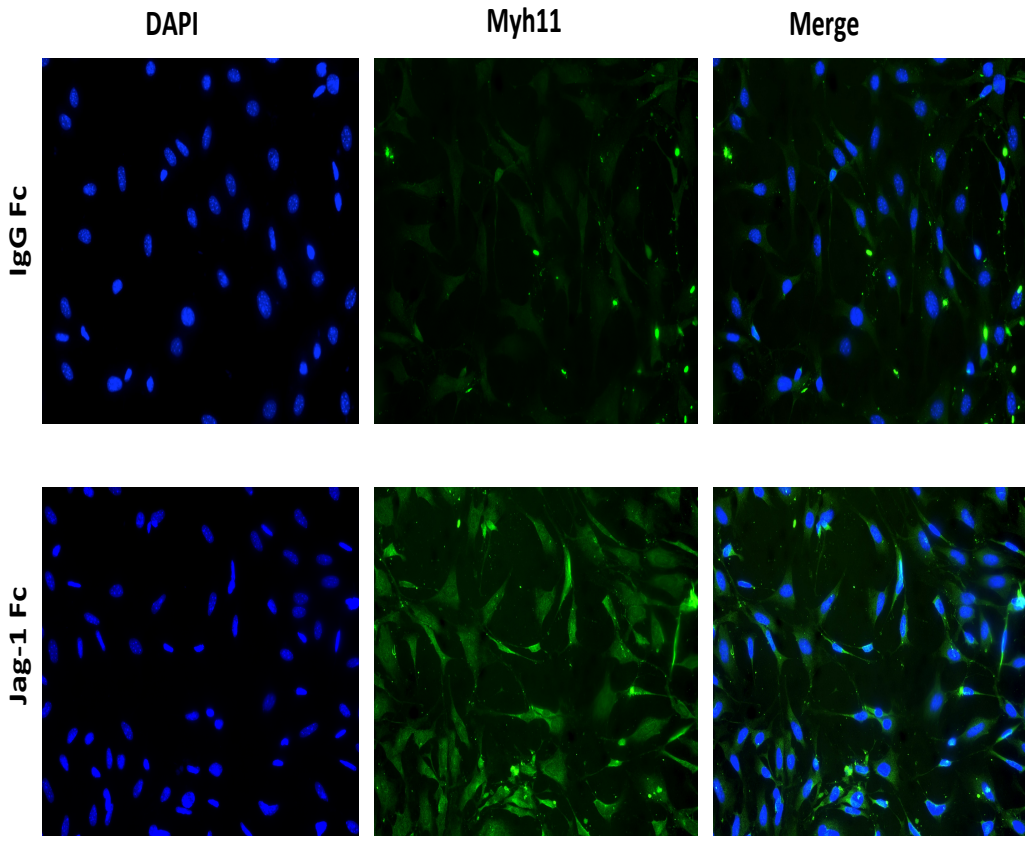

Figure 5.6. Myh11 expression in MVSCs exposed to Jag-1. Mouse MVSCs were treated with Jag-1-Fc or control IgG-Fc in growth media containing 5% FBS. Cell were fixed in formaldehyde and stained with antibody against smooth Myh11 and probed with Alexafluor 488 anti-mouse IgG secondary antibody. DAPI was used for nuclear visualisation, 20 X magnification. The images are representative of 12 images from two independent experiments.

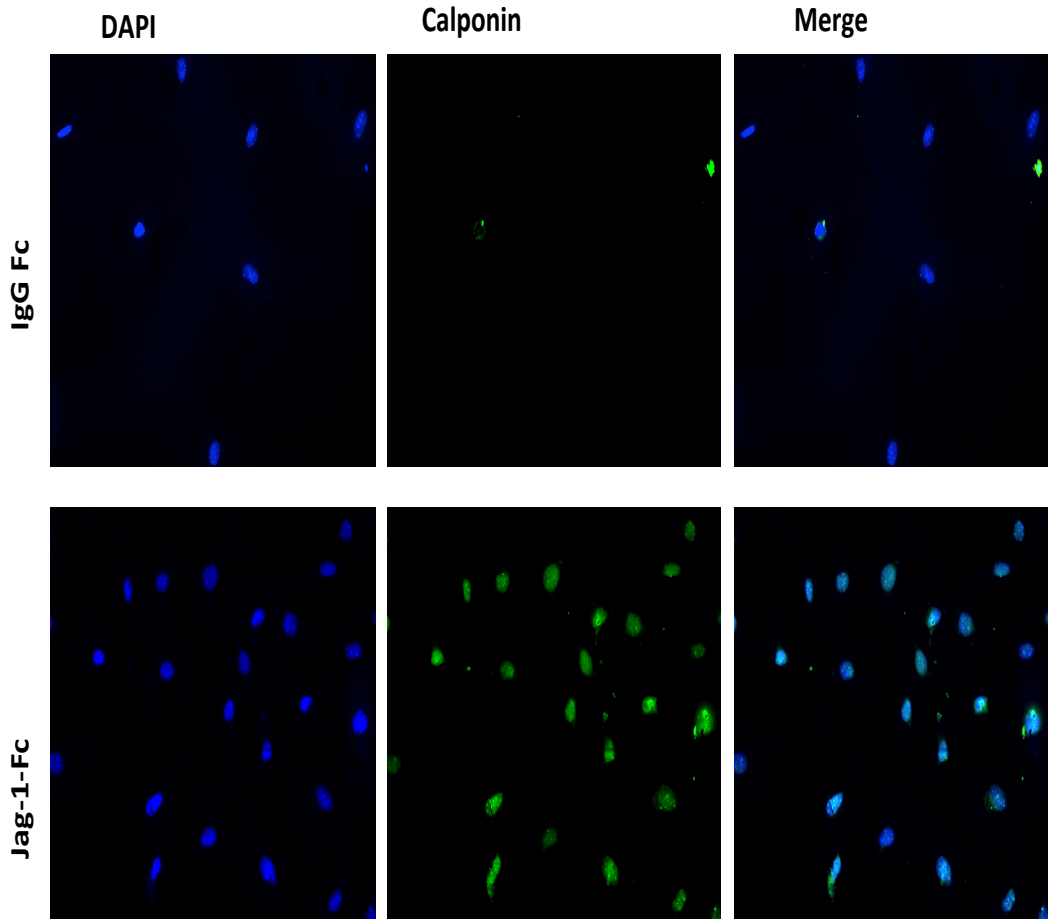

Figure 5.7. Cnn1 expression in MVSCs exposed to Jag-1. Mouse MVSCs were treated with Jag-1-Fc or control IgG-Fc in growth media containing 0.5% FBS. Cell were fixed in formaldehyde and stained with antibody against smooth Cnn1 and probed with Alexafluor 488 goat anti-rabbit IgG secondary antibody. DAPI was used for nuclear visualisation, 20 X magnification. The images are representative of 12 images from two independent experiments.

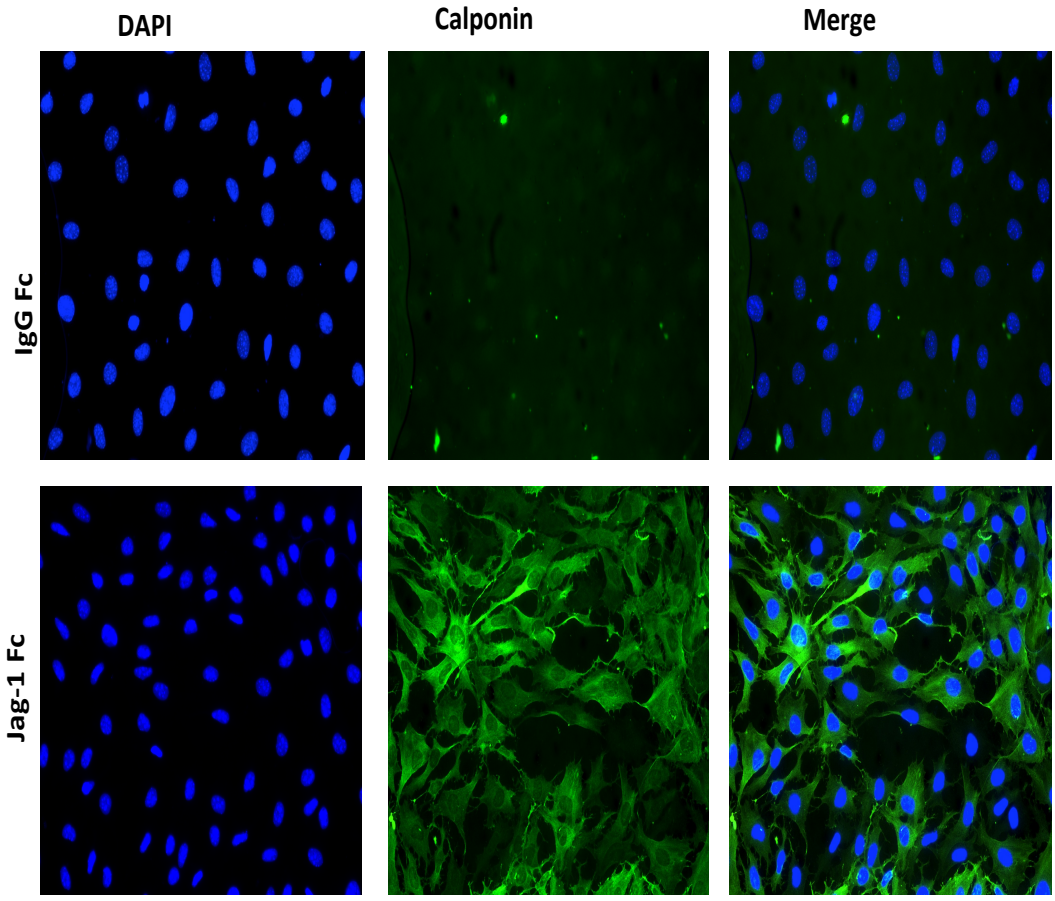

Figure 5.8. Cnn1 expression in MVSCs exposed to Jag-1. Mouse MVSCs were treated with Jag-1-Fc or control IgG-Fc in growth media containing 5% FBS. Cell were fixed in formaldehyde and stained with antibody against smooth Cnn1 and probed with Alexafluor 488 goat anti-rabbit IgG secondary antibody. DAPI was used for nuclear visualisation, 20 X magnification. The images are representative of 12 images from two independent experiments.

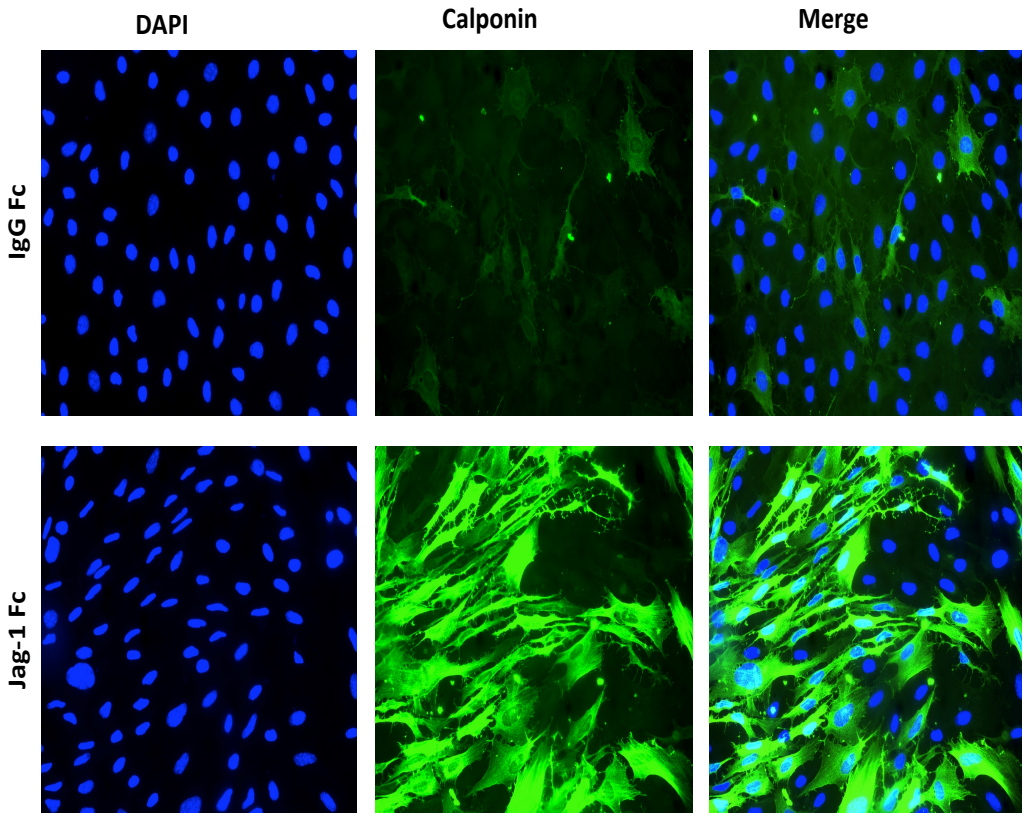

Figure 5.9. Cnn1 expression in MVSCs exposed to Jag-1. Mouse MVSCs were treated with Jag-1-Fc or control IgG-Fc in growth media containing 10 % FBS. Cell were fixed in formaldehyde and stained with antibody against smooth Cnn1 and the appropriate secondary antibody. DAPI was used for nuclear visualization, 20 X magnification. The images are representative of 12 images from two independent experiments.

The effect of overexpression of NICD on smooth muscle gene expression in MVSCs was also investigated. Following overexpression of constitutively active form of Notch in MSVCs, a significant increase in the levels of Cnn1 was detected by qRT-PCR analysis (2.8 ± 0.64 fold, n = 3) (Figure 5.10).

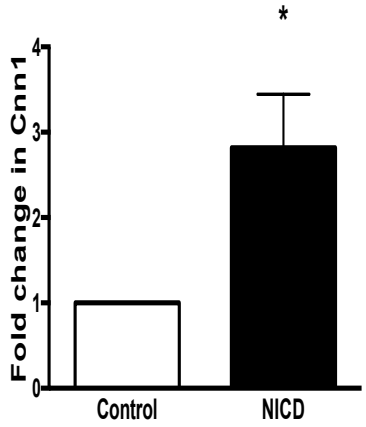

Figure 5.10. The effect of NICD over-expression on myogenic differentiation of MVSCs. RT-PCR analysis of Cnn1 mRNA following transfection of MVSCs with NICD plasmid for 48h. The experiment was performed in triplicate, chart is a representative of three independent experiments. Data are ± SEM *$p < 0.05$ as compared to control (students t test).

5.6 Notch1 is required for Jag-1-induced Notch signalling

To demonstrate that the Notch receptor is required for Jagged-induced Notch signalling and as a result, stem cell differentiation towards vascular smooth muscle cell lineage, Notch1 siRNA knockdown was performed on MVSCs while inducing differentiation with Jag-1. Jagged treatment resulted in an upregulation of Hey1 mRNA expression (10.37±1.50 fold, n=3). siRNA knockdown of Notch1 ((30%, Figure 5.11)) was found to reduce the expression of Notch target gene Hey 1 (4.69 ± 2.14 fold, n=3) (Figure 5.12).

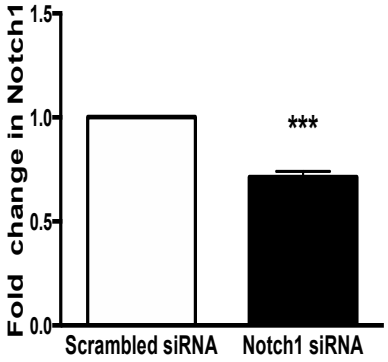

Figure 5.11. Notch1 knockdown by siRNA. MVSCs were seeded at a density of 200,000 cells /well in a 6well plate. 18-24h later, the cells were transfected using 10nM scrambled negative control duplex or Notch1 siRNA. 48h post-transfection, RNA was isolated, and Notch1 levels were measured using qRT-PCR. More than one siRNA was tested, relative expression was normalized to internal control mRNA using scrambled control as baseline (100%). The experiment was performed in triplicate, chart is a representative of two independent experiments. Data are ± SEM ***p<0.001 as compared to scrambled siRNA control (Students t test).

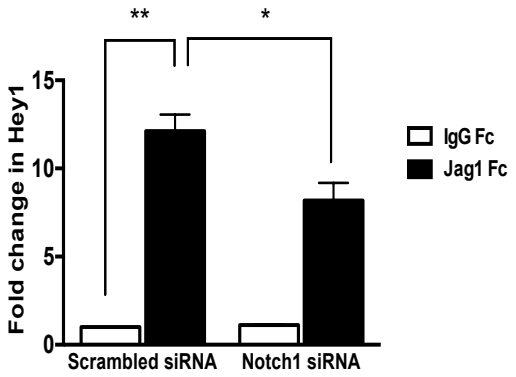

Figure 5.12. Hey 1 expression in MVSCs following Notch1 knockdown and Jag-1 treatment. MVSCs were seeded at a density of 200,000cells /well in a 6well plate precoated with Jag-1 Fc. 18-24h later, the cells were transfected using 10nM scrambled or Notch1 siRNA. 48h post-transfection, RNA was isolated, and Hey1 levels were measured using qRT-PCR. The experiment was performed in triplicate, chart is a representative of two independent experiments. Data are ± SEM **p< 0.01 as compared to scrambled siRNA-Fc treated control. *p < 0.05 as compared to scrambled siRNA-Jag-1 Fc treated control (ANOVA).

There was a modest increase in vascular smooth muscle cell Cnn1 mRNA in Jag-1 treated cells (1.88 ± 0.38 fold, n=3). Depletion of Notch1 reduced the expression of Cnn1 mRNA (0.70 ± 0.40 fold, n=3) (Figure 5.13). Immunocytochemistry confirmed an increase in Cnn1 protein expression (3.21 fold, 33.8%) in Jag-1-treated cells. Notch1 knockdown reduced the levels of Cnn1 protein (0.75 fold, 7.9%) (Figure 5.1).

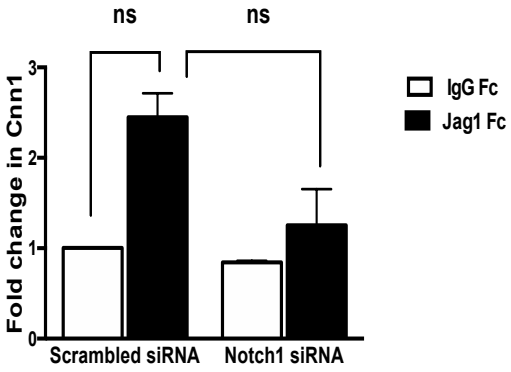

Figure 5.13. Cnn1 expression in MVSCs following Notch1 knockdown and Jag-1 treatment. MVSCs were seeded at a density of 200,000 cells /well in a 6well plate precoated with Jag-1 Fc. 18-24h later, the cells were transfected using 10nM scrambled or Notch1 siRNA. 48h post-transfection, RNA was isolated, and Cnn1 mRNA levels were measured using qRT-PCR. The experiment was performed in triplicate, chart is a representative of three independent experiments. Data are ± SEM.

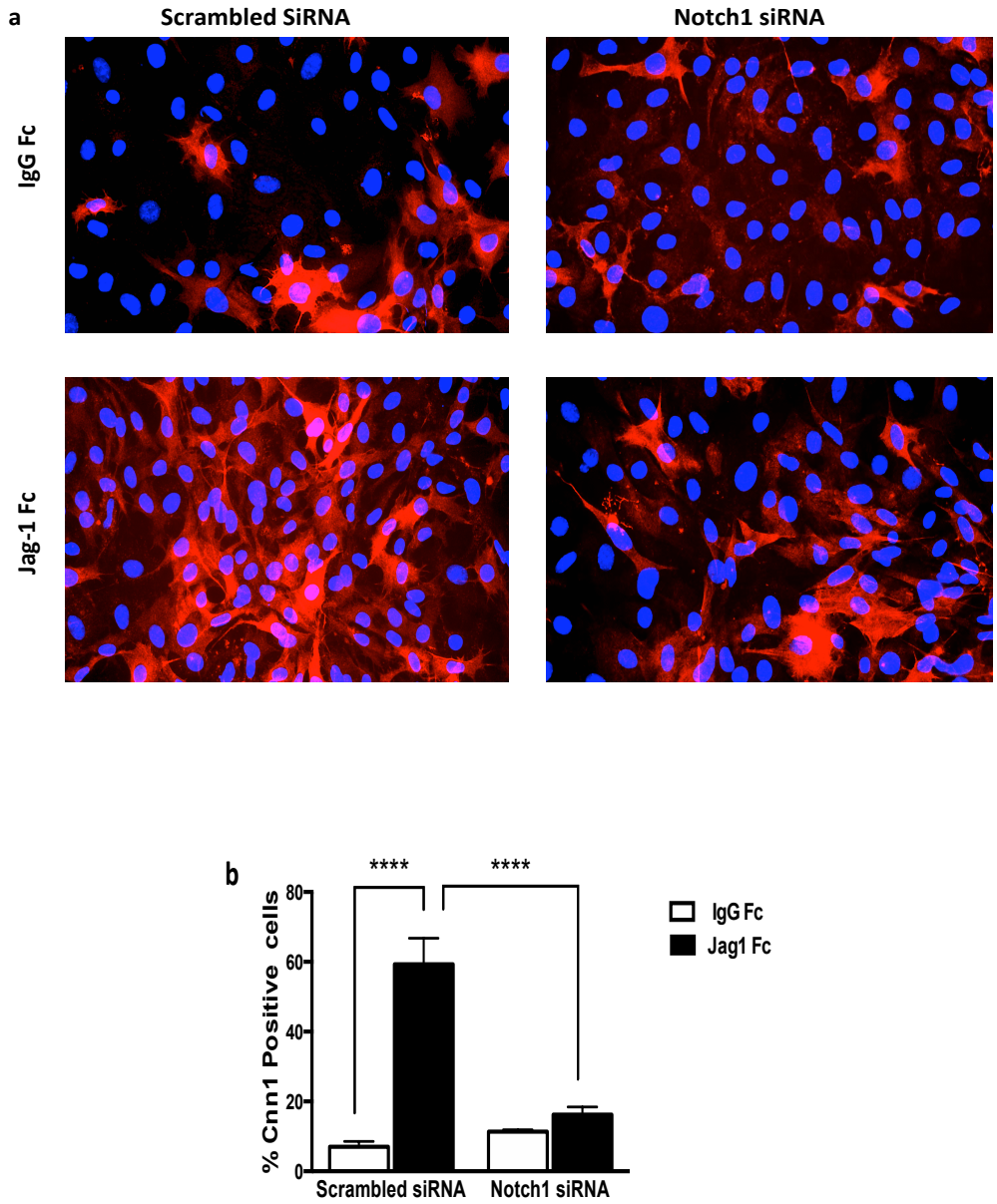

Figure 5.14. Cnn1 protein expression in Notch1 depleted MVSCs. (a)ICC analysis of MVSCs following transient transfection with scrambled siRNA or Notch1 siRNA and concurrent Jagged1 treatment. 10,000 cells were seeded on wells pre-coated with Jag-1 or Fc control, followed by 48h siRNA transfection, media was replaced with growth media and cell were incubated for additional 12 days. Cells were fixed with formaldehyde and stained with antibody against calponin1 and probed with Alexafluor 546 goat anti-rabbit IgG secondary antibody. DAPI was used for nuclear visualization, 20 X magnification. The images are representative of 12 images from two independent experiments. (b) Representive percentage of Cnn1 positive cells from (a). Data are ± SEM **** $P < 0.0001$ as compared to scrambled siRNA-Fc treated control, **** $P < 0.0001$ as compared to scrambled siRNA-Jag-1 Fc treated control (ANOVA). The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM.

Jag-1 treatment resulted in the upregulation of vascular smooth muscle cell Myh11 mRNA level (3.81 ± 0.68 fold, n=3), and effect attenuated following Notch1 knockdown (1.03 ± 0.56 fold, n=3) (Figure 5.15).

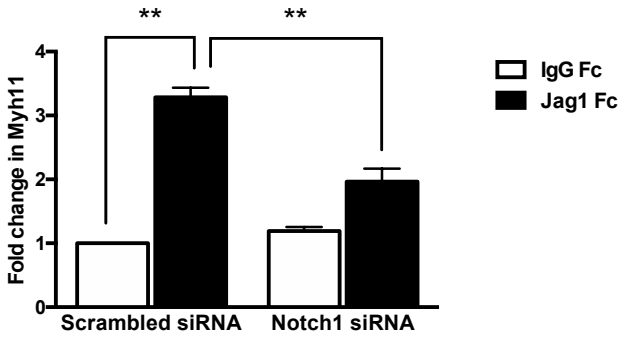

Figure 5.15. Myh11 expression in MVSCs following Notch1 knockdown and Jag-1 treatment. MVSCs were seeded at a density of 200,000cells /well in a 6well plate precoated with Jag-1 Fc. 18-24h later, the cells were transfected using 10nM scrambled or Notch1 siRNA. 48h post-transfection, RNA was isolated, and MYH11 levels were measured using qRT-PCR. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. **P<0.01 as compared to scrambled siRNA-Fc treated control. **P<0.01 as compared to Notch1 siRNA-Fc treated control (ANOVA).

5.7 The effect of N-glycosylation inhibition by tunicamycin on Notch activation and myogenic differentiation

Having established the effect of tunicamycin on N-glycosylation of the MVSC lysate containing over-expressed Notch1 receptor by ELLA analysis (chapter 4), modulation of Notch activation of Notch target genes and specific SMC differentiation gene transcripts by tunicamycin treatment was determined.

Treating MVSC with tunicamycin while inducing differentiation with Jagged1 was found to have no effect on the Notch target gene Hey1 expression. There is a significant increase in Hey1 expression in Jagged treated cells in comparison to Fc treated cells, 8.72 ± 0.99 fold. The Hey1 expression did not change in the tunicamycin treated cells, 8.40 ± 0.21, n=3 fold (Figure 5.16).

There is a modest increase in Cnn1 expression in Jag-1 treated cells in comparison to Fc treated cells. Modification of the N-glycosylation of Notch receptors with tunicamycin did not affect the expression of smooth muscle cell marker Cnn1 in Jag-1 treated MVSCs. Cnn1 expression in Jag-1 only treated cells and Jag-1-tunicamycin treated cells were 2.31 ± 0.07 fold, n=3 and 0.41 ± 0.02 fold, n=3 respectively. Decrease in Cnn1 expression was observed in Fc treated cells (0.14 ± 0.01 fold, n=3) (Figure 5.17) RNA extracted from mouse aorta was used a positive control for Cnn1 expression.

The effect of tunicamycin on Mhy11 expression in Jag-1 treated MVSCs was determined. There is a significant increase in Myh11 expression in Jag-1 treated cells in comparison to Fc treated cells. The Myh11 expression is less in the tunicamycin treated cells. Myh11 expression in Jagged treated cells was 4.06 ± 0.07

fold, n=3. N-glycosylation inhibition reduced the level of smooth muscle Myh11 to 2.90 ± 0.14 fold, n=3 (Figure 5.18). RNA extracted from mouse aorta was used a positive control for Myh11 expression. It was observed that the level of Cnn1 and Myh11 mRNA is significantly greater in the aorta.

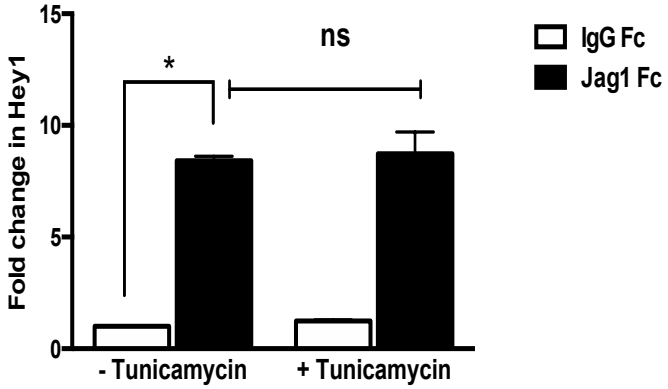

Figure 5.16. Hey1 expression in MVSCs after treatment with Jag-1 and tunicamycin. Cells were seeded on wells pre-coated with 5 μg/ml Jag-1 or Fc control and incubated for 48h, followed by 24h tunicamycin (0.5μg/ml) treatment to inhibit N-glycosylation. RNA samples were prepared from the cells and analysis was carried out by PCR to measure the level of expression of Hey1. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. *P < 0.05 as compared to tunicamycin-Fc treated control (ANOVA).

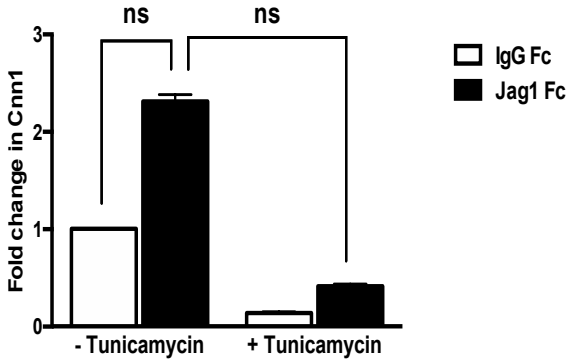

Figure 5.17. Cnn1 expression in MVSCs after treatment with Jag-1 and tunicamycin. Cells were seeded on wells precoated with Jag-1 or Fc control and incubated for 48h, followed by 24h tunicamycin treatment (0.5μg/ml) to inhibit N-glycosylation. RNA samples were prepared from the cells and analysis was carried out by PCR to measure the level of expression of Cnn1. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. Not significant when compared to (-)Tunicamycin Fc control. Not significant when compared to (-) tunicamycin-Jag-1 treated control (ANOVA). Representation of two experiments.

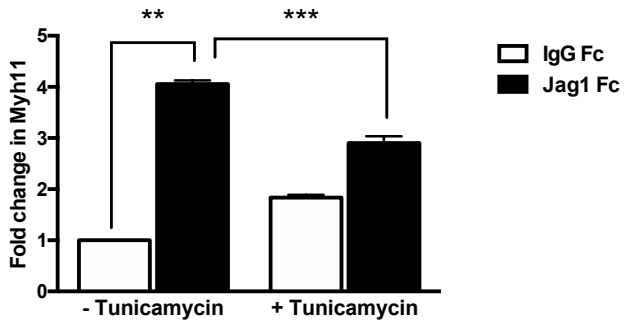

Figure 5.18. Myh11 expression in MVSCs after treatment with Jag-1 and tunicamycin. Cells were seeded on wells precoated with Jag-1 or Fc control and incubated for 48h, followed by 24h tunicamycin (0.5μg/ml) treatment to inhibit N-glycosylation. RNA samples were prepared from the cells and analysis was carried out by PCR to measure the level of expression of Myh11. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. **P < 0.01 as compared to (-) Tunicamycin-Fc treated control. ***P < 0.001 as compared to (-) Tunicamycin-Jag-1 treated control (ANOVA).

5.8 Lfng down-regulation inhibits Jag-1-induced Notch activation and myogenic differentiation

The effect of Lfng knockdown on Jagged's ability to stimulate myogenic differentiation of MVSCs was determined. Cells were seeded on wells pre-coated with Jag-1 or Fc control, followed by 48h treatment with Lfng siRNA. RNA samples were obtained from the cells and analysis was carried out by PCR to measure the level of Notch target gene Hey1, vascular smooth muscle genes, Cnn1 and Myh11. The siRNA knockdown resulted in a modest but significant decrease in the expression of Hey1 (4.57± 0.71 fold, n=3) (Figure 5.19). The ability of Lfng to modulate Notch activity, hence myogenic differentiation was assessed by Immunocytochemistry. siRNA knockdown of Lfng did not affect the levels of Cnn1 protein expression and also no adverse effect was observed on the Cnn1 mRNA level (Figure 5.20). However, transfection of cells with Lfng siRNA resulted in reduced Myh11 mRNA level in comparison to cells transfected with scrambled siRNA (Figure 5.21).

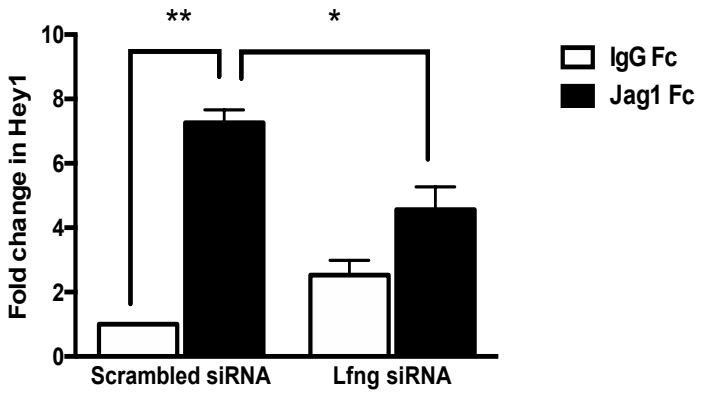

Figure 5.19. Down-regulation of Lfng decreases Notch target gene Hey1 mRNA levels. Real-time PCR analysis of Jag-1/Fc stimulated MVSCs mRNA following transfection with 10nM Lfng targeted siRNA. The experiment was performed in triplicate. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. **P<0.01 as compared to scrambled siRNA-Fc treated control. *P < 0.05 as compared to scrambled siRNA-Jag-1 treated control (ANOVA).

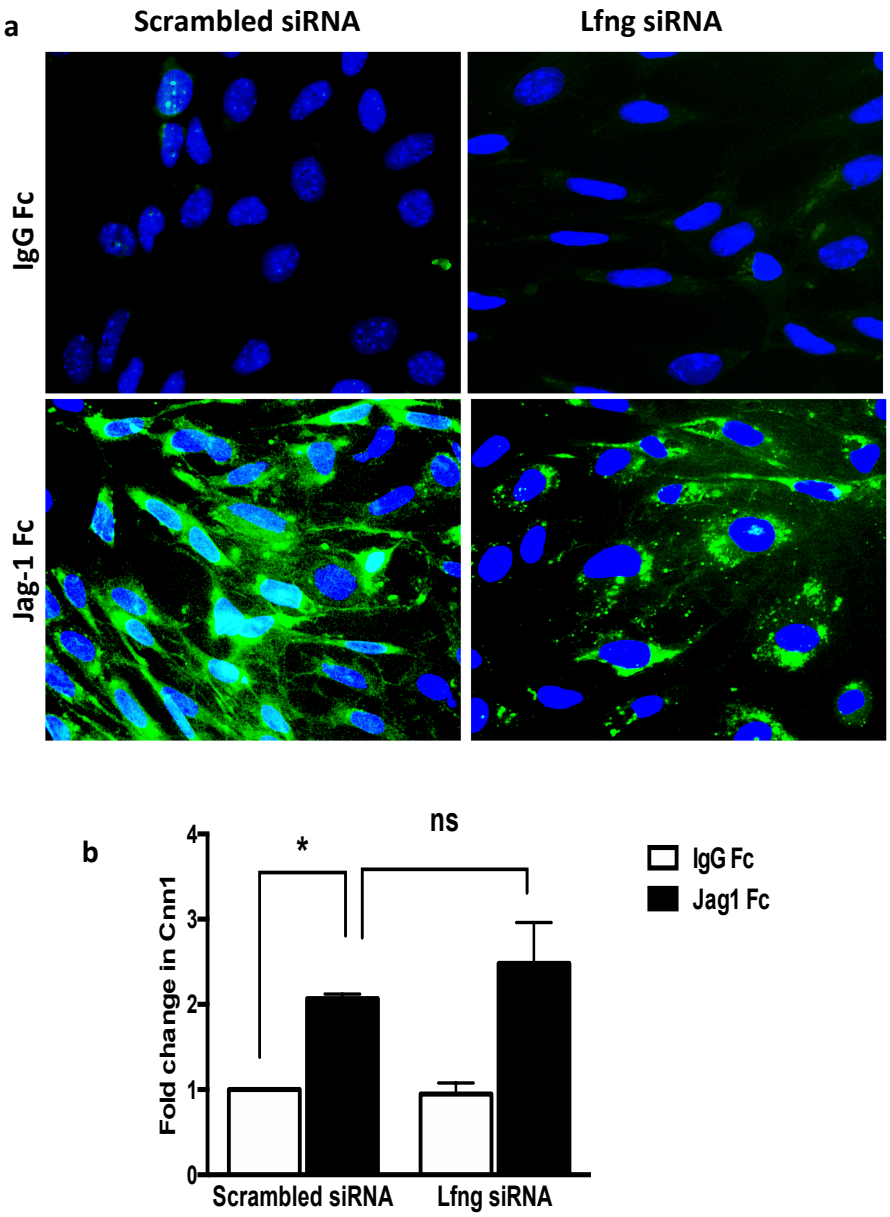

Figure 5.20. The effect of Lfng down-regulation on smooth muscle marker Cnn1 expression. (a) Following transfection of Jag-1 / Fc treated MVSCs with 10nM scrambled or Lfng siRNA, cells were fixed with formaldehyde and stained with antibody against calponin1 then probed with Alexafluor 488 goat anti-rabbit IgG secondary antibody. DAPI was used for nuclear visualization, 20 X magnification. Representation of 12 images from two independent experiments. (b) Real-time PCR analysis of Jag-1/Fc stimulated MVSCs mRNA following transfection with 10nM Lfng targeted siRNA. The experiment was performed in triplicate. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. *P < 0.05 as compared to scrambled siRNA-Jag treated control (ANOVA).

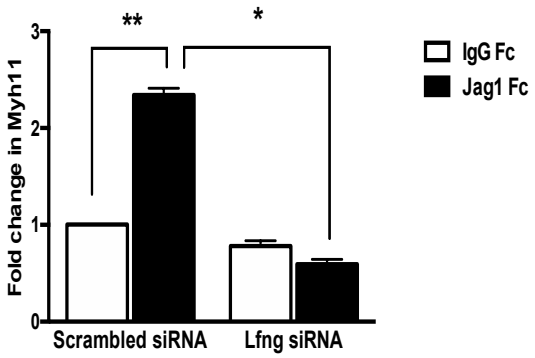

Figure 5.21. Knockdown of Lfng decreases smooth muscle Myh11 mRNA levels. Real-time PCR analysis of Jag-1/Fc stimulated MVSCs mRNA following transfection with 10nM Lfng targeted siRNA. The experiment was performed in triplicate, chart is a representative of two independent experiments, Data are ± SEM. **P<0.01 as compared to scrambled siRNA-Fc treated control, *P < 0.05 as compared to scrambled siRNA-Jag treated control (ANOVA).

Summary

- Activation of Notch receptors with Jag-1 resulted in a significant increase in the expression of Notch target genes, Hey1 and Hey2 in MSC and MVSC.
- Jag-1 induced the expression of SMC differentiation markers smooth muscle cell alpha actin (SMA, ACTA2), calponin1 (Cnn1) and smooth muscle myosin heavy chain 11 (Myh11) and the percentage of Cnn1 and Myh11 positive cells.
- Jag-1 promoted the expression of SMC differentiation marker, transgelin (SM-22a) using mass spectrometry analysis.
- Activation of Notch receptors with Jag-1 resulted in a significant increase in the expression of SMC differentiation markers (Cnn1, Myh11) in MSC and MVSC.
- Partial Notch1 knockdown attenuated Jag-1-induced Notch target gene expression (Hey1), Cnn1 protein expression and Myh11 gene expression.
- Tunicamycin treatment had no effect on Jag-1 induced Notch target gene expression (Hey1) but significantly inhibited SMC differentiation marker expression (Myh11).
- Lunatic Fringe (Lfng) knockdown significantly inhibited Jag-1-induced Notch target gene expression (Hey1) and SMC differentiation marker expression (Myh11).

Discussion

The biological process involved in stem cell transition to smooth muscle cell has been a complicated topic within the literature, particularly in establishing the various factors that influence this process. Notch signalling pathway plays an important role in the differentiation and function of vascular smooth muscle cells. Active Notch signalling can either inhibit (Havrda et al. 2006) or promote differentiation and contractile phenotype (Boucher et al. 2011; Wang et al. 2015) in some smooth muscle cells in culture.

Different types of resident SMC-related vascular progenitors have so far been identified within the tunica media. These include mesenchymal stem cell (MSC)-like cells, a side population of $Sca1^{+}$ Adventitial progenitor cells (APCs) and MVSCs (Tintut et al. 2003). Notch signalling generally supports SMC differentiation of stem cell populations (Kurpinski et al. 2010; Doi et al., 2006). Vascular stem cells become activated following injury, transition to vSMC and subsequently differentiate into smooth muscle cells and play a significant role in vascular development and remodelling (Tang et al. 2012; Gomez and Owens 2012; Xu et al. 2008).

The first major finding in this study is that Notch activation is sufficient to induce smooth muscle gene and protein expression in MSCs, therefore reinforcing the hypothesis that Notch activation is involved in smooth muscle differentiation. Two methods were used to activate Notch signalling; over-expression of NICD and immobilised Jag-1 ligand. Over-expression of NICD increased Notch target gene Hey1 and Hey2 expression in MSCs.

MSCs stimulated with Jagged1 promoted Notch activation with increase in Notch target gene Hey1 and Hey2 and myogenic differentiation of MSCs towards vascular smooth muscle cells lineage. Notch activation by Jagged alone was enough to induce smooth muscle markers, alpha actin, Cnn1 and especially the smooth muscle Myh11 in MSCs, therefore supporting the role of Notch signalling activity in myogenic differentiation of MSCs. The Notch ligand, Jagged1 has been shown previously to have a direct influence on activating Notch and the expression of smooth muscle cells markers in MSCs (Kurpinski et al. 2010).

Proteomic analysis was performed by mass spectrometry to study global protein expression in different cell type: cultured MSCs, SMCs and freshly isolated aortic mouse SMCs. In order to understand what kind of intracellular changes occur during Jagged-induced myogenic differentiation of stem cells, global proteome of MSCs treated with Jag-1 or Fc control were analysed by mass spectrometry. Proteomic studies have been done previously by mass spectrometry to determine protein expression of vascular SMCs in culture (Baykal et al. 2013) and in human mesenchymal stems cells and with embryonic stem cells (Roche et al. 2009). The MSCs in culture were treated with Jag-1 to induce myogenic differentiation and transition to vascular smooth muscle like cells. Distinct smooth muscle cell markers, smooth muscle actin, Cnn1 and myosin 11 were all expressed in the aortic SMCs as expected. RT-PCR analysis of mouse aorta corresponds with mass spectroscopy data since Cnn1 and Myh11 are expressed in high abundance in aortic SMCs. Interestingly, aortic smooth muscle alpha actin was also detected in Fc treated MSCs, Jag-1 treated MSCs and untreated MSCs and surprisingly not in the cultured SMCs. Cultured SMCs was found to express alpha cardiac muscle actin.

Although other myosin isoforms were identified in the cultured SMCs and MSCs, however, they are not of significance. Notch1 could not be detected in the 50ng/ml Notch1 standard or in the proteomes from the different cell type, possibly due to low concentration. Although quantification of endogenous Notch1 in cultured cells by ELISA analysis indicated very low-level expression of Notch1 in stem cells, it was still surprising that Notch1 could not be detected or quantified by mass spectrometry.

However, some important glycosyltransferases were detected across the proteomes (Table 5.3), one of which is Pofut 1 identified in Fc treated MSCs, which has a distinctive role of adding O-fucose to the EGF repeats of Notch receptor. The other glycosyltransferases are also involved in glycosylation processes but not directly linked to Notch receptor.

A further study was conducted on the resident stem cells MVSCs to verify if Notch activity influences their myogenic differentiation. Previous studies have shown that MVSCs can differentiate to MSC-like cells that subsequently differentiate and transition to SMCs (Sainz et al. 2006; Xu. 2008). NICD overexpression was sufficient to at least induce increased expression of smooth muscle Cnn1 in MVSCs. Similar to the findings from MSC studies, Jagged1 induced Notch activation as confirmed by an increase in Hey1 gene hence promoting MVSC transition towards SMCs lineage. Both immunocytochemistry and RT-PCR analysis revealed an increase in the smooth muscle markers Cnn1 and Myh11 expression in the Jagged1 treated MVSC. These results again indicate that the Notch pathway is an important factor in the MVSC transition to SMC. Varying amounts of serum was tested to establish the optimum condition for Notch

activation using ligand Jagged1, the level of expression of both Cnn1 and Mhy11 protein was directly proportional to increasing amount of serum in the growth media.

To determine whether Notch1 is involved in Jag-1-induced Notch activation, hence myogenic differentiation, we investigated the changes in MVSCs when exposed to Jag-1 with a concurrent knockdown of Notch1 gene expression. Down-regulation of Notch1 expression leads to a decrease in Notch target gene Hey1. Although the Notch1 gene was only knocked down by approximately 30%, it still significantly reduced the levels of Cnn1 protein expression and Myh11 gene in comparison to treatment with Jag-1 and scrambled siRNA, thereby suggesting the importance of Notch1 in Notch activation and myogenic differentiation of MVSC in response to Jag-1.

The Notch receptor is O- and N-glycosylated. Post-translational modifications of Notch receptors with O-fucose and O-glucose are known to control Notch signalling pathway (Stanley 2007;Okajima, Matsuura and Matsuda 2008; Luther and Haltiwanger 2009). Little is known about the role of N-glycosylation in regulating Notch activity. The role of N-glycosylation in Notch activity and stem cell myogenic differentiation was investigated by treating MVSCs with appropriate dose of tunicamycin, an N-glycosylation inhibitor while inducing differentiation with Jag-1. RT-PCR analysis to assess smooth muscle cells markers before and after differentiation demonstrated significant down regulation of Myh11 expression. Although Hey1 expression was not affected by the exposure to tunicamycin, indicating that inhibition of N-glycosylation did not impact Notch activation but Jagged's ability to stimulate myogenic differentiation of MVSCs

towards a SMCs lineage was compromised in the tunicamycin treated cells as was determined by the reduction in Myh11 mRNA level.

The Notch protein is fucosylated following translation by the GDP protein O-fucosyltransferase (O-fut 1in *Drosophila*: Pofut 1 in mammals). Fringe proteins elongate O-fucose by adding a single N-acetyl glucosamine on EGF repeat 12 in *Drosophila* Notch. This modification is enough to cause an increase in receptor binding to Delta while reducing receptor binding to Serrate (Jagged) *in vivo* and *in vitro* (Xu et al., 2007). The role of Lunatic Fringe in differentially modulating Notch signalling has been studied by several groups (Hick et al. 2000; Yang et al. 2004; Tsukumo et al. 2006).

In this study, the role of Lunatic Fringe (Lfng) in controlling Notch activity, hence stem cell myogenic differentiation was investigated. This was done by monitoring changes in Notch target gene and activation of SMC specific gene transcripts in MVSC when exposed to Jagged with a concurrent knockdown of Lfng. With reduced Lfng expression, Notch target gene Hey1 and smooth muscle Myh11 gene expression were diminished but no effect was observed on the smooth muscle Cnn1 gene and protein expression as confirmed by RT-PCR and immunocytochemistry analysis. This finding from this part of the study supports the hypothesis that Lfng modification of Notch regulates Jag-1 ability to promote myogenic differentiation.

Chapter 6:

General Discussion

Cell fate alterations in the vasculature are crucial to the pathogenesis of vascular diseases, including, arteriosclerosis, atherosclerosis and restenosis after angioplasty (Li et al. 2009). Notch signalling is known to be highly involved in regulating cell fate in vasculature development during embryogenesis, controlling various mechanisms such as stem cell proliferation, cell-cell interaction and differentiation state (Jafar-Nejad, Leonardi and Fernandez-Valdivia 2010). Growing evidence suggests that Notch signalling amongst other signalling pathways plays a major role in altered cell fate decisions leading to vascular disease (Li et al. 2009).

The Notch pathway is conserved and highly regulated through several mechanisms including signal transduction and post-translational modifications of its ligand and receptors. A major post-translational modification that Notch receptor undergoes is glycosylation, primarily O-glycosylation on the EGF-like repeats of the Notch proteins (Moloney et al. 2000; Matsuura et al. 2008). N-glycosylation occurs to a lesser extent. Evidence from several reports has indicated the significance of glycosylation of the Notch receptors in regulating Notch signalling (Stanley 2007;Okajima, Matsuura and Matsuda 2008; Luther and Haltiwanger 2009).

The overall hypothesis is that Notch activation regulates myogenic differentiation in stem cells and that N-glycosylation of the extracellular domain of Notch1 has a putative role in this process.

6.1 Notch1 N-glycans can be detected using validated platforms

To establish platforms for detecting and measuring N-glycosylated proteins, a system was designed to generate pure N-glycosylated protein, anti-IL-8 monoclonal antibody, an IgG1. The model N-glycosylated protein was isolated from the supernatant of CHO DP-12 cells in culture using a protein affinity chromatography purification method. Size exclusion HPLC was developed to detect the N-glycoprotein and measure changes in N-glycosylation of the protein after treatment with N-glycosylation modifiers. HPLC technique was successfully applied to measure the glycoprotein following the glycosylation inhibition treatment, however, due to limitation in this method of analysis it was not possible to determine the glycan structure that may have been modified by the treatment.

These data strongly suggests that the size of glycoprotein is not significantly altered following treatment, and any individual differences in glycan composition may be too subtle to detect. The difficulties encountered with SEC application in determining changes in N-linked glycosylation made it impossible to continuously use it for glycan analysis. To overcome this difficulty, an alternative means was sought for easier and faster analysis of glycoproteins.

Enzyme-linked lectin assay (ELLA), a simple model for glycoanalysis, was developed to support the analysis of glycoproteins and lectin interactions, in a standard microtiter plate arrangement. The major benefit with this method is that it does not require prior glycan removal from glycoprotein and derivatisation procedure as would be required for HPLC analysis, hence making the analysis easy to perform (Pilobello et al. 2005; Chen et al. 2007). To validate the ELLA method

for the analysis of N-glycosylated proteins, groups of lectins of known glycan-binding specificities were analysed with anti-IL-8 mAb glycoprotein and human serum IgG standard. In doing so, more knowledge was acquired about the types of sugars present on the glycoprotein.

The focus of this research project was mainly on defining a putative role of N-glycosylation of Notch1 receptor and the influence on resident vascular stem cell fate. To investigate the functional role of the N-glycans in Notch signalling activity, first, native Notch1 was detected and quantified in cultured mesenchymal stem cell lysate using ELISA to ensure that Notch1 is expressed in these cells. Although the ELISA method was useful in determining the levels of endogenous Notch1 present in MSCs, the technique was not used routinely, as there was no means of easy isolation of Notch1 from the total protein in the lysate to study its glycosylation.

The validated ELLA method was employed to examine the glycosylation state of Notch1 using Notch1 Fc, a representative of Notch extracellular domain and human Notch1 standard. To verify that N-glycans are present on Notch1 Fc and Notch1 overexpression lysate, they were treated with PNGase F and resulting glycoproteins displayed a significant reduction in binding to N-glycan specific lectin Con A, suggesting that N-glycans had been modified. This finding demonstrates the potential use of ELLA technique for N-glycosylation analysis of protein of interest Notch1.

6.2 Notch ligand Jagged1 promotes myogenic differentiation of stem cells *in vitro* through activation of Notch1 receptor

Medial progenitor stem cells that display phenotypic markers related with neuroectoderm stem cells are able to differentiate into SMCs following injury and as a result contribute to intimal thickening of the artery and may be partly responsible for causing vascular disease (Tang et al. 2012). It is speculated that MVSC become mesenchymal stem cell (MSC)-like while transitioning into smooth muscle cells. These cells are thought to be involved in restenosis and that the "de-differentiated" SMC do not play any role in the process (Tang et al. 2012). The involvement of Notch activity in Jagged-induced stem cell differentiation was investigated *in vitro* using MSC and MVSC. It was confirmed that Jagged-induced Notch activation promotes myogenic differentiation of MSC and MVSC where an increase in smooth muscle gene and protein expression were detected.

Previous *in vivo* studies have shown that Notch1, rather than Notch3 in SMCs play a major role in mediating neointimal formation and vascular remodelling after injury in mice models (Li et al. 2009). Notch1 knockdown in mice with injury induced remodelled artery significantly inhibited medial thickening and neointimal formation (Redmond et al. 2013). To verify that Notch1 and not Notch 2,3,4 is required for Jag-1-induced Notch activation hence myogenic differentiation, the effect of depletion of Notch1 receptor in MVSC myogenic differentiation was determined. Notch1 depleted cells expressed a significant reduction in smooth muscle gene and protein expression. This finding demonstrated that Jagged1 activated Notch1 *in vitro*, however, there are four Notch genes (Notch 1-4) in mammals which encode receptors for several Notch ligands (Jagged1, Jagged2,

Delta like 1,3,4) and it is not fully understood whether specific ligand –receptor interactions are similar *in vivo*.

6.3 Notch1 receptor N-glycans can be removed/inhibited and validated using IP ectopic Notch1 studies

Given that the level of endogenous Notch1 detected in the culture MSC lysate is too low for any significant interaction with the lectin for glycan analysis, Notch1 was ectopically expressed in MSC and isolated by immunoprecipitation. N-glycosylation state of immunoprecipitated Notch1 was verified and changes detected following PNGase F treatment. It is evident from literature and findings from this study that Notch1 is indeed N-glycosylated. The effect of tunicamycin treatment or Lfng depletion on Notch1 N-glycosylation could not be assessed due to immunoprecipitation of overexpressed Notch1 in MVSC being unsuccessful. Still, the evaluation of overexpressed Notch1 lysate glycome revealed reduced binding with N-glycan specific lectins thus indicating N-glycans had been modified. It is believed that Fringe proteins attach complex N-glycan to the O-fucose sites on Notch EGF repeats. Interestingly, ELLA profile showed 50% reduction in N-acetyl glucosamine in Notch lysate glycome. This corresponded with the PCR analysis of Lunatic Fringe mRNA where 50% depletion was observed in MVSC depleted of Lfng. It is speculated that the N-glycans on Notch might have been modified by the Lunatic Fringe knockdown. It is however not clear whether the reduced binding observed with the N-glycan lectin has any significance to the N-glycans on the Notch receptor.

The N-glycosylation profile of MVSCs before and after differentiation to SMC lineage using Jag-1 as an inducer was examined by ELLA (data not shown). No significant differences were observed across N-glycan specific lectins tested. This suggests that ELLA might not be sensitive enough to pick up any slight change that might have occurred. The signal determined by ELLA on total proteins from stem cells rather than immunoprecipitated Notch1 is an overall contribution from all glycoproteins in lysate rather than individual proteins. Although the method is sensitive and specific to changes in total abundance of glycosylation, it cannot distinguish differences in glycan structure at the individual protein level.

The correlations between protein glycosylation and many biological processes and diseases are increasing the demand for quantitative glycomics strategies enabling sensitive monitoring of changes in the abundance and structure of glycans. Most of the studies carried out previously on stem cells and glycosylation employ lectin and antibodies staining and techniques such as flow cytometry and western blotting. Lectin and antibody staining are easy to perform, however, they are not as specific and could result in loss of vital information of the carbohydrates structure (Baykal et al. 2013). Other strategies that are employed include techniques such as capillary electrophoresis, liquid chromatography and mass spectrometry (Mechref et al., 2013).

The use of only optical detection methods to quantify individual glycan species has limitations such as susceptibility to operator subjectivity and is slowly being replaced by mass spectrometric detection. The need for subjective and standardised quantitation methods is currently best addressed using label-free glycomics. Recently, labelling with stable isotopic reagents has also been presented as a very viable strategy enabling relative quantitation. Most isotopic

labelling techniques involve chemical derivatisation, though metabolic and enzymatic incorporation are also used. This technique allows for multiple samples analysis in a single instrumental injection and due to this, there is minimisation of technical variation for comparative studies (Smith et al., 2017). Although, mass spectrometry is time consuming and generally not suitable for routine use for rapid evaluation of glycosylation state, still the tool is a lot more sensitive and highly specific making the application desirable for this project for studying the N-glycome profile of stem cells and their differentiated progeny and to investigate glycan structures related to differentiation. With this, distinct signatures can be established for identifying smooth muscle cells and stem cells. The application of gene editing strategies such as CRISPR/Cas9, zinc finger nucleases is another exciting development aimed at simplifying glycosylation and generating homogenous glycoforms. This development combined with the rapid advancement in the field of analytical glycomics, could enable and empower future developments in attaining greater knowlegde of the functional role of complex glycoproteins structure (Smith et al., 2017).

6.4 Removal of specific N-glycans decorating Notch1 receptor results in diminution Jag-1 induced myogenic differentiation.

The focus of this study has been the regulation of Notch signalling by N-glycosylation at the level of receptor modulation. The final study examined the role of N-glycosylation of Notch1 and Notch signalling in myogenic differentiation. Having shown that Notch activity controls stem cell myogenic differentiation and established the effect of tunicamycin on N-glycosylation of Notch1 overexpression in the MVSC lysate, the effect of tunicamycin in controlling myogenic differentiation was determined. This was established by treating stem cells with N-glycosylation inhibitor tunicamycin with concurrent Jagged1 treatment. Tunicamycin treatment lead to down regulation of smooth muscle gene Myh11. A previous study has shown that tunicamycin could be employed in targeting breast cancer stem cells inside tumour by inducing endoplasmic reticulum stress. Tunicamycin disrupts the correct folding of protein in eukaryotic cells, which leads to accumulation of unfolded proteins in the endoplasmic reticulum causing ER stress (Nami, Donmez and Kocak 2016). Although many studies have not been reported on N-glycosylation inhibitor tunicamycin and Notch signalling in stem cells, it is thought that a similar approach may be applied to cardiovascular related cells.

The concluding part of the study focused on establishing a role for Lunatic Fringe (Lfng) in Notch activation and myogenic differentiation. Lfng adds N-acetyl glucosamine (GlcNAc), a monosaccharide and a principal element of complex N-glycan structure to O-fucose. Notch1 is made up of 36 EGF repeats in its extracellular domain, 17 of which contain O-fucose sites (Kakuda and Haltiwanger

2017). The glycosyltransferases enzymes are conserved between flies and mice, still the repercussions of Notch glycosylation in flies are not always reflected in mammals (Lei et al., 2003). Understanding the impacts of Fringe on Notch activity can be problematic due to the presence of several receptors, ligands, and Fringe proteins. Vertebrate glycosyltransferases appear to contribute to Notch biology in a different manner to that of flies. In T cells, Lfng modification of Notch promotes Delta-to-Notch signalling and inhibits Jagged-to-Notch signalling. A different pattern is witnessed in the somite where Lfng limits Delta-to-Notch signalling (Dale et al., 2003).

Having identified the effect of Lunatic Fringe (Lfng) knockdown on overexpressed Notch1 receptor N-glycosylation by ELLA analysis, the role for Lfng in the regulation of Notch signalling was investigated. A 50% reduction in Lfng gene was achieved following transient transfection of MVSCs (without Jag-1 or Fc treatment) with Lfng siRNA, and as a result, significant reduction in Notch activity (reduced Hey1 expression) and myogenic differentiation (reduced Myh11 expression) was observed following Jag-1 treatment. This finding supports the hypothesis that Lfng has a modulatory effect on Notch activity and myogenic differentiation.

A recent study using mouse Notch1 model evaluated which predicted O-fucosylation sites are modified by Lunatic, Manic or Radical Fringe and which of these are required for Notch1 activation by delta ligand 1 or Jag-1. There is an indication that Fringe proteins select different regions of the Notch1 ECD and as a result activate or inhibit Notch1 activity. Lfng elongation enhanced binding of

Jagged1 at EGF12 while it inhibited Jag-1 induced Notch1 activation at EGF 6 or EGF 36 (Kakuda and Haltiwanger 2017).

It is not known if the biochemical effects of Lfng protein on Notch signalling is as a result of intracellular modifications of Notch1 or an element of the Notch signalling transduction pathway. It might be acting via a direct, physical interaction with Notch1 and its ligand Jagged1 on the surfaces. There is an indication that differential modulation of the Notch extracellular domain by Lfng controls downstream events with respect to ligand binding either by inhibiting or promoting Notch receptor activation and signalling (Hicks et al., 2000). There is a possibility that the Jagged1 might be interfering indirectly with the Lfng or Notch1 expression when cells are transfected with siRNA and concurrent exposure to Jagged1. It is suggested for future work to transfect cells with siRNA prior to introducing on Jagged1 coated surfaces.

One of the questions that needed to be addressed in this project is what role does the glycosylation of the ligand Jagged1 play in Notch signalling. The glycosylation state of Jagged1 was examined since the Notch activation assays involve incubating stem cells on Jag-1 Fc coated surfaces. ELLA analysis of Jag-1 Fc indicated the presence of both O and N-glycans suggesting that Jagged1 is glycosylated. However, there are limited studies on the modulation of Notch ligand by glycosyltranferases and this could not be further addressed.

Summary

- Jagged1 stimulated Notch activation induced myogenic differentiation of both MSCs and MVSCs.
- Myogenic differentiation is compromised when N-glycosylation of Notch1 receptor is inhibited by tunicamycin or Lunatic Fringe knockdown.
- These findings have significant implications for the role of N-glycans in mediating Notch1 regulation of smooth muscle differentiation *in vitro*.

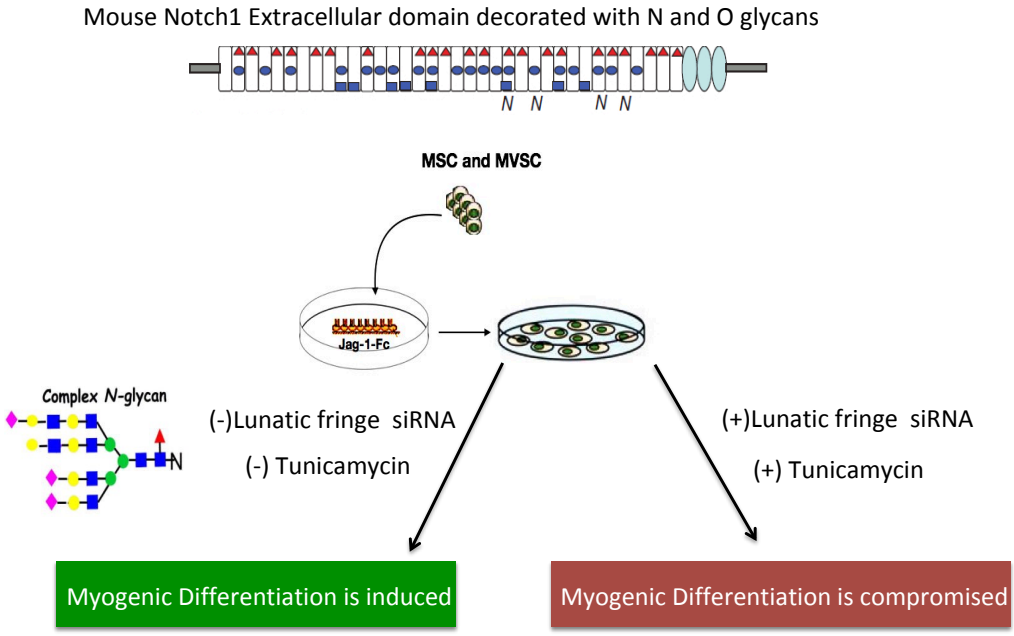

Figure 6.1. Summary diagram of findings from the thesis.

Conclusion

The overall purpose of this research project is to study the putative role of N-glycosylation of Notch1 receptor and the influence on resident vascular stem cell fate. Employing ligand Jagged-induced Notch signalling assays has enabled us to gain insights into how alterations in the N-glycans of the Notch1 regulate Notch signalling and myogenic differentiation. The findings in this study are significant in terms of understanding how MSCs and MVSCs can transition to a SMC lineage.

The results showed that Jagged1 stimulation and Notch activation were sufficient to induce myogenic differentiation of MSCs and MVSCs. The finding that myogenic differentiation is compromised when N-glycosylation of Notch1 receptor is inhibited by tunicamycin or Lunatic Fringe siRNA is novel. These findings have significant implications for the regulation of smooth muscle differentiation *in vitro*.

If Notch receptors control stem cell maintenance and are the source of stem-cell derived myogenic progeny that leads to intimal medial thickening, then targeting the glycans on the extracellular domain of these receptors using fusion proteins might offer a potential therapeutic strategy in the treatment of cardiovascular disease.

Future work

A more detailed biochemical and structural analysis of Notch1 is necessary for a better understanding of how these carbohydrates regulate ligand binding and subsequent Notch activation. Optimisation of the immunoprecipitation procedure to enable isolation of Notch1 receptor following glycosylation inhibition and analysis of the modifications using a more sensitive technique such as mass spectrometry, with the view of detecting small changes in protein-glycan composition. Optimise Lfng and Notch1 knockdown studies and assess protein levels by immunoblot following transfection.

Flow cytometry application might possibly detect change in surface N-glycan modification following treatment of cultured cells with PNGase F using FITC-conjugated N-glycan specific lectins (Hamanoue and Okano 2011) and mass spectrometry technique to study the N-glycome of stem cells before and after PNGase F treatment of cultured cells (Hamouda et al. 2013).

Site-directed mutagenesis is a promising tool that may possibly be employed to identify the putative role of targeted N-glycans on Notch receptor extracellular domain. A previous study compared Protein O-fucosyltransferase 1 (Pofut 1) sequences found in the database, it was discovered that mammalian Pofut 1 enzyme has two putative N-glycosylation sites. The first site is highly conserved among species. By site-directed mutagenesis, they showed that the loss of N-glycan at this specific site slightly affected the O-fucosyltransferase activity (Loriol et al. 2007). This technique used in combination with mass spectrometry should further clarify a role for N-glycans in Notch signalling and identify differentiation associated glycan structures.

Although we were able to profile stem cells proteome by mass spectrometry before and after Jagged-induced differentiation and identified some important glycosyltransferases, however, further work is required to determine the N-Glycan profile before and after transition of resident stem cells to SMC.

Following the extensive study on the glycosylation of Notch receptor and the role the N-glycans play in Notch signalling, it is expected that one should have a better understanding of how N-glycosylation influences stem cell myogenic differentiation towards vascular smooth muscle cells. These findings could potentially serve as a means of monitoring smooth muscle differentiation and could be applied to development of therapeutics for treatment of cardiovascular diseases.

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