The Role of GSK-3β in Notch Signalling in Vascular Smooth Muscle Cells

A dissertation submitted for the degree of Ph.D

by

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Declaration

I hereby certify that this material which I now submit for assessment on the program of study leading to the award of Doctor of Philosophy is entirely my own work 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 own work.

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Dedication

To my grandmother, Eilish Carter, who passed away, following a stroke, while I was completing my thesis, and was an inspiration to me and a reminder of the importance of the work carried out in the vascular labs in DCU.

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To the most patient man in Ireland, Mark Tully, thank you for putting up with all the tears and tantrums over the last three years ◡̈. I hope I can be as supportive of you in the future.

Abstract

Vascular smooth muscle cell (VSMC) fate decisions are critical to the pathogenesis of vascular diseases, including hypertension, arteriosclerosis and atherosclerosis and restenosis after angioplasty. Crosstalk between the developing pathways of Hh, VEGF and Notch are crucial during vascular morphogenesis and modelling of the embryonic vasculature. These pathways may be recapitulated in adult cells resulting in altered fate decisions leading to vascular disease. The Notch signalling pathway is tightly regulated by a number of mechanisms including post-translational modification. The serine-threonine kinase, GSK-3β has been shown to bind to and phosphorylate NotchIC. Using a number of strategies including ectopic expression, siRNA technology and pharmacological inhibition, it is demonstrated that GSK3β positively regulates Notch receptors, Notch downstream targets and Notch downstream target promoter activity, while inhibiting Hh downstream target gene expression. Using recombinant Shh protein, recombinant VEGF-A protein and siRNA targeted against VEGF-A, it is demonstrated that Hh signalling positively modulates Notch signalling via VEGF-A.

Notch signalling is pro-proliferative and anti-apoptotic in VSMCs. GSK-3β is shown to positively modulate VSMC proliferation. Moreover, GSK-3β inhibits VSMC apoptosis, downstream of Notch signalling.

Altered haemodynamic forces have been implicated in the changes in cell fate associated with vascular disease and Notch signalling modulates VSMC fate in response to cyclic strain. Notch signalling in VSMCs is downregulated following strain in a MAPK-dependent manner. It is demonstrated that GSK-3β activity is decreased following strain. Moreover, MAP-kinases, p44/42 and p38 are shown to be activated in

VSMCs in a GSK-3β-dependent manner following cyclic strain. In-stent restenosis occurs in some cases following balloon angioplasty. Stent expansion causes a low amplitude/high mean strain environment that promotes proliferation of VSMCs. We demonstrate that GSK-3β activity and Notch1 levels are increased in a perfused mock coronary artery. Similarly, Notch, Hh and VSMC proliferation are increased in a ligated carotid artery (LCA) *in vivo* model of biomechanically-induced vascular remodelling. It is identified that GSK-3β expression is regulated in this model and that GSK-3β, Notch and Hh downstream targets are regulated in human arteriosclerosis. Collectively, these studies suggest that GSK-3β is involved in impaired Notch signalling leading to altered vascular cell fate and remodelling in disease.

Publications

Guha S, Morrow D, Cullen JP, Walls D, Redmond EM, Cahill PA. GSK-3β controls Notch Regulation of Vascular Smooth Muscle Cell Growth. 2009, in preparation.

Morrow D, Cullen JP, Liu W, **Guha S**, Sweeney C, Birney YA, Collins N, Walls D, Redmond EM, Cahill PA. Sonic Hedgehog Induces Notch Target Gene Expression in Vascular Smooth Muscle Cells via VEGF-A. *Arterioscler Thromb Vasc Biol.* 2009 Jul; **29(7)**: 1112-8.

Morrow D, **Guha S**, Sweeney C, Birney Y, Walshe T, O'Brien C, Walls D, Redmond EM, Cahill PA. Notch and vascular smooth muscle cell phenotype. *Circ Res.* 2008 Dec;**103(12)**:1370-82. Review.

Morrow D, Sweeney C, Birney YA, **Guha S**, Collins N, Cummins PM, Murphy R, Walls D, Redmond EM, Cahill PA. Biomechanical regulation of hedgehog signaling in vascular smooth muscle cells in vitro and in vivo. *Am J Physiol Cell Physiol.* 2007 Jan;**292(1)**:C488-96.

Morrow D, Sweeney C, **Guha S**, Walshe T, O'Brien C, Walls D, Redmond EM, Cahill PA. Notch signaling and vascular cell fate - how vascular cells respond to stress. *Wien Klin Wochenschr.* 2007 Jun;**119**:(11-12 Suppl 1)7-10.

Morrow D, Scheller A, Birney YA, Sweeney C, **Guha S**, Cummins PM, Murphy R, Walls D, Redmond EM, Cahill PA. Notch-mediated CBF-1/RBP-Jkappa-dependent regulation of human vascular smooth muscle cell phenotype in vitro. *Am J Physiol Cell Physiol.* 2005 Nov;**289(5)**:C1188-96.

Poster Presentations

Guha S, Murphy R, Walls D, Redmond EM, Cahill PA. Inhibition of GSK-3β decreases vascular smooth muscle cell growth *in vitro* through modulation of Notch signaling. Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference (ATVB), Chicago, April 2007.

Morrow D, **Guha S**, Sweeney C, Birney YA, Murphy R, Cummins PM, Walls D, Redmond EM, Cahill PA. Hedgehog Regulates Notch Signaling via VEGF in Vascular Smooth Muscle Cells *in vitro*. American Heart Association Scientific Session, Dallas, November 2005.

Morrow D, **Guha S**, Sweeney C, Birney YA, Cummins PM, Walls D, Redmond EM, Cahill PA. Hedgehog Regulation of Notch Signaling in Vascular Smooth Muscle Cells *in vitro*. European Molecular Biology Organization (EMBO) Workshop on Notch Signaling in Development and Cancer, Rome, April 2005.

Abbreviations

ANK	Ankyrin
ApoE	Apoliprotein E
BCA	Bicinchoninic Acid
BHLH	Basic helix-loop-helix
BMM	Bone-marrow derived macrophages
BSA	Bovine Serum Albumin
BTD	β-trefoil domain
CADASIL	Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts And Leukoencephalopathy
CAM	Calmodulin
CFDA	Carboxy-fluorescein diacetate succinimidyl ester
Ci	Cubus Interruptus
Cos2	Costal2
CSL	CBF1/RBP-$_{J\kappa}$, Su(H), Lag-1 protein
CKII	Casein kinase II
CTD	C-terminal domain
DES	Drug-eluting Stent
Dhh	Desert Hedgehog
DISC	Death Inducing Signaling Complex
Dll	Delta-like
DMSO	Dimethylsulfoxide
DNA	Deoxyribose Nucleic Acid
dsRNA	double stranded RNA
DTX1	Deltex-1
EC	Endothelial Cell

EDTA	Ethlyenediamine Tetracetic Acid
EGF	Epidermal Growth Factor
ERK	Extracellular Signal Regulated Kinase
ET-1	Endothelin-1
FACS	Fluorescence Activated Cell Sorting
Fl-Notch	Full-length Notch
Fu	Fused
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GLUT1	Glucose transort isoform
GPCR	G protein-coupled receptor
GSK-3α	Glycogen Synthase Kinase-3α
GSK-3β	Glycogen Synthase Kinase-3β
Hac	Histone Acetyltransferase
HDAcCoR	Histone Deacetylase Co-repressor
HDL	High Density Lipoprotein
HERP	Hes Related Repressor Protein
Hes	Hairy Enhancer of Split
Hey	Hairy/E(spl)-related with YRPW motif
Hh	Hedgehog
HRT	Hairy Related Transcription Factor
Ihh	Indian Hedgehog
Jag	Jag1
JNK	C-Jun N-Terminal Kinase
LB	Luria Bertani
LCA	Ligated carotid model
LDL	Low Density Lipoprotein

L-Fg	Lunatic Fringe
LNR	Lin-Notch repeats
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MCA	Mock coronary arteries
Neur	Neuralised
NO	Nitric Oxide
Notch ECD	Notch Extracellular Domain
Notch IC	Notch Intracellular
Notch ICD	Notch Intracellular Domain
NTD	N-terminal domain
OPNG	o-nitrophenyl-β-D-galactopyranoside
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PI-3 Kinase	Phosphoinositide 3 Kinase
PKC	Protein Kinase C
PS	Phosphatidylserine
Ptc	Patched
PTCA	Percutaneous transluminal coronary angioplasty
RISC	RNA-induced signaling complex
RNA	Ribonucleic Acid
ROCK	Rho Kinase
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase PCR

RVSMC	Rat Vascular Smooth Muscle Cell
SDS	Sodium Dodecyl Sulphate
Shh	Sonic Hedgehog
ShRNA	small hairpin RNA
SiRNA	small interfering RNA
SKIP	Ski-interacting Protein
SMC	Smooth Muscle Cell
TAD	Transactivation Domain
TE	TRIS-EDTA
TGF-β	Transforming Growth Factor-β
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
VSMC	Vascular Smooth Muscle Cell

Units

bp	Base Pairs
cm	Centimeters
cm^2	Centimeter Squared
^oC	Degree Celsius
g	Grams
h	Hours
kDa	Kilodaltons
L	Litre
M	Molar
mg	Milligrams
min	Minute
ml	Mililitre
mm	Milimetre
mM	Milimolar
ng	Nanogram
OD	Optical Density
pM	Picomolar
rpm	Revolutions Per Minute
sec	Seconds
U	Enzyme Units
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar
V/v	Volume per volume

W/v	Weight per volume
V	Volts
W	Watts
x g	G force

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Chapter 1

Introduction

1.1 Cardiovascular Disease

1.1.1 The Vasculature

The vasculature is essential in all vertebrates to distrubute oxygen and nutrients to tissues. It is comprised of a network of blood vessels called arteries, arterioles, veins, venules and capillaries. Blood, rich in oxygen, is carried in arteries from the heart into the tissue capillary bed, via the arterioles and capillaries. Exchange occurs and blood is carried back to the heart through the venous system. The structure of a blood vessel consists of three layers: the tunica adventia, the tunica media and the tunica intima (Figure 1.1).

The innermost layer, the intima, is a monolayer of endothelial cells (EC). These are the first cells of the vasculature to form during embryogenesis (Eichmann *et al.*, 2005). The endothelium plays an important function in regulating vascular tone, mediating inflammatory response, and forming a barrier for the transport of molecules into the

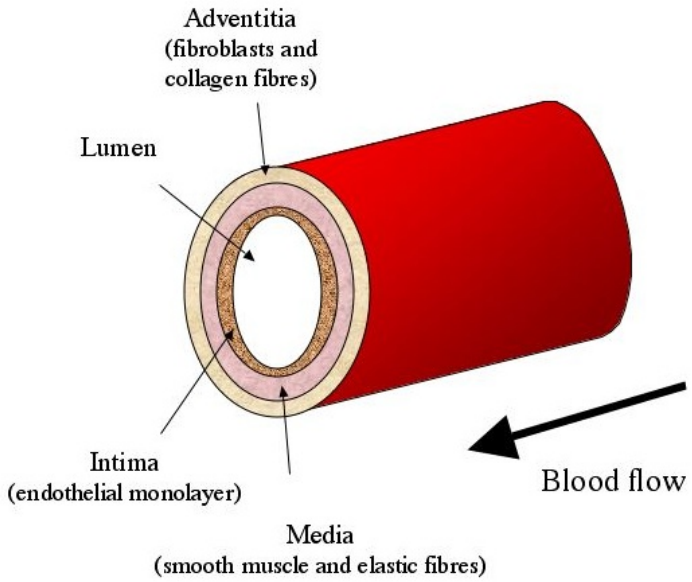


Figure 1.1: Arterial Structure

second layer, the media. The media is comprised of a compact mass of vascular smooth muscle cells (VSMC) and elastic fibres. The endothelium responds to a number of stimuli to regulate vascular tone. It can produce a number of vasotransmittors such as the vasodilator nitric oxide (NO). NO signals to the underlying smooth muscle cells, allows the opening of calcium channels and results in smooth muscle cell relaxation. Vascoconstrictors such as endothelin-1, operate in a similar manner to allow vascular smooth muscle contraction.

The primary function of an adult VSMC is to contract and dilate to regulate blood pressure and flow. VSMC contraction is triggered by an increase in $[Ca]^{2+}$ ions, due to Ca^{2+} release from the sarcoplamic reticulum and Ca^{2+} entry from the extracellular space through Ca^{2+}channels. Ca^{2+} binds calmodulin (CAM) to form a Ca^{2+} -CAM complex, which activates myosin light chain kinase and causes myosin

light chain contraction (Salamanca and Khalil, 2005).

During blood vessel formation, the phenotype of smooth muscle changes such that secretion of ECM proteins is reduced and the formation of intracellular myofilaments is increased. These contractile smooth muscle cells show an exceedingly low rate of proliferation and are largely nonmigratory. Adult VSMCs have been shown to convert reversibly between this noncontractile and contractile phenotypes (Li *et al.*, 1999b) in response to several physiological and pathological stimuli and there is evidence to show that there is heterogeneties between SMCs within a given blood vessel (Owens *et al.*, 2004).

The adventitial layer is a solid layer of fibroblasts and collagen fibres, which give structure to the blood vessel. Adventitial fibroblasts synthesize extracellular matrix,collagen and fibrin, and play a significant role in arterial repair (Strauss and Rabinovitch, 2000).

Blood vessels differ in their size and structure, depending on their position and function within the vascular system. Capillaries do not contain smooth muscle cells and are composed primarily of endothelium and the basement membrane upon which the endothelium rests. Pericytes are a relatively unspecialized cell that is derived from the same precursor cells that form endothelial cells in new vessels and can give rise to smooth muscle cells during vessel growth (Armulik *et al.*, 2005).

Arterioles contain scattered or one complete layer of SMCs and are surrounded by an elastic net, rather then internal membrane, Reticular fibers and collagenous fibers surround individual smooth muscle fibers and condense externally to form the tunica adventitia. Small arteries are unnamed and have at least two layers of smooth muscle cell. Small arteries and arterioles are the determinants of vascular resistance. They

are the primary regulators of blood pressure and blood flow within the system. They respond to changes in nerve activity and circulating hormones by constricting or dilating and are, therefore, referred to as resistance vessels (Moore *et al.*, 2003).

Large arteries, or 'elastic arteries', have sheets of elastic tissue in their thicker media. This is because the larger arteries, which include the aorta, carotid and coronary arteries, are subjected to high systolic pressures and must be compliant. Large arteries are distensible and temporarily store blood during systole before being propelled onwards by the elastic recoil of the arteries during diastole. Therefore, the compliancy of the aorta and large artery is of great significance (Moore *et al.*, 2003). The venous system, on the other hand, is a low resistance capacitance system which contains more then half of the total blood volume (Moore *et al.*, 2003).The media is thinner than in arteries as they do not need to adapt to pressure changes (Yamboliev *et al.*, 2002). Valves are formed by loose, pocket-shaped folds of the tunica intima, which extend into the lumen of the vein to prevent back flow of blood (Bianchi *et al.*, 2007).

The aorta is the largest artery, beginning at the root of the aortic valve, extending into the ascending artery, curving into the aortic arch and descending into the descending thoracic aorta and ending at the abdominal aorta. The coronary arteries originate from the root of the aorta, immediately above the aortic valve. The common carotid artery is a paired structure and the left and right common carotid arteries follow the same course with the exception of their origin. The right common carotid originates in the neck from the brachiocephalic trunk (which comes out of the ascending aorta). The left arises from the aortic arch in the thoracic region (Tortora and Grabowski, 1996).

1.1.2 Vascular Disease

Cardiovascular disease (CVD) is the most common cause of death in the USA and Europe and accounts for 36% of all deaths in Ireland (Irish Heart Foundation Website, 2009).

Cardiovascular disease refers to the class of diseases that involve the heart and/or blood vessels. Disease of the large arteries includes coronary artery disease leading to myocardial infarction, carotid artery disease, which leads to stroke, and peripheral artery disease, disease of the lower limbs, which may result in amputation. These diseases have a similar pathophysiology, including arteriosclerosis, atherosclerosis and aneurysm. Arteriosclerosis refers to hardening of the medium and large arteries and is a term that is used to describe a number of vascular disease states. Atherosclerosis is the most common form of arteriosclerosis, but the term arteriosclerosis also refers to a non-inflammatory vascular remodelling event in which hardening of the arteries occurs. An aneurysm is a localized, blood-filled dilation (balloon-like bulge) of a blood vessel caused by disease or weakening of the vessel wall. Aneurysms most commonly occur in the aorta. As the size of an aneurysm increases, there is an increased risk of rupture, which can result in severe hemorrhage or other complications including sudden death (Tortora and Grabowski, 1996).

Risk Factors

Risk factors for cardiovascular disease include those classed as modifiable such as diet, smoking exercise and weight. Poor nutrition plays an important role and a diet high in saturated fat and cholesterol is a high risk factor for cardiovascular disease. Cholesterol is insoluble in

blood. Very-low density lipoproteins (VLDLS) are secreted by the liver and converted to low-density lipoproteins (LDLs). LDLs carry cholesterol to other tissues where they can be oxidized, forming a pro-atherogenic environment. High-density lipoproteins, on the other hand, carry cholesterol back to the liver for degradation. A diet, therefore, high in HDLs and anti-oxidants and low in LDLs and tryglycerides is considered appropriate for a healthy lifestyle. Increased levels of homocysteine have also been linked to cardiovascular disease (Baccarelli *et al.*, 2007). A diet rich in folic acid and vitamin B6 is thought to be important in reducing the risk of cardiovascular disease.

A figure of 11% of total cardiovascular deaths have been attributed to smoking, mainly manifesting as chronic obstructive pulmonary disease (Perlstein and Lee, 2006). This may be mediated by increased inflammation and oxidative stress (Ambrose and Barua, 2004). Obesity is another major risk factor for cardiovascular disease. Excess free fatty acid liberation and reduced insulin action in peripheral tissues of the liver, adipose tissue and skeletal muscle, results in endothelial dysfunction and the onset of vascular disease (Caballero, 2003). This endothelial dysfunction and reduced vasoconstricitive response along with abnormality and pro-thrombotic response are also hallmarks of the diabetic state, a major contributor to cardiovascular disease.

While these risk factors may be controlled through specific lifestyle choices, other factors such as age, sex and genetic predisposition also determine the likelihood of developing cardiovascular disease. There is an age-dependent rise in hypertension and atherosclerotic disease. Ageing in itself confers a greater risk for disease than the conventional risk factors such as lipid levels, smoking, diabetes and sedentary lifestyle

(Plante, 2002).

Hypertension

Hypertension is a cardiovascular risk factor. Systemic hypertension is defined as chronically elevated blood pressure and uncontrolled high blood pressure can lead to a number of serious complications, including increased peripheral vascular resistance and atherosclerosis. Normal blood pressure is considered to be 120/80 mm Hg, whereas blood pressure of above 140/90 mm Hg is considered to be high (Owens *et al.*, 2004). The term 'essential hypertension' is used if there is no definitive cause of the condition, whereas secondary hypertension occurs as a result of other diseases such as renal problems.

1.1.3 Arteriosclerosis

Arteriosclerosis refers to any disease involving hardening of the arteries but is commonly used to describe age-related non-inflammatory hardening of the conduit or 'larger' arteries. To understand the ageing effect on the artery, we must consider the importance of the compliance or distensibility of the artery. Vessel compliance is inversely related to the stiffness of the vessel wall and is related to the material content of the wall and the relative thickness of the wall compared to its lumen diameter (Greenwald, 2007).

The vascular wall is complex and varies according to location within the arterial tree. The aorta is the largest and most compliant artery in the body as it contains the largest amount of elastin. It is similar to elasticated rubber, in that systolic pressure from the heart is absorbed passively by a "ripple effect" along the vessel wall. The ratio of elastin to collagen falls

with increasing distance from the heart, while the number of VSMCs per unit volume increases (Zieman Susan *et al.*, 2005).

The compliance of an artery may be measured by its pulse wave velocity. The more rigid the wall of the artery, the faster the wave moves. When the wave hits the major branching points, such as at the renal and femoral arteries, these waves are reflected back so that they reverse direction and travel back to their point of origin. As stiffness increases, reflected wave amplitude increases and augments pressure in late systole (Nichols *et al.*, 2008). The compliance of the aorta decreases with age (Zieman Susan *et al.*, 2005). With ageing, an increase in the ratio of collagen/elastin content is observed in the larger arteries (Cattell *et al.*, 1996). Increased MMP-2 and inhibition of its inhibitors localized primarily in the thickened intima, perhaps due to an exaggerated VSMC response to cytokines, is linked to the decrease in elastin (Li *et al.*, 1999c). The structure of the elastic lamellae become sparse with age and show signs of fragmentation and disorganization. It is hypothesised that this may occur due to the cumulative effect of mechanical stress and that disintegration may be mediated by integrins and metabolic factors such as oxidative stress (Atkinson, 1998). Non-atherosclerotic calcification of the remaining elastin in the media also further enhances arterial stiffness (Greenwald, 2007). Cross-linking of collagen is a major contributor to stiffening of the artery, Advanced glycation endproducts (AGEs) are formed by a reaction between reducing sugars and biological amines. Because of their marked stability, glycated proteins accumulate slowly over a person's lifespan (Zieman and Kass, 2004). The formation of AGEs on vascular wall and myocardial collagen causes cross-linking of collagen molecules to each other and other proteins (Aronson, 2003). All these

factors contribute to a decrease in the compliancy of the vessel, leading to an increase in pulse pressure or hypertension. Indeed, aortic rigidity precedes the development of arterial hypertension in the spontaneously hypertensive rat (SHR) model, as it does in individuals with borderline hypertension (Plante, 2008).

Age-related features also includes intimal thickening, decreased NO production and increased endothelin production (Jani and Rajkumar, 2006). Intimal thickness increases considerably with aging (Virmani *et al.*, 1991). SMC proliferation and migration into the developing neointima, and synthesis and secretion of ECM proteins, including collagen, elastin, and proteoglycan causes the intima of the conduit arteries to thicken and can predict higher risk for developing atherosclerosis (Orlandi *et al.*, 2006). Impaired NO and endothelin production impacts on the vasoactive resistance arteries. However, peripheral vascular resistance is the main determinant and a better predictor of cardiovascular risk in subjects younger than 50, but large artery stiffness is the more important in older subjects (Gerhard *et al.*, 1996).

1.1.4 Atherosclerosis

Atherosclerosis is a form of arteriosclerosis, distinguished by the formation of an atheroma or plaque. It is a slow, progressive disease that occurs mainly in large and medium sized vessels. It is characterised by a chronic inflammatory response in the walls of arteries and involves the generation of an obstructive plaque that can result in additional cardiovascular disorders, including a critical reduction in coronary blood flow, and subsequent myocardial ischaemia (Spyridopoulos and Andrés, 1998).

Endothelial dysfunction is considered an early marker of atherosclerosis. A damaged endothelium, either physical or biochemical, results in increased endothelial permeability, a chronic inflammatory process accompanied by a loss of antithrombotic factors and an increase in vasoconstrictor and prothrombotic products (Hadi *et al.*, 2005). Increased levels of endothelin-1, thrombin and platelet-derived growth factors (PDGF), along with reduced levels of NO and prostayclin result in platelet adhesion and aggregation and exacerbated SMC proliferation and migration. These responses are accompanied by accumulation of new extra cellular matrix (ECM) by the VSMCs (Davignon and Ganz, 2004).

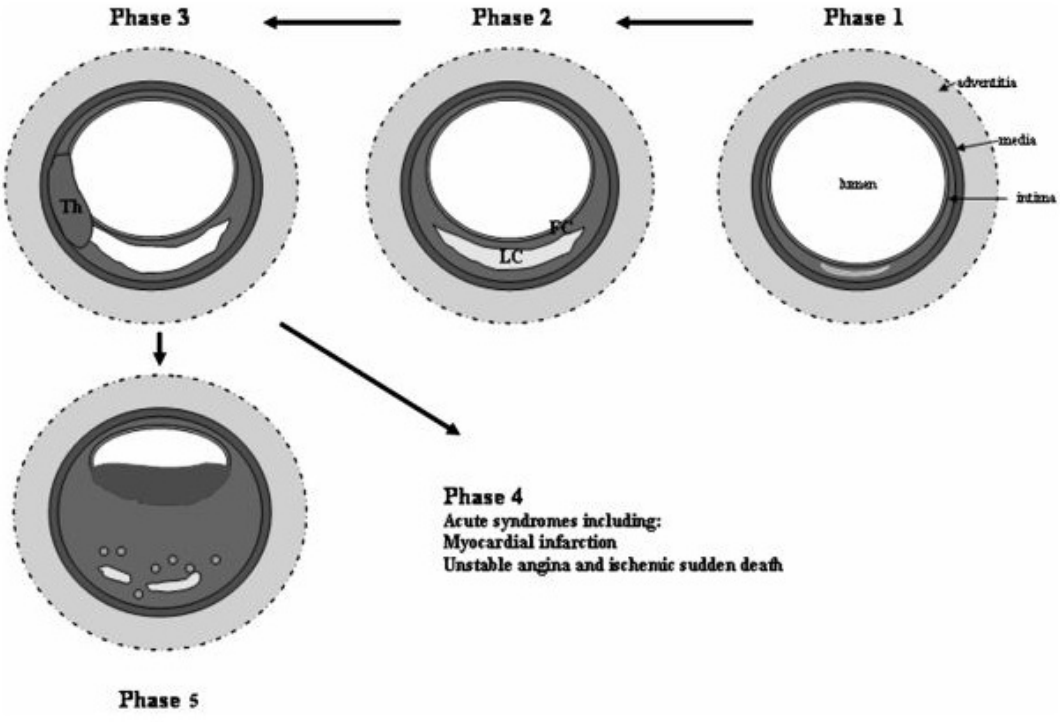


Figure 1.2: Development of Atherosclerotic Lesion (Stoneman and Bennett, 2004) (LC- lipid core, FC- fibrous cap, Th-thrombus)

Fatty streaks are the earliest visible atheroschlerotic lesions and consist of a region of intimal thickening (Figure 1.2: Phase 1). Intimal thickening is due to the accumulation of cellular and extracellular substances in the space between the EC lining and the underlying medial VSMCs (Spyridopoulos and Andrés, 1998). Droplets of lipids are also present in these intimal lesions, which can be found in children at an early age. However, not all initial lesions develop into fatty streaks, and not all fatty streaks develop into advanced lesions (Consigny, 1995). Low-density lipoproteins (LDLs) penetrate the subendothelium due to the increased permeability of the damaged endothelium. The LDLs may bind to proteoglycans and undergo oxidation by free radicals. These oxidised LDLs (oxLDLs) attract circulating monocytes. Further lipid accumulation in the cytoplasm of macrophages gives rise to lipid-laden foam cells. Different cell types,including ECs, platelets, and inflammatory cells release mediators, such as growth factors and cytokines that induce multiple effects including phenotype change of vascular smooth muscle cells (VSMC) from the quiescent contractile phenotype state to the active synthetic state, that can migrate and proliferate from media to the intima (Cai, 2006).

As a lesion develops, smooth muscle cells recruited from the media and subendothelium, embedded in connective tissue, form a fibrous cap over the lesion (Figure 1.2: Phase 2). Plaques can be defined as stable or vulnerable. Stable plaques have a high VSMC and collagen content and a limited amount of lipid-laden inflammatory cells. This subsequently forms a thick, protective fibrous cap, and while their may be an increase of stenosis or narrowing of the blood vessel, a stable plaque is clinically not as serious as a vulnerable plaque (Figure 1.2: Phase 5). Vulnerable

plaques, containing a large lipid core and a thin fibrous cap with less number of VSMCs, are prone to rupture on the the shoulder region of the lesion, in which macrophages are concentrated (Stoneman and Bennett, 2004) (Figure 1.2: Phase 3). The components are released, where they are free to contact with blood clotting factors and form a thrombus. This thrombus can form an embolism, which is carried elsewhere, causing acute coronary syndromes (Figure 1.2: Phase 4) (Stoneman and Bennett, 2004). Together with apoptosis (programmed cell death), proliferation and migration of VSMC are vital to the pathogenesis of atherosclerosis and plaque rupture. Rupture of the plaque is associated with increased fibrous cap macrophage, increased VSMC apoptosis, and reduced fibrous cap VSMC. VSMCs are the only cells capable of synthesizing structurally important collagen isoforms, and the apoptosis of VSMC might promote plaque rupture (Walsh *et al.*, 2000).

Angioplasty and Restenosis

Angioplasty is the technique of mechanically widening a narrowed or obstructed blood vessel, typically as a result of atherosclerosis. While generally successful in the short-term, restenosis or re-occlusion of the vessel is a major obstacle in maintaining an open lumen and may result in the need for further surgery. Mechanical injury to the vessel leads to a thrombogenic and proliferative phase which is typified by VSMC proliferation, neointimal hyperplasia and eventual remodelling with pathological changes in the cellular and protein content of the media and adventitia (Ellozy and Carroccio, 2003).

During angioplasty, plaques are compressed against the arterial wall by an angioplasty balloon (Figure 1.3: left vessel). To maintain the result

of the opened lumen and to prevent the closure of the lumen by fragmented or dissected plaques, stents, which are tubular metallic scaffolding tubes, are usually placed in the arteries (Figure 1.3: right vessel.). However, restenosis may still possibly occur. The use of drug-eluting stents (DES) is a significant development in the prevention of restenosis. The DES is designed to release pharmacological agents after deployment to inhibit the response to injury mainly responsible for restenosis. A coating on the surface of the stent struts which contains some drugs can inhibit the growth of neointimal tissues (Luscher *et al.*, 2007).

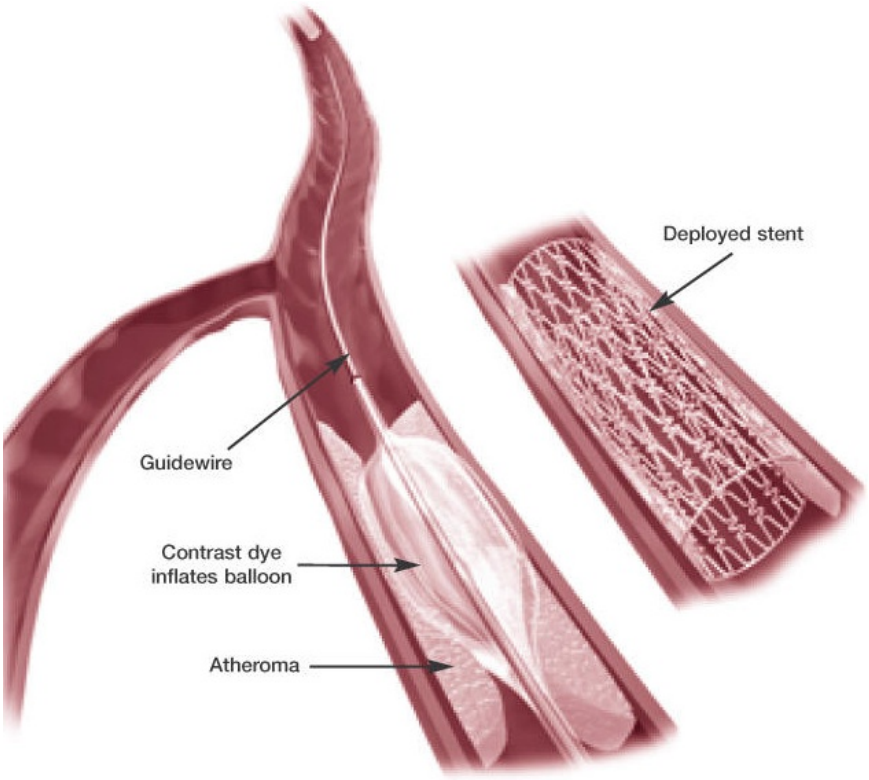


Figure 1.3: Stent Angioplasty (Ashley and Niebauer, 2004)

1.2 Cell Fate

1.2.1 Vascular remodelling

Vascular remodelling describes any enduring change in the size or composition of a blood vessel and may occur to accommodate to changes in haemodynamic forces or as a response to inflammation or injury. Remodelling is usually an adaptive process, but it may subsequently contribute to the pathophysiology of vascular diseases and circulatory disorders (Gibbons and Dzau, 1994). Altered VSMC contraction and relaxation, along with changes in cell fate (proliferation, apoptosis, migration and differentiation) result in structural remodelling. VSMC proliferation can cause medial thickening, while the adventitial layer is modulated by changes in fibroblast growth. Impaired production and degradation of extracellular matrix and stimulation of inflammatory responses also induce a remodelling response and compromise the compliancy of the vessel (Touyz, 2005).

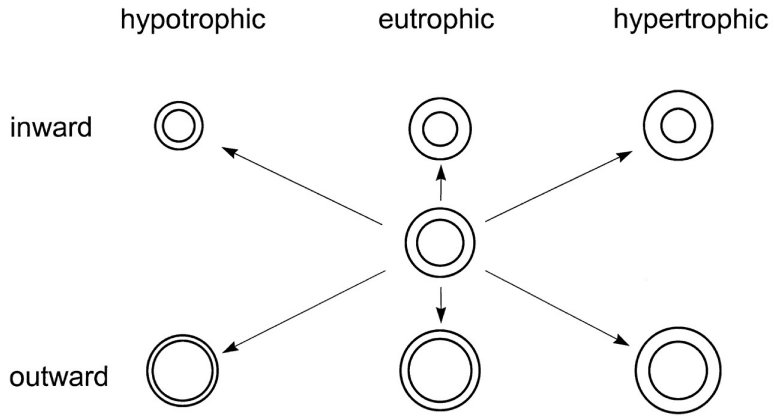


Figure 1.4: Vascular remodelling (Mulvany, 1999)

In inward eutrophic remodelling, in which outer and lumen

diameters are decreased, the media cross-sectional area is unaltered (Figure 1.4). Therefore, while stenosis occurs, stiffening of the artery is avoided.These vessels have the same number of smooth muscle cells and little evidence of cell hypertrophy. It is possible that VSMC growth is offset by levels of apoptosis (Schiffrin, 2004). However, with inward hypertrophic remodelling (Figure 1.4), the media growth encroaches on the lumen, causing stenosis. Inward remodelling, as described above, is generally observed in the resistance vessels of mild hypertensive patients and differs from that seen in large arteries (Mulvany, 1999). Increased medial thickness, due to remodelling results in a decrease in compliance of larger arteries and the progression of arteriosclerosis (Intengan and Schiffrin, 2001).

Following vascular injury, neointimal hyperplasia of conduit arteries occurs. VSMCs migrate into the neointima and play a principal role in atherogenesis by proliferating and synthesizing a cascade of molecules. Adaptive outward remodelling, however, where the medial growth is outward with lumen size increase, can compensate for progressive growth of atherosclerotic plaques, thus postponing the development of flow-limiting stenosis (Ward *et al.*, 2000).

1.2.2 Proliferation

Altered cell fate decisions, including proliferation and apoptosis, are critical in a number of disease states. Adult VSMCs are not terminally differentiated and have the ability to change phenotype in response to changes in local environment through the expression of contractile and synthetic genes. Proliferation of VSMCs contribute to the development of neointimal lesions in atherosclerosis, and hypertension-related

arteriosclerosis. Restenosis after vascular injury is also characterised by a VSMC hyperplastic response. Production of extracellular components by VSMCs contribute further to the remodelling event (Touyz, 2005). Growth factors that induce VSMC proliferation and are upregulated in atherosclerotic lesions and the neointima following angioplasty include PDGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor(IGF)-1 (Abid *et al.*, 2005). The ability of VSMCs to respond to growth factors may be dependent on changes in specific ECM components and integrins through regulation of cyclin dependent kinases inhibitors (CKIs) (Spyridopoulos and Andrés, 1998). MMPs are upregulated in atherosclerotic lesions and after angioplasty and have been shown to regulate growth factor availability, thereby promoting proliferation (Newby, 2006)

A number of transcription factors that are known mediators of VSMC proliferation have been linked to neointimal formation. Activation of NF-κB is thought to play a major role in atherosclerosis and inflammatory diseases (Monaco and Paleolog, 2004) and is known to induce proliferation of VSMCs (Hoshi *et al.*, 2000). Thrombin has been shown to mediate activation of NF-κB leading to proliferation of human VSMCs (Nakajima *et al.*, 1994). Other transcription factors involved in VSMC proliferation and that have been identified as inducing neointimal hyperplasia include c-myc (De Nigris *et al.*, 2003) and mitochondrial transcription factor A (mTFA) (Yoshida *et al.*, 2005).

An important inhibitor of VSMC proliferation is NO (Costa and Assreuy, 2005). In animal models of arterial injury and vein grafts, NOS activity and NO release are markedly altered (Jeremy *et al.*, 1999). NO is a flow-dependent inhibitor of VSMC proliferation, highlighting the

importance of haemodynamic forces on changes in VSMC proliferation (Boo and Jo, 2003).

The Cell Cycle

The cell-cycle consists of an orderly set of phases, during which specific subsets are transcribed and assembled. The mitotic (M) phase of the cell cycle is relatively brief and involves nucleur and cytoplasmic division. Interphase begins with the Gap 1 (G_1) phase, where biosynthesis within the cell increases at a high rate. This includes the synthesis of enzymes required for the S phase of interphase. At this point, DNA synthesis occurs, resulting in replication of the chromosomes. The cell enters G_2 phase, where rapid protein synthesis is carried out. This is the last stage in the interphase, before the M phase begins. Depending on environmental and developmental signals, cells may enter the G_0 Phase, directly afer G_1 phase. This is a quiescent state and cells may remain in this state for long periods of time (van den Heuvel, 2005). The passage of a cell through the cell cycle is controlled by cytoplasmic proteins called cyclins, which bind to and activate specific-cyclin dependent kinases (CDKs). The cell cycle is therefore carefully regulated through the levels of cyclins at specific stages of the cycle (Koledova and Khalil, 2006). The cyclin levels are controlled both transcriptionally and by ubiquitin-dependent proteolytic machinery (Spyridopoulos and Andrés, 1998). The D-type cyclins (D1, D2, and D3) and their catalytic partners CDK4 and CDK6 act early in G_1 phase. These are controlled by mitogen signals, including ERK1/2 (Sherr and Roberts, 2004). Cyclin A/CDK2 complexes are required for DNA synthesis (S Phase). Cyclin A/CDK1 and cyclin B/CDK1 pairs are then assembled and activated during the G_2

phase and M phase. CDK inhibition may occur through association with CKIs that include p15, p16, p18. p19 and p21 among others (Spyridopoulos and Andrés, 1998).

1.2.3 Apoptosis

Apoptosis, or programmed cell death, is vital for normal cell turnover. Impaired apoptosis, however, where there is too much or to little, is a factor in many conditions including neurodegenerative diseases and cancers, and is critical in the pathophysiology of atherosclerosis, vascular remodelling, and other cardiovascular disorders. Cell growth, the balance between proliferation and apoptosis, of VSMCs is an important factor to consider in studying vascular disease.

In atherosclerosis, apoptosis is a double-edged sword and SMCs isolated from atherosclerotic plaques have been shown to be more susceptible to apoptosis (Bennett *et al.*, 1995). Initially, apoptosis can prevent lesion progression. However, in an advanced plaque apoptosis of VSMCs promotes coagulation and inflammation. Plaque SMCs undergoing apoptosis have the same potency to generate thrombin as platelets. Modified LDLs can hinder phagocytosis of apoptotic VSMCs which release and pro-coagulant factors inflammatory cytokines, as they undergo secondary necrosis (McCarthy and Bennett, 2000). Apoptotic cells expose phosphatidylserine on the surface early in the process. In the presence of factors V and VII, exposed PS can then act as a substrate to thrombin generation. Thus, apoptosis of smooth muscle cells in primary atherosclerotic plaque could be detrimental for plaque stability and increase the risk for thrombosis (Kockx and Herman, 2000). Apoptosis of VSMCs within a progressive plaque also reduces the stability of the

fibrous cap due to the loss of ECM, particularly collagen type I, produced by the VSMCs and, thus, potentially leads to thrombosis and occlusion of the vessel (Davies, 1996). Macrophages may promote apoptosis of VSMCs through production of death ligands on their surface (Boyle *et al.*, 1998) or secretion of pro-apoptotic cytokines, including TNF and IL-1 (Rakesh and Agrawal, 2005). TGF-β and the transcription factor p53 are important players in promoting apoptosis of VSMCs. Conversely, bFGF and other growth factors, including PDGF. bFGF and IGF-1 and transcription factors, such as c-myc, promote survival of VSMCs (Rakesh and Agrawal, 2005).

In arterial injury in animal models, apoptosis of medial VSMCs occurs rapidly within 30 min of injury (Perlman *et al.*, 1997). This first wave of apoptosis may be associated with the redox-sensitive activation of stress-activated protein kinases (Pollman *et al.*, 1999). As repair occurs, intimal VSMC apoptosis is induced and medial VSMC apoptosis is also observed (8-21 days in a rat carotid artery model (Bochaton-Piallat *et al.*, 1995)). It has been hypothesized that the early wave of medial VSMC apoptosis may exacerbate late neointima formation by provoking a greater wound healing response to overcome the cellular deficit and that late apoptosis may limit lesion growth (Walsh *et al.*, 2000). Specimens from human patients with in-stent restenosis also display abundant apoptotic cells (Kearney *et al.*, 1997) and neointima cells are more sensitive to the induction of apoptosis than media VSMCs (Erl, 2005). Moreover, populations of SMC are phenotypically distinct within a given blood vessel and also between blood vessels of the same vascular bed and in vivo, sub-populations of SMCs within the same tissue express different levels of caspase 3 and show different sensitivities to apoptosis

(Chan *et al.*, 2000).

Overall, our understanding of the mechanisms of VSMC apoptosis can aid us in finding new therapeutic targets to combat vascular disease.

The Apoptotic Pathways

During early apoptosis, chromatin compaction, nuclear condensation and fragmentation and also cell shrinkage occurs (Mignotte and Vayssiere, 1998). This is followed by the separation of cell fragments into apoptotic bodies. Phosphatidylserine (PS) is exposed on the surface of the cell, allowing recognition by non-inflammatory macrophages and phagocytosis of the apoptotic body. No inflammatory reaction occurs as there is no production of anti-inflammory cytokines and no release of cellular constituents. This differs from necrosis, or oncosis which is the process leading up to necrosis. Cell swelling, disruption of the cell membrane, release of cytoplasmic contents and recruitment of inflammatory cells are all hallmarks of oncosis (Majno and Joris, 1995) .

The activation stage of apoptosis can be triggered by a number of stimuli that are either extrinsic or intrinsic. The extrinsic signalling pathway involves death receptors of the tumor necrosis factor receptor (TNFR) family that are activated by ligand binding, resulting in the transmission of a death signal from the cell surface to the intracellular signalling pathway (Figure 1.5). Extracellular signals may include hormone, growth factors, nitric oxides or cytokines. Examples of the ligand-receptor interaction are FasL/FasR and TNF-α/TNFR1. These both involve recruitment of cytoplasmic adapter proteins e.g. FADD to FasR, which bind to the receptor through their death domain. FADD then associates with pro-caspase 8, resulting in the formation of a

death-inducing signalling complex (DISC), and allowing activation of pro-caspase 8 and the beginning of the execution pathway (Elmore, 2007).

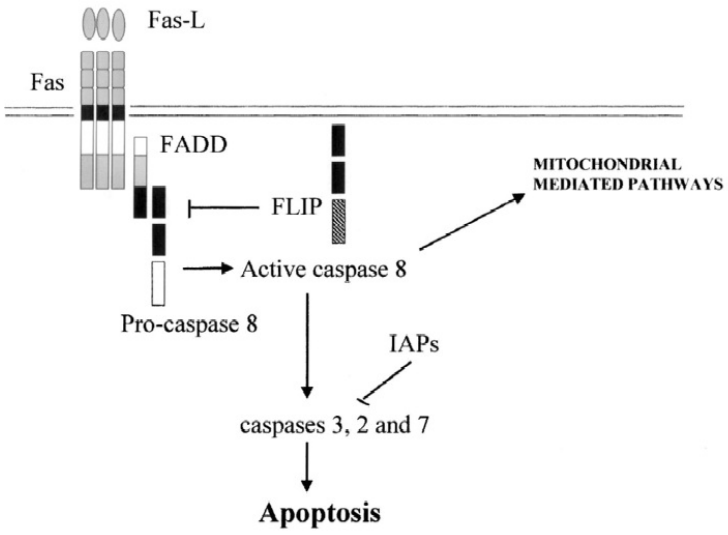


Figure 1.5: Extrinsic Apoptotic Pathway (McCarthy and Bennett, 2000)

The intrinsic pathway is non-receptor meditated, but results in an intracellular signal. Radiation, hypoxia and viral stimulants that cause DNA damage can positively induce apoptotic signalling through the intrinsic pathway. Removal of growth factors, cytokines and hormones can remove apoptotic suppression and allow transduction of the intracellular signal. Changes in the inner mitochondrial membrane occur that allow the release of cytochrome c and other pro-apoptotic proteins from the mitochondria into the cytosol (Figureřefintrinsic). Cytochrome c binds to Apaf-1 and caspase-9, allowing its activation and the beginning of the execution pathway, similar to the extrinsic apoptotic pathway (Rossi and Gaidano, 2003).

The execution pathway involves the activation of caspase-3, caspase-6 and caspase-7 (Nicholson and Thornberry, 2003). This pathway is tightly

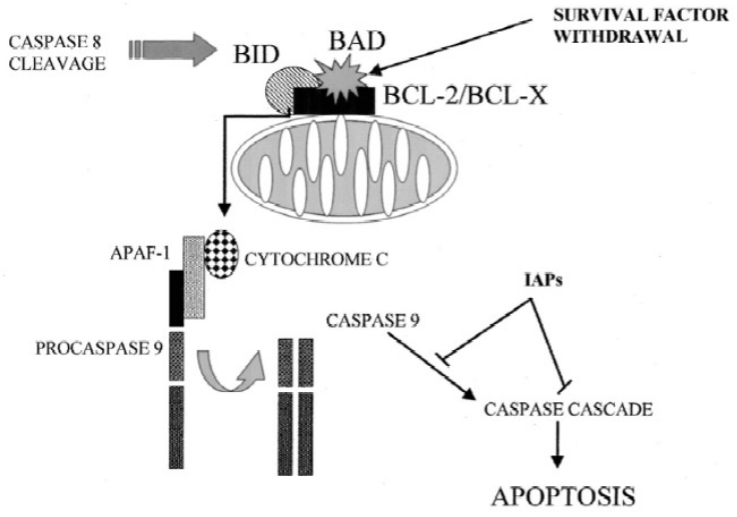


Figure 1.6: Intrinsic Apoptotic Pathway (McCarthy and Bennett, 2000)

regulated by the Bcl-2 protein family, which includes a group of antiapoptotic proteins, such as Bcl-2, and proapoptotic proteins, such as Bax. These proteins act upstream of caspase-3 and the relative levels of the pro-and anti- apoptotic proteins determine the susceptibility of cells to a death signal (Burlacu, 2003). Activation of the proteolytic caspases results in the organised degredation of cellular organelles by the caspases, and subsequent phagocytosis by macrophages.

1.3 Haemodynamic Forces

Blood vessels are permanently subjected to the haemodynamic forces of pulse pressure and shear stress. Shear stress, the frictional force of blood flow against the vessel wall, acts in parallel to the surface of the vessel and is primarily sensed by endothelial cells (Figure 1.7). Shear stress is known to be atheroprotective and plaques are known to occur in areas where there

is reduced shear stress (Cunningham and Gotlieb, 2004).

Shear stress also impacts indirectly on VSMCs through the release of vasoactive mediators and growth factors by the endothelium (Kraiss *et al.*, 1991). In diseased states, the intima may be denuded of the endothelium and in this instance, shear stress may exert a direct effect on VSMC proliferation and migration (Ueba *et al.*, 1997), (Qi *et al.*, 2008).

Pulse pressure creates radial and tangential forces and effects all cell types within the vasculature. (Lehoux *et al.*, 2006) (Figure 1.7). An increase in mean and/or pulse pressure stretches the vessel wall, a change which is sensed directly by the VSMC, leading to changes in its contractile state and/or its synthetic activity. While biomechanical stretch at physiological levels is essential to develop and maintain organic structure and function, altered mechanical stretch may result in pathological conditions. Pulsatile shear stress and uniaxial circumferential stretch exists in the straight part of the arterial tree. Disturbed flow and relatively undirected stretch are seen at branch points and other regions of complex geometry (Chien, 2007). Atherosclerotic plaques occur preferentially at bifurcations and curvatures of arterial blood vessels, where there is irregular hemodynamic shear stresses (Wernig and Xu, 2002).

Remodelling of arteries as they grow, age or become diseased leads to changes in their composition, their geometry and the manner in which mechanical forces are distributed within their walls. These changes can lead to altered haemodynamics and possible further remodelling, with an exacerbation of disease states.

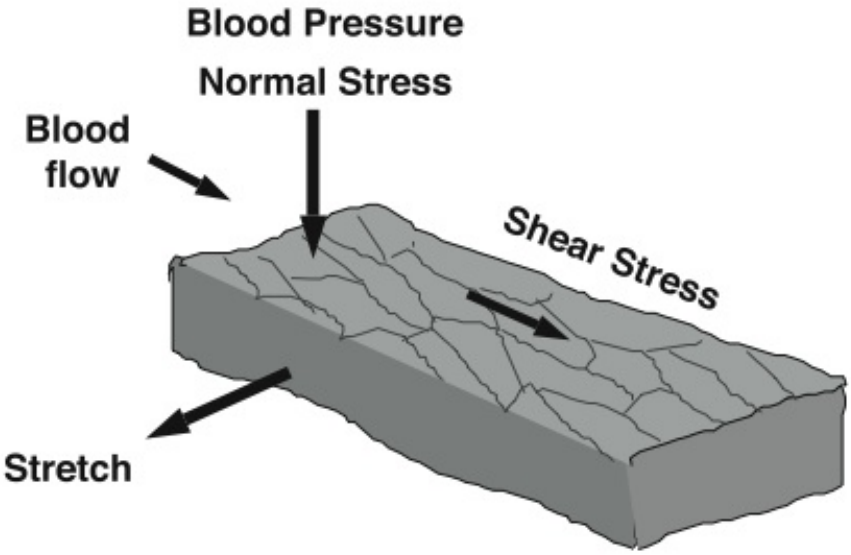


Figure 1.7: Diagram of Haemodynamic Forces (Chien, 2007)

1.3.1 Cyclic Strain

The pulsatile nature of the cardiac output imposes a cyclic pressure and strain on the arterial wall. Arteries undergo small pulsatile circumferential deformations relative to the deformed state caused by the mean pressure and *in situ* axial extension (Cowin and Humphrey, 2001), and physiological stretch of the human aorta has been reported to be 9-12% under normotensive conditions (Chapman *et al.*, 2000). .

The cyclic stretch in response to pulse pressure, ΔP, is determined by arterial compliance defined as

$$C = \frac{\Delta r_i}{r_i \Delta P}$$

where r_i is the inner radius at mean pressure and *in situ* length, and Δr_i is the change in radius (Cowin and Humphrey, 2001).

In hypertensive patients, increased mean blood pressure may occur

through altered compliance of the conduit arteries and impaired vasodilation of the resistance arteries. Both of these factors are hallmarks of ageing (Nicita-Mauro *et al.*, 2007), (Dohi *et al.*, 1995) and contribute to greater stretch of the conduit arteries. Additionaly, localised abnormalities such as bifurcations and plaques can also lead to altered mechanical stretch (Kaazempur-Mofrad *et al.*, 2003).

Altered mechanical forces can contribute to pathological changes of the vessel by inducing VSMC proliferation or apoptosis. Cyclic strain can impact both indirectly on VSMCs, by signalling through endothelial cells, or by directly effecting the biomechanical sensors of VSMVCs (Lacolley, 2004). There have been conflicting reports, however, on the effect of stretch on proliferation of VSMCs. A number of studies have shown an anti-proliferative effect of stretch on VSMCs (Sumpio and Banes, 1988), (Morrow *et al.*, 2005b). Rhythmical stretch was found to be important to keep the rate of DNA synthesis and thereby the proliferation of VSMCs at a low level (Hipper and Isenberg, 2000). Exposure of rat aortic VSMCs to cyclic stretch blocked the proliferative effect of PDGF and thrombin (10%, 1 Hz) in a p21-dependent manner and caused an arrest of cells in the G1 phase of the cell cycle (Chapman *et al.*, 2000). Interestingly, this same study, found that both types of cyclic stretch, uniaxial, and equiaxial caused an increase of cells in the fraction of cells in the G_0/G_1 phase of the cell cycle. SMCs in the walls of most arteries are orientated in the circumferential direction, whereas the motion of the wall occurs predominantly in the radial direction so that SMCs experience a type of unaxial stretch. Other studies have found the opposing effect of stretch on VSMC proliferation. Li, et al. and Birukov et al. both found a positive effect of stretch on proliferation in rabbit aortic VSMCs (Li *et al.*, 1997),

(Birukov *et al.*, 1995). Bovine aortic smooth muscle cells also showed enhanced proliferation with 0-7% strain compared to static control (Mills *et al.*, 1997).

A number of factors have been identified as critical to the proliferative response of VSMCs to stretch. The proliferative response due to cyclic stretch of SMC obtained from small resistance arteries was significantly greater then that of VSMCs obtained from large conduit arteries (Nakamura *et al.*, 1998). Studies have also found that stretch potentiates proliferation in VSMCs obtained from venular but not arterial blood vessels (Dethlefsen *et al.*, 1996). Opposing results have also been found from arterial cells cultured from newborn and adult animals (Wilson, 1993), (Seifert *et al.*, 1984). Additionally, the phenotype of VSMCs has been found to dictate the growth response to pulse pressure in vitro (Cappadona *et al.*, 1999). As there is evidence to show that there are heterogeneties between SMCs within a given blood vessel (Owens *et al.*, 2004), this is of important significance when considering the proliferative profile of VSCMs. Together, these studies suggest that the source of the VSMCs must be considered when studying the prolfiferative response of VSMCs to changes in haemodynamic stretch.

Lack of haemodynamic forces triggers apoptosis in vascular endothelial cells (Kaiser *et al.*, 1997). However, a number of studies have shown that stretch induces apoptosis in VSMCs *in vitro* (Sedding *et al.*, 2008), (Morrow *et al.*, 2005a), (Sotoudeh *et al.*, 2002). Increased apoptosis occurs in SMC cultured from spontaneously hypertensive rats compared with normotensive control rats (Hamet *et al.*, 1996). Further study is required, however, to understand the mechanisms involved in stretch-induced apoptosis.

1.3.2 Mechanotransduction

Endothelial and vascular smooth muscle cells have the ability to convert mechanical signals into a biochemical response and do so through receptors on the cytoskeleton and other structural components. Mechanical stresses may directly perturb the cell surface or alter receptor conformation, thereby initiating signalling pathways usually used by growth factors (Zou *et al.*, 1998). In vascular smooth muscle cells, these mechanosensors include integrins, G-protein and G protein-coupled receptors, tyrosine kinase receptors and ion channels. The intracellular pathways of protein kinase C and mitogen-activated protein kinases (MAPK) are also activated in response to mechanical stimuli (Li and Xu, 2000).

Integrins

Integrins allow attachment of a cell to the extracellular matrix and other cells. Their covalently bound subunits, α and β, consists of a single transmembrane domain, a large extracellular (EC) domain and a small cytoplasmic domain. The EC domain binds to a wide variety of ECM ligands, including fibronectin, vitronectin, laminin and collagen (Katsumi *et al.*, 2004). Twenty different integrin receptors can be formed. Heterodimerization of the different combinations of the fifteen α and eight β subunits allows the formation of twenty different integrin receptors. Clustering with other bound integrins results in the formation of highly organized intracellular complexes, known as focal adhesions, that are connected to the cytoskeleton. $\beta 1$ and $\beta 3$ integrins have been shown to have a role in adhesion and migration of vascular smooth muscle cells (Clyman *et al.*, 1992), (Lee *et al.*, 2006) and are sensitive to

mechanical strain in VSMCs (Wilson *et al.*, 1995). In contrast to the unidirectional nature of GPCRs and receptor PTKs, integrin signalling is bidirectional with ”outside-in” signalling involving interaction of integrins with the extracellular matrix (ECM) rather than with soluble ligands (Sugden, 2003).

Ion Channels

Cyclic strain-sensitive ion channels, including Ca^{2+} and Na^{+} channels increase their frequency of opening following cyclic strain. This results in a transient increase in intracellular Ca^{2+}, Na^{+} and other divalent cations, and membrane depolarization. This may have implications of vascular remodelling where changes in cell fate of vascular smooth muscle cell occurs. Increased cytosolic levels of calcium stimulates quiescent cell entry into the cell cycle, and drives proliferating cells (Landsberg and Yuan, 2004).

Receptor Tyrosine Kinsases

Receptor tyrosine kinases (RTKs) are high affinity cell surface receptors that include the VEGF, PDGF, and insulin receptor families. Mechanical stretch has been shown to increase both vascular endothelial growth factor (VEGF) expression and phosphorylate the VEGF receptor in VSMC (Smith *et al.*, 2001). PDGF receptors α and β are reported to be up-regulated by mechanical stress. Binding of platelet-derived growth factors (PDGF)s to their plasma membrane receptors leads to auto-phosphorylation of the tyrosine residues in the the PDGF receptor kinase domain. This leads to sequential phosphorylation events and activation of MAPK cascades (Li and Xu, 2000).

G protein-coupled receptors

G protein-coupled receptors are characterised by seven transmembrane segments. G protein-coupled receptors are activated by an external signal in the form of a ligand or other signal mediator such as cyclic strain. This creates a conformational change in the receptor, causing activation of a G protein (Weis and Kobilka, 2008). Small guanine nucleotide binding proteins (small G proteins) or GTPases include five families of Ras, Rho, Rab, Sar1/ADP ribosylation factor and Ran, which oscilate between inactive GDP-bound G-proteins and active GTP-bound G proteins. Growth factors, cytokines and G protein-coupled receptors (GPCRs) allow activation of G proteins, and GDP association inhibitors and GTP-ase activating proteins cause the small G protein to remain in its inactive GDP-bound state. G-proteins have been shown to have important downstream effects in VSMCs. Ras promotes cell proliferation through activation of the Raf-MEK-ERK cascade (McCubrey *et al.*, 2007). RhoA, through activation of ROCK (Rho kinase), is involved in increased sensitivity of VSMC (Loirand *et al.*, 2006) and reduced p21Cip1 or p27Kip1 results in Rho-induced cell proliferation in the vasculature of hypertensive rats (Seasholtz *et al.*, 2001).

Intracellular signalling Pathways

There are a number of second messengers that are activated following changes in haemodynamic forces. These include activation of the MAPK, Protein Kinase C, PI3K/Akt and NF-$\kappa\beta$ Pathways. There are five families of MAPKs, but each have similar features in the pathway. A MAPKs is activated, through phosphorylation by a MAPKK (MAPK kinase). MAPKKs are also known as MEK or MKK and examples of a MAPKKs

are MKK1 and MKK2. MAPKKs are activated through dual phosphorylation on a threonine and tyrosine residue adjacent to a proline, by a MAPKK kinase such as A-RAF, B-Raf and Raf-1. These are serine/threonine kinases, which are stimulated by extracellular stimuli and react with Ras/Rho and PKC (protein kinase C). The five families of MAPK are ERK 1/2 (extra-cellular signal regulated kinases), JNKs 1,2,3,(c-Jun amino-terminal kinases), p38/SAPK (stress activated protein kinase) isoforms (α,β,γ, δ), ERKs 3,4 and ERK 5. ERK 1/2 mainly respond to growth factors, while JKNs and p38 are mainly sensitive to stress stimuli. Mechanical strain activates p38, JNKs and ERK1/2 in vascular smooth muscle cells (Tock *et al.*, 2003) and ERK 1/2 and p38 have been shown to be pro-proliferative in VSMC in a Ras/rac dependent manner (Li *et al.*, 2000). Atherosclerotic lesions show requirement for JNK2 (Ricci *et al.*, 2004) and treatment of macrophages with JNK or p38 MAPK inhibitors blocks oxLDL-induced foam cell formation (Muslin, 2008). Acute hypertension and balloon angioplasty induces expression and activation of ERK 1/2, JNKs, in rat vascular models, (Hu *et al.*, 1997), (Xu *et al.*, 1996). p38 has also been implicated in the response to balloon angioplasty and neointimal hyperplasia after vascular injury (Ohashi *et al.*, 2000), (Ju *et al.*, 2002).

The protein kinase C (PKC) is a large family of serine/threonine kinases containing several isoforms divided into three groups based on their requirements for Ca^{2+} and diacylglycerol (DAG) for activation (Yan *et al.*, 2006). These groups are the conventional (cPKCs), the novel (nPKCs) and the atypical (aPKCs) The protein kinase C (PKC) family is activated by a large number of extra-cellular signals and, in turn, modify the activity of numerous targets such as cytoskeletal proteins, MAPK and

transcription factors (Li and Xu, 2000). Cyclic strain activation of PKC has been shown to promote VSMC proliferation (Mills *et al.*, 1997) and migration (Li *et al.*, 2003). Another study demonstrated that PKC-δ promotes VSMC proliferation and migration through the activation of ERK1/2 (Liu *et al.*, 2007). A number of PKC isoforms have been linked to hypertension (Salamanca and Khalil, 2005) and PKC-β has been shown to have a central role in atherosclerosis and restenosis after injury (Andrassy *et al.*, 2005), (Harja *et al.*, 2005).

1.4 Notch Signalling Pathway

The Notch Signalling Pathway is a developmental pathway, that has since been identified as an important regulator of vascular smooth muscle cell fate (Morrow *et al.*, 2005a). Tight control of Notch Signalling is essential to maintain physiological levels of downstream activity. Shaggy, the Drosophila homolog of GSK-3β, is known to act downstream of Notch (Ruel *et al.*, 1993) and GSK-3β has been shown to phosphorylate and regulate Notch IC. In this study, we examine the role of GSK-3β in Notch Signalling in VSMCs (Foltz *et al.*, 2002).

1.4.1 The Role of the Notch signalling Pathway

The Notch gene was originally identified in Drosophila where it was isolated originally as a neurogenic gene. Embryos lacking Notch gene function showed an increased number of neuroblasts at the expense of epidermal precursors (Artavanis-Tsakonas *et al.*, 1999) and much of our understanding of the Notch signalling Pathway comes from studies on Drosophila Notch, as well as the Notch-related genes lin-12 and glip-1 in

Caenorhabditis elegans (Weinmaster, 1997). Notch regulates multiple cell-cell interactions and such diversity is generated through the activation of different notch like receptors. In 1992, a second mammalian gene, Notch2, which encoded a protein that contained all the structural motifs characteristic of a Notch protein, was identified (Weinmaster, 1992). Notch 3 and Notch 4 were later identified, showing specificity in vascular smooth muscle and endothelial cells, respectively. These Notch proteins display both overlapping and distinct tissue distributions as well as both redundant and distinct functions (Wu and Bresnick, 2007)

Notch in the Vasculature

Notch3 is predominantly expressed in adult arterial smooth muscle cells in humans (Joutel *et al.*, 2000) and genetic disorders such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) and Alagille Syndrome demonstrate a crucial role of the Notch pathway in vascular development. A Notch3-null mouse is viable and fertile but and absence of Notch3 results in enlarged arteries with abnormal distribution of elastic laminae (Mason *et al.*, 2005). CADASIL is caused by mutations in the Notch receptor, Notch 3, gene and is the most common form of inherited stroke and vascular dementia in humans (Gridley, 2007) and is characterised by systemic VSMC degeneration (Wang *et al.*, 2008). The mutations are located within the EGF repeats in the extracellular domain of the Notch3 gene with a strong clustering of the mutations observed within exons 3 and 4 (Joutel *et al.*, 1997). Cerebral vessels are consistently narrowed by intimal thickening and expansion of the extracellular matrix in CADASIL. This is accompanied by widespread disruption and degeneration of smooth

muscle cells in vessel wall and the deposition of the granular osmiophilic material (GOM). Karlstrom et al. has suggested that Notch3 mutations causes reduced S1 site cleavage, which results in lower amounts of mature heterodimeric mutant receptor to be presented at the cell surface (Karlstrom *et al.*, 2002) CADASIL-mutation carrying cells in culture maintained in viable conditions undergo normal physiological signalling. It is possible that differences in mutant and wild type would surface if the cells were subjected to mechanical or chemical stress, as it constantly occurs in the cerebral vasculature with increasing age (Kalaria *et al.*, 2004).

In humans, mutations in the Notch1 locus result in a spectrum of heart defects. The most prevalent malformations are bicuspid aortic valve disease and calcification of the aortic valve (Garg, 2006). Alagille Syndrome is an autosomal dominant disorder characterized by development abnormalities of a number of organs including the liver, heart, and eye and most cases are caused by the Notch ligand, Jagged 1, mutations (Kamath *et al.*, 2004).

Angiogenesis

The Notch signalling pathway is important in the regulation of blood vessel sprouting and branching during both normal development and tumor angiogenesis. The Notch ligand Dll4 has been shown to regulate blood vessel sprouting and branching through the vascular endothelial growth factor A (VEGFA) pathway in endothelial cells. During angiogenesis, supporting cells such as pericytes and smooth muscle cells are recruited to the vessels to provide structural support and stability for the vascular walls (Suchting *et al.*, 2007).

Arterial-Vein Differentiation

Notch signalling also has a critical role to play in arterial-venous differentiation (Figure 1.8). Arterial specification of embryonic stem cells has been shown to be VEGF and Notch dependent, while inhibition of Notch signalling or low VEGF levels is permissive for venous differentiation (Lanner *et al.*, 2007). Loss of Notch5 activity, the zebrafish form of Notch, or its target gene gridlock, leads to a loss of the arterial marker EphrinB2 and increase expression of the venous marker EphrinB4 (Wu and Bresnick, 2007). Suppression of Notch signalling by the COUP-TFII transcription factor has been shown to regulate vein identity (You *et al.*, 2005).

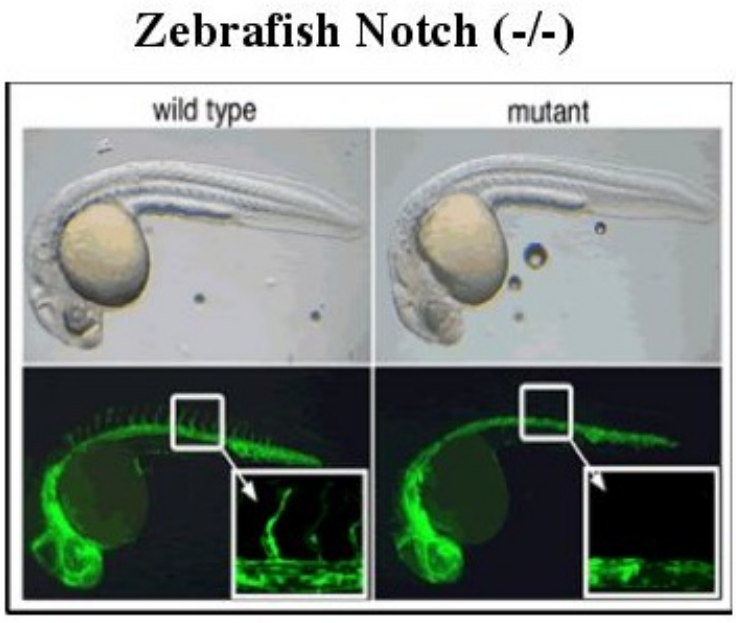


Figure 1.8: Arterial Differentiation in Knockout Notch Zebrafish (Lawson *et al.*, 2002)

Regulation of Cell Fate

Notch signalling has been shown to have an important part to play in the regulation of vascular cell fate in embryogenesis and in adult cells. A role for Notch signalling in repressing smooth muscle cell differentiation during *in vitro* cell culture has been established (Proweller *et al.*, 2005) and expression of the constitutively active form of Notch3 IC was shown to down-regulate the differentiation markers smooth muscle α-actin (SMA), myosin, calponin and smoothlin in human aortic SMCs (Morrow *et al.*, 2005a). Also, Notch1IC or HRT2 expression inhibits myocardin-activated SMC marker gene transcription (Proweller *et al.*, 2005). However, some studies have shown NotchIC/CBF-1 activity inducing SMA expression in a number of cell types. Expression of the constitutive active ICD of human Notch1, Notch2, or Notch4 receptors increased SMA levels in primary human smooth muscle cells. Concurrently this study found that the Notch downstream targets HRT1 or HRT2 repressed basal SMA expression. A negative-feedback pathway, therefore, has been proposed by which expression of HRT1 or HRT2, alone or in combination with Notch activation, suppresses SMA transcript and protein levels. SMA regulation by Notch signalling may depend on a balance between Notch activity and HRT activity (Tang *et al.*, 2008b).

Notch has been shown to be pro-proliferative and anti-apoptotic in vascular smooth muscle cells (Morrow *et al.*, 2005a) and to be modulated by both biomechanical stimuli *in vitro* and following vascular injury *in vivo* (Morrow *et al.*, 2005a), (Wang *et al.*, 2002). Notch may be, therefore, a player in the altered vascular cell phenotype observed in pathophysiological environments, such as vunerable plaques and restenosis after injury.

1.4.2 Canonical Notch signalling

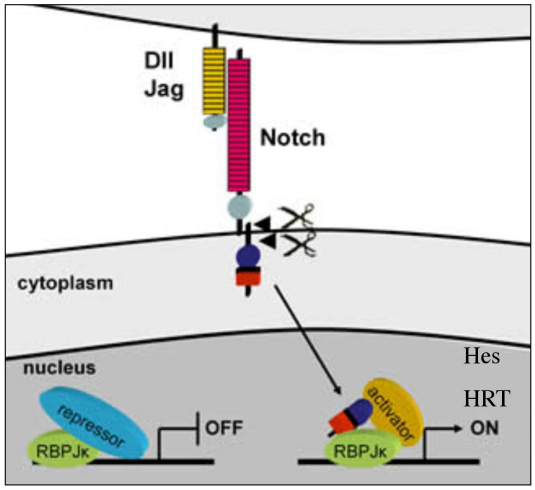


Figure 1.9: Canonical Notch signalling (Fischer and Gessler, 2007)

The canonical Notch signalling pathway involves binding of a Notch ligand from a neighbouring cell to the transmembrane Notch receptor (Figure 1.9). Two proteolytic cleavages release the Notch intracellular domain (NICD) from its plasma membrane tether, allowing it to translocate to the nucleus. In the absence of ligand binding, the extracellular LIN-12/Notch Repeats (LNRs) prevent S2 cleavage. Ligand-induced cleavage at a distinct site (S2 cleavage site) within the Notch extracellular domain (NECD) by transmembrane proteases of the ADAM/TACE family, leaves an activated membrane bound form of Notch. This is followed by further cleavage at an S3 cleavage site, by a γ-secretase/presenilin complex, allowing a release of active NICD, which translocates to the nucleus (Figure 1.9). The NICD forms a complex with a CSL(CBF1/RBP-$_{J\kappa}$, Su(H), Lag-1) protein, displacing a histone deacetylase (HDAc)-co-repressor (CoR) complex from the CSL protein.

Components of an activation complex, including MAML and histone acetyltransferases (HAc), are recruited to the NICD-RBPJ complex, leading to the transcriptional activation of Notch target genes (Gridley, 2007).

Structure of Notch Receptors

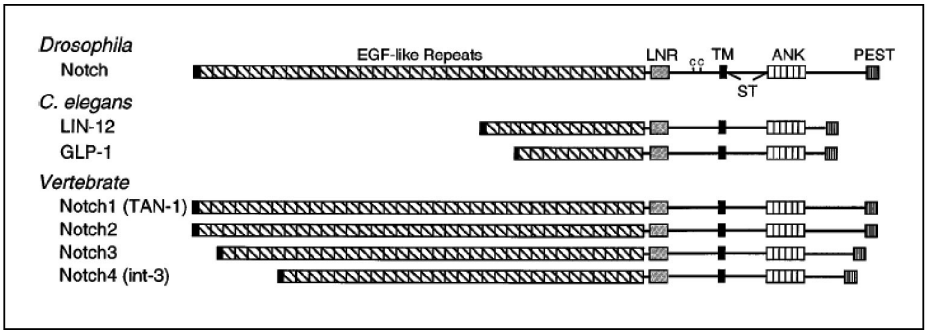


Figure 1.10: Schematic Diagram of Notch Receptor (Weinmaster, 1997)

Notch receptors are heterodimeric proteins that are synthesized as single chain pre-cursors in the Golgi apparatus. Following cleavage of full-length notch by a furin-associated convertase, the NECD associates non-covalently with the membrane tethered NICD. The Notch receptor proteins contain extracellular arrays of epidermal growth factor (EGF) repeats which are involved in ligand interaction (Figure 1.10). The EGF-repeat 11/12 are essential for ligand binding (Rebay *et al.*, 1991). Three Lin-Notch repeats (LNR) modulate interaction between the extracellular (NotchECD) and the membrane-tethered intracellular domains (NotchIC). The Notch IC includes seven ankyrin repeats (ANK), that mediate the interaction between Notch and CSL. These are flanked by nuclear localization signals, a praline-, glutamate-, serine, threonine-rich sequence (PEST domain) that is involved in degradation and a

transcription activation domain (TAD) (Fiúza and Arias, 2007).

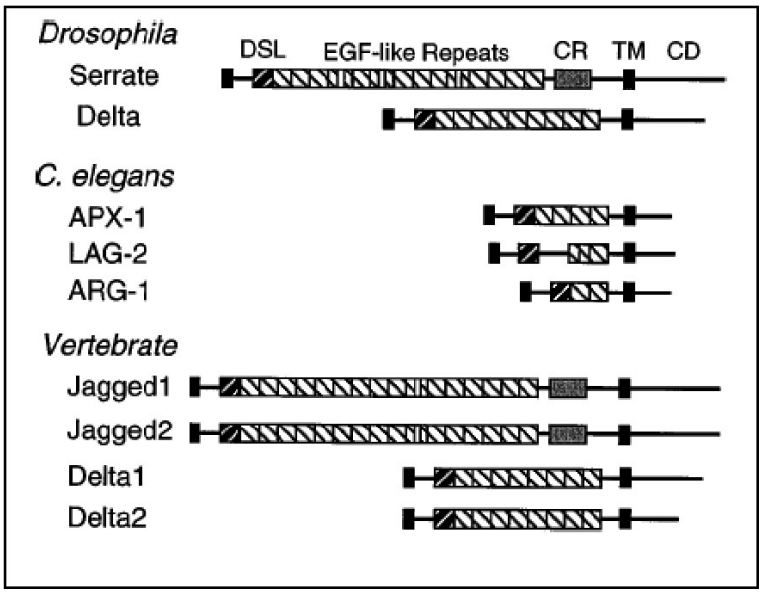


Figure 1.11: Schematic Diagram of Notch Ligands (Weinmaster, 1997)

Structure of Notch Ligands

There are five Notch ligands in vertebrate, Delta-like1(Delta1), Delta-like3(Dll-3), Delta-like 4(Dll-4), Jagged 1 and Jagged 2. They are collectively called DSL ligands after there *Drosophila* Delta/Serrate and *C.elegans* Lag-2 counterparts. The Delta ligands are similar to *Drosophila* Delta and the Jagged ligands are most similar to *Drosophila* Serrate. The intracellular tails of these DSL ligands are poorly conserved, as are the size and composition.They are composed of a DSL region responsible for the interaction with the Notch receptor and several EGF-like repeats (Figure 1.11). Jagged ligands contain, in the extracellular region, a greater number of EGF repeats insertions and a cysteine-rich region closer to the membrane. Interaction of Notch with the ligand is believed to lead to a

conformational change that exposes the S2 site of cleavage, causing Notch signalling activation.However, there is very little evidence that Dll-3 physically binds to the Notch receptors or that it truly functions as a Notch ligand (Chiba, 2006).

CBF-1 Protein

CBF-1(C Promoter Binding Factor), also known as RBP-$_{J\kappa}$, is the mammalian orthologue of *Drosophila* Su(H) (suppressor of Hairless) and and *C.elegans*Lag-1. These three proteins are collectively known as CSL. CBF-1 is a DNA-binding transcription factor that binds to the DNA sequence GTGGGAA and is required for both repression and activation of Notch target genes (Zhou *et al.*, 2000). In the absence of Notch signalling, CBF-1 interacts with Notch target gene DNA in the nucleus and recruits co-repressors to form transcriptional co-repressor complexes. These co-repressors include CtBP, SMRT, SHARP, CoREST, Sin3A, CIR and MeCP2 (Fryer *et al.*, 2002). This is followed by the recruitment of HDACs to the site which converts the target gene into a transcriptionally silent form .The transcriptional co-regulator, Ski-interating protein (SKIP) may bind to the CBF1: corepressor complex and may also play a role in the recruitment of the Notch ICD in signalling (Zhou *et al.*, 2000). On binding of the active Notch IC, the co-repressors are displaced and MaM forms a ternary complex with CBF-1 and Notch. Formation of the ternary complex on DNA requires the N-terminal domain of MAM and the ankyrin (ANK) repeats of the ICD. This is folowed by recruitment of transcription factors PCAF/GCN5 and CBP/p300, which allows transcription of the target to occur (Kovall, 2007).

CBF-1 is composed of the N-terminal domain (NTD), the β-trefoil

domain (BTD) and the C-terminal domain (CTD). CBF-1 binds to the major groove of DNA through the NTD and the BTD contributes to minor groove DNA binding. Both Notch IC and co-repressors bind to CBF-1 in the BTD of CBF-1. NotchIC interacts strongly with CBF-1 through its RAM domain (Kovall and Hendrickson, 2004).

1.4.3 CBF-1 Independent signalling

Notch mutant phenotypes in Drosophila have been shown to be slightly stronger than that of Su(H) mutants. This suggests that Su(H)-dependent signalling does not mediate all functions of Notch (Arias *et al.*, 2002). Since this was initially observed, evidence has arisen to show CBF-1 independent Notch signalling in vertebrates, including inhibition of differentiation of myoblasts by a constitutively active form of Notch1 Shawber (1996). The most commonly described mediator of CBF-1 independent signalling is deltex. While most studies have been performed in *Drosophila*, Deltex-1 (DTX1), a mammalian homolog of Drosophila Deltex, has been shown to mediate a Notch signal to block differentiation of neural progenitor cells (Yamamoto *et al.*, 2001) . Deltex protein has been shown to interact with the Notch intracellular domain (Diederich, 1994). Deltex binds to the conserved ankyrin repeats within Notch and only the most carboxyl (sixth) ankyrin repeat has been shown to be non-essential for this binding activity (Matsuno, 1995). Deltex-Notch interaction prevents the cytoplasmic retention of the Suppressor of Hairless Su(H) protein, which otherwise is sequestered in the cytoplasm via association with the Notch ankyrin repeats and translocates to the nucleus when Notch binds to its ligand Delta (Matsuno, 1995). Notch IC has also been shown to antagonize signalling

through a JNK MAP kinase pathway, in a deltex dependent manner (Ordentlich *et al.*, 1998).

Other proteins that are involved in CBF-1-independent Notch signalling include NF-$_{\kappa B}$ and Nurr 77 and there is also evidence to show that ligands such as F3/concactin can activate Notch (Miele, 2006). Notch-1 has been reported to induce NF-κB promoter activity and induce expression of NF–κB subunits (Wang *et al.*, 2006). The Drosophila abl gene, and its vertebrate homologue, the protooncogene, c-abl encode cytoplasmic tyrosine kinases with a role in Notch signalling in neurons. This effect is mediated by the abl accessory protein, disabled, which binds directly to Notch IC, and exerts an effect on axon guidance (Giniger *et al.*, 1998). Additionally, Notch 1 IC has been shown to directly interact with nur 77, resulting in an inhibition of nur 77-dependent transcription (Jehn *et al.*, 1999).

1.4.4 Effectors of Notch signalling

The primary targets of the Notch signalling pathway are the *Hes* and *HRT* genes. The HES family are the mammalian forms of the *Hairy* and *Enhancer of split*(*E(spl)*) genes in Drosophila (Iso *et al.*, 2003) and are Class C proteins of the basic helix-loop-helix(bHLH) transcription factor family of proteins. There are seven *Hes* genes, where only *Hes 1*, *Hes 5* and *Hes 7* are Notch induced. The *HRT* genes, *HRT 1-3 (hairy-related transcription factor 1-3)*, also know as *Hey 1,2 and L* (Hairy/Enhancer-of-split related with YRPW motif 1, Hairy/Enhancer-of-split related with YRPW motif 2 and Hairy/Enhancer-of-split related with YRPW motif-like) , or *HERP* (Hes-related repressor protein genes), encode three related bHLH transcription factors.

Structure of Target Genes

The HES and HRT genes contain highly conserved Orange and bHLH domains within their respective family, but are less conserved among the two families. A YRPW sequence in the last amino acids are conserved in HRTs (Figure 1.12). This is a WRPW motif in HES at the C-terminal tetrapeptide. A conserved proline residue at a specific site of the basic domain of HES confers unique DNA-binding activity and this is replaced by a glycine in the HRT family (Iso *et al.*, 2001).

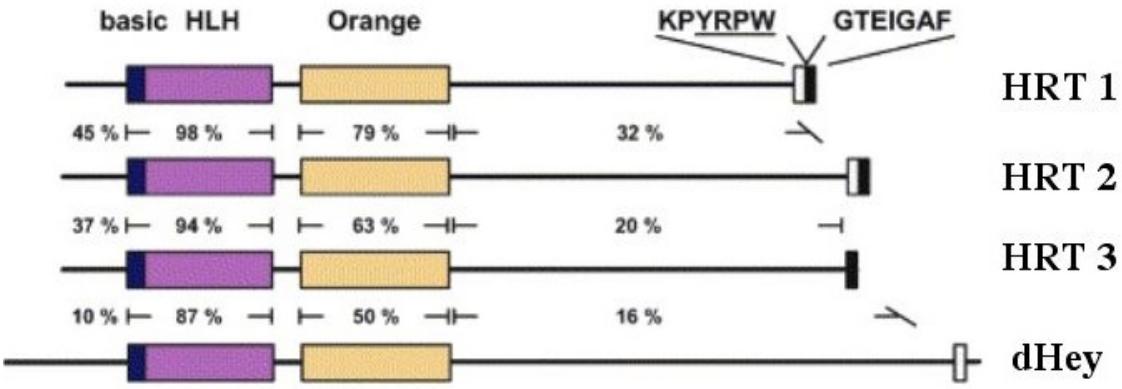


Figure 1.12: Schematic Diagram of HRT Family Protein Structures (Fischer and Gessler, 2003) HLH = helix loop helix; HRT = Hairy-related transcription factor, dHEY= Drosophila HRT

HRTs are detected in both HES-expressing and non-HES-expressing tissues. There is much evidence to show that HRTs are effectors of Notch signalling. All HRT promoters contain binding sites for CBF-1 and all three genes are induced by Notch. Indeed, mutations of CBF-1 binding sites in the promoters of HRT1, HRT2 and HES1 genes abolish activity of their promoters (Maier and Gessler, 2000).

Both Hey and Hes proteins mainly act as transcriptional repressors by

binding to DNA-bound activators and turning them into repressors. Repression can occur through DNA binding, formation of homo- and heterodimers and recruitment of co-repressors.

Mechanism of Repression

HES have different repression mechanisms then HRTs but both show evidence of acting through both active and passive repression. Recruitment of co-repressors is an example of active repression. HES proteins recruit the corepressor TLE though its WRPW motif (Paroush *et al.*, 1994). The HRT family's repressive activity is primarily due to the bHLH domain rather then the YQPW motif. Their bHLH domain can interact with mSin3 and N-CoR co-repressors (Iso *et al.*, 2001). The HES family may act passively through sequestration of proteins. HES1 can form a non-functional heterodimer with other bHLH factors such as E47 and therefore disrupt the formation of functional heterodimers such as MyoD-E47 and Mash1-E47 (Hirata *et al.*, 2000). Similarly, HRT1 binds the aryl hydrocarbon receptor nuclear translocator (ARNT) and inhibits ARNT-dependent transcription of the VEGF promoter by dissociating the ARNT complex from DNA (Chin *et al.*, 2000)

The HES and HRT family also differ in their binding capability. Both, however, have their basic helix-loop-helix (bHLH) domain, which contains a basic domain capable of DNA binding. Hes 1,5, and 7 can bind to N-box sequences (CACNAG) and class C sites (CACGNG) present in the promoter region of their target genes, while Hey proteins show preference for a class B E-box sequence (CACGTG) (Nakagawa *et al.*, 2000).

Protein dimerisation is an important mechanism for repression.

Hydrophobic residues in the HLH motif allow them to form a hetero-or homo-dimer (Murre *et al.*, 1994). The Orange domain contains two amphipathic helices that regulates the selection of bHLH heterodimer partners (Taelman *et al.*, 2004). HES-HRT heterodimers show a marked increase in DNA binding activity compared to homodimers and are, therefore, able to cooperate for transcriptional repression in in cells co-expressing them (Iso *et al.*, 2001).

Targets of the Effectors

Although few targets of the effectors have been identified, Hes1 has been shown to negatively regulate its own transcription. The Orange domain of HES1 was shown to repress transcription of its own gene and $p21^{WAF}$ promoters (Castella *et al.*, 2000). Overexpression of HES1 gene downregulated Mash1 transcription by directly binding to the promoter region (Chen *et al.*, 1997). HRT2 has also been shown to downregulate its own gene expression (Nakagawa *et al.*, 2000).

Effectors of Notch signalling in the Vasculature

HES and HRT factors are differentially expressed in different tissues. HES factors have been shown to be predominantly involved in neurogenesis and development of sensory organs, and HRT factors play critical roles in the cardiovascular system (Fischer and Gessler, 2007). HRT2 is the homologue of zebrafish gridlock (grl) and is the only HRT gene expressed in blood vessels in zebrafish. Knockout *HRT2* mice show cardiac impairment and failure to thrive (Donovan *et al.*, 2002). HRT2 and 3 are predominantly expressed in VSMCs in the dorsal aorta during development , with HRT1 transcripts restricted to the endothelial layer

(Fischer and Gessler, 2003). HRTs are the primary transducer molecules for the Notch Pathway in the vasculature and Notch 3 can induce HRT1, HRT2 and HRT3 in VSMCs (Sweeney *et al.*, 2004). HRT1 has been shown to protect against serum deprivation and FasL-induced apoptosis in VSMCs (Wang *et al.*, 2003). HRT3 inhibits myocardin-induced vascular smooth muscle cell differentiation by interfering with SRF Binding to a CArG Box (Doi *et al.*, 2005) .

1.5 Regulation of Notch signalling

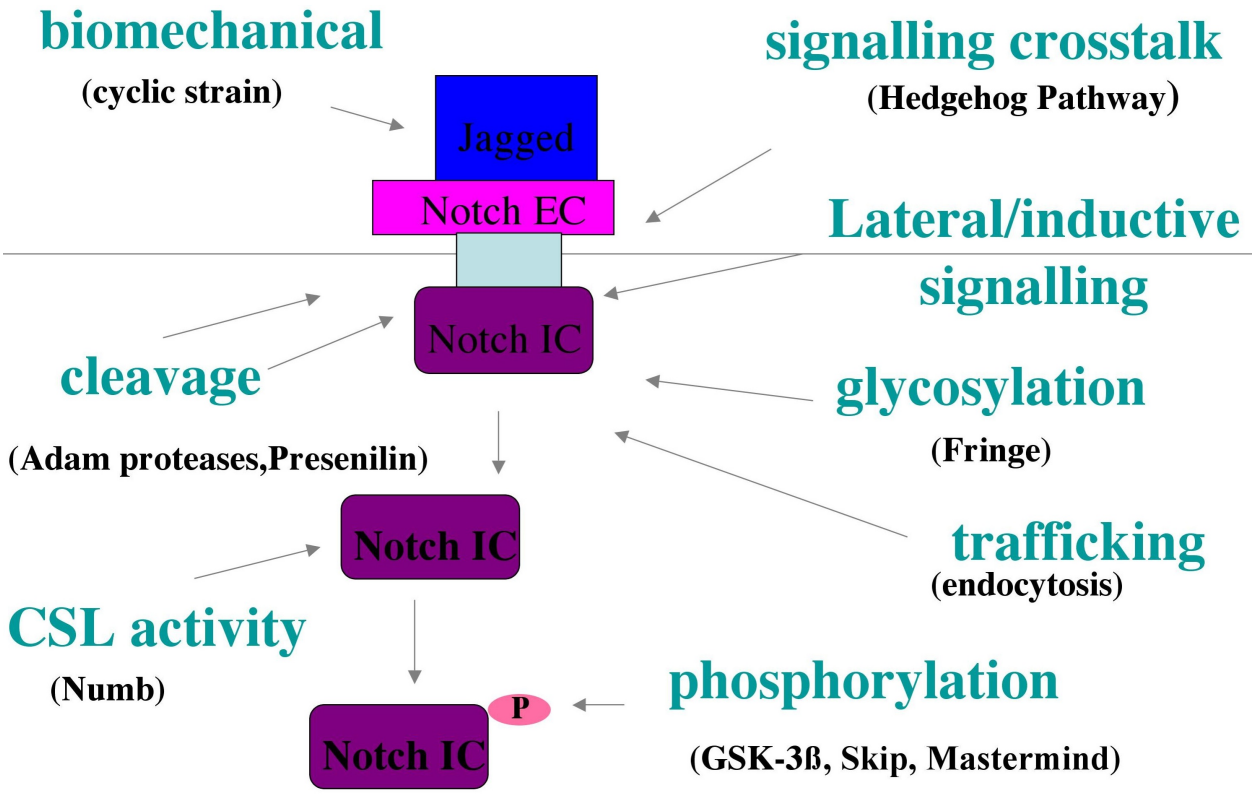


Figure 1.13: Primary Regulatory Mechanisms of Notch signalling

Lateral and Inductive signalling

Notch signalling is regulated at a number of levels, as summarised in Figure 1.13, including cell to cell interaction. This may occur through a process know as lateral signalling that is dependent on the relative concentration of Notch in neighbouring cells. A signal can change the relative concentration of ligand in two initially equivalent cells, which then take up different fates and differentiate to different tissues (Figure 1.14). Each of the cells is able to send and receive a weak signal, but with time one cell becomes a signalling cell and the others become receiving cells. This is caused by a regulatory loop within the cells, which causes activation of the receptor and results in down-regulation of the ligand (Katsube and Sakamoto, 2005). Inductive signalling, however, occurs between non-equivalent cells and a specific response at the interface between the two. The signal is sharply defined across two populations producing a different ligand. Activation of the Notch receptor promotes production of Notch ligand, thus causing increased Notch activation in the adjacent cell and renders cells insensitive to the ligand produced by equivalent cells (Simpson, 1998). Notch allows differentiation of neural precursors cells into epidermal cells by inhibiting the proneural genes achaete(ac) and scute (sc). Mutations of Notch and Delta show an increase in the number of precursor cells at the expense of epidermis (Ramain *et al.*, 2001)

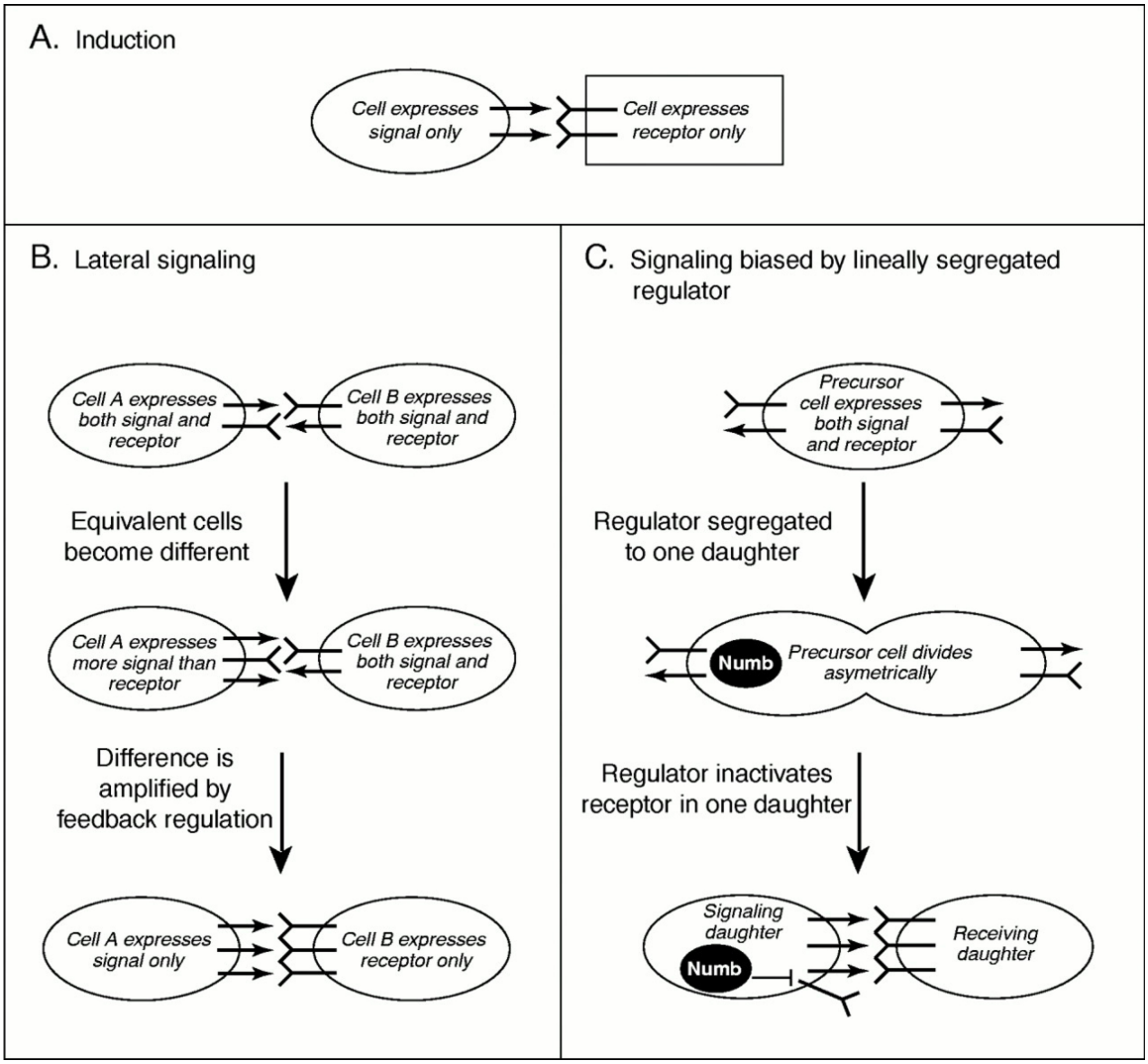


Figure 1.14: Schematic of Lateral and Inductive Notch signalling (Kimble and Simpson, 1997)

Numb

Numb, a negative regulator of Notch signalling, is a phosphotyrosine-binding (PTB) domain protein that binds via its PTB domain to two regions of Notch, the RAM domain and at the C-terminus (Baron *et al.*, 2002). In Drosophila, Numb blocks Su(H) protein translocation to the nucleus in response to Notch activation, but does not interfere with Su(H) cellular localization in the absence of Notch receptor-ligand interactions (Frise *et al.*, 1996). Numb and Notch act antagonistically in specifying correct cell fate in the bristle lineage. During asymmetric cell division in Drosophila sensory organ precursor cells, the Numb protein localizes where it influences cell fate by repressing signal transduction via the Notch receptor (Figure 1.14 C). Numb acts upstream of of the S3 cleavage of Notch in this process (Guo *et al.*, 1996). Numb antagonizes Notch activity by recruiting α-adaptin. This probably stimulates endocytosis of Notch and, thus, serves as a mechanism to downregulate signalling (Berdnik *et al.*, 2002). (Brou, 2008)

Endocytosis

Trafficking of receptor and ligand has been recognised in the last number of years as being a key part of the regulation of Notch signalling. Multi-ubiquitylation of DSL ligands by two E3 ubiquitin ligases, Neuralised (Neur) and Mindbomb(Mib) is proposed to serve as a signal for DSL ligand internalisation by promoting their interaction with cytosilic adaptor proteins that contain ubiquitination-binding domains. Direct ubiquitination of Delta by Neuralized allows the ligand to enter an Epsin-dependent endocytic pathway which is required for its activation (Kanwar and Fortini, 2004). Epsins are multidomain adaptor proteins

that bind phosphoinositol, ubiquitin, clathrin and other accessory proteins, and they might serve to link monoubiquitinated cell-surface proteins to the endocytic complex. Trafficking of endocytosed-ligand back to the surface, following these post-translational modifications, increases the efficacy of the ligand (Wang and Struhl, 2004). Endocytosis of NECD-bound Delta in the signal-sending cell may create pulling forces on Notch that induce conformational changes associated with the unmasking of the S2 cleavage site. In this model, the dynamic membrane invaginations that take place during endocytosis are utilized to generate the forces that dissociate the Notch heterodimer and induce its proteolytic activation. Dynamin-regulated endocytosis is required both in signal-sending cells, to allow activation of DSL signalling and in signal-receiving cells to promote ligand-dependant Notch activation (Parks, 2000). Mono-ubiquitination of the receptor is a crucial event to target it to endocytic vesicles, where it is cleaved by γ-secretase. Notch receptor accumulation in endosomal compartments may enhance its exposure to γ-secretase (Gupta-Rossi *et al.*, 2004). .

Cleavage of Receptor and Ligand

Cleavage of receptor and ligand is also an important factor in Notch signalling regulation. As discussed, Notch receptor cleavage at S2 and S3 sites is essential for notch signalling to occur. In the absence of ligand binding, the ADAM-protease cleavage site is buried, suggesting that ligand-binding provokes a conformational change exposing the cleavage site (Gordon *et al.*, 2007). Most Notch receptors at the cell surface are heterodimeric, single-pass transmembrane molecules and the heterodimer is generated in the secretory pathway by a cleavage called

S1. In mammalian cells, this additional furin-based S1 cleavage, during trafficking of Notch to the plasma membrane, is also required for canonical notch signalling prior to ligand binding (Logeat *et al.*, 1998). In the absence of furin processing, ligand-induced signalling can occur through activation of uncleaved Notch1 at the plasma membrane. This uncleaved receptor signals in a CBF-1-independent manner (Bush *et al.*, 2001). Low amounts of the uncleaved form of the Notch 2 and Notch 3 receptor has also been detected on the plasma membrane by other studies (Blaumueller *et al.*, 1997), (Joutel *et al.*, 2000).

Additionally, ligands can be similarly cleaved at S2 and S3 sites by ADAM proteases and γ-secretase and these cleaved intracellular fractions are important in activating ligand transcription (Dyczynska *et al.*, 2007). Ligand processing generates soluble, extracellular forms that can bind Notch receptors. DSL ligands expressed at high levels have been observed to act as antagonists of Notch present in the same cell in both Drosophila and vertebrates (Zolkiewska, 2008). It is possible that the intracellular domains of Delta and Jagged may signal intracellularly, and may be, therefore, involved in bidirectional signalling (Bland *et al.*, 2003).

Glycosylation

The extracellular domain of Notch receptors are glycosylated with *O*-fucose, *O*-glucose glycans and N-glycans on their EGF-like repeats. O-fucosyltransferase (O-Fut) allows the addition of a first fucose to the EGF repeats of the Notch IC within the endoplasmic reticulum (ER).(Wu and Bresnick, 2007). Fringe protein is a glycosyltransferase which is responsible for transfer of *N*-acetylglucosamine to fucose on the EGF-repeats in the Golgi apparatus. This has been shown to modulate

Notch-Delta interactions (Bruckner *et al.*, 2000) and may also be a form of modification for Notch ligands (Panin *et al.*, 2002). Serrate has a higher affinity for fucosylated Notch, whereas subsequent Fringe-mediated modification potentiates Delta-mediated Notch activation. Notch receptors lacking *O*-fucose are inactive and disruption of the glycosylation event leads to severe Notch signalling defects in Drosophila and mammals (Lu and Stanley, 2006). Three O-fucose sites (within EGF repeats 12, 26, and 27) are invariantly conserved in all Notch homologues with 36 EGF repeats. The most highly conserved O-fucose sites in Notch1 were shown to be important in Notch 1 for both processing and ligand-mediated signalling (Rampal *et al.*, 2005). In an interesting study by Sakamoto et al. (2002) vertebrate Notch 1, Dl-1 and Ser-1 were shown to form homomeric or heteromeric complexes in a cell autonomous manner. They found that the ligand/receptor complexes did not appear on the cell surface but had a dominant-negative effect on Notch signalling within the cell, thus supressing the cells competence to receive the notch signal. Lunatic fringe (L-fg) was found to inhibit the cell-autonomous association of the ligand and receptor (Sakamoto *et al.*, 2002).

Phosphorylation

A considerably low-level of Notch IC in the nucleus is required to initiate transcription of downstream target genes (Schroeter *et al.*, 1998). For this reason, tight regulation through phosphorylation is important in maintaining this low-level of Notch IC within the nucleus. Notch IC has been shown to be hyperphosphorylated and phosphorylated forms of Notch have been identified in the nucleus (Foltz and Nye, 2001). Fractionation of Drosophila embryos as well as mammalian cells in

culture reveals that the phosphorylated IC domain localized primarily to the nucleus (Kidd *et al.*, 1998).

Ski-interacting protein (SKIP) and Mastermind (MAM) are able to recruit kinases that specifically phosphorylate NICD in the TAD and PEST domain. This marks the phosphorylated sites of Notch IC for ubiquitination and degradation by Fbw7/Sel10 (Fryer *et al.*, 2004). This may stall signalling and post-activation of target genes, by coordinating activation with turnover of the Notch IC.

Nuclear ICD may also be phosphorylated by the serine-threonine kinase, glycogen synthase kinase-3β (GSK3β). However, this regulation may occur off DNA because GSK-3β did not bind the HES1 promoter at the same time as Notch in ChIP experiments (Fryer *et al.*, 2004). This suggests a mechanism whereby phosphorylation by GSK-3β occurs prior to activation of transcription by NotchIC. Phosphorylation by GSK3β has been found to downregulate Notch 2 activity by directly binding and phosphorylating Thr-2068 and/or Ser-2070, Thr-2074, and Thr-2093 at the C-terminal of the Notch 2 ankyrin repeat (Espinosa *et al.*, 2003). However, phosphorylation of Notch1IC by GSK-3β has been shown to aid stablilty of the active receptor within the nucleus, leading to increased signalling activity (Foltz *et al.*, 2002). Both studies suggest a role for GSK-3β in the complex regulation of Notch signalling, perhaps in a receptor-specific manner.

Regulators of Notch signalling

Along with GSK-3β, many proteins have been identified as regulators of Notch signalling, including Numb, Fringe, Deltex, among others already discussed. Additionally, Scabrous, a secreted fibrinogen-related protein,

acts as a negative regulator of the Notch signalling pathway by binding to the EGF-like repeats and modulating receptor-ligand binding (Lee *et al.*, 2000). Itch, the mammalian form of suppressor of deltex Su(dx), controls the degradation of the non-activated Notch 1 receptor and targeting it, after early endocytosis, for lyosomal degredation (Chastagner *et al.*, 2008). A number of Notch signalling repressors within the CBF-1 complex, including Hairless and Groucho, have been identified (Maier, 2006). Altogether, tight control of an important signalling pathway, such as Notch, is demonstrated by the vast number of regulators that have been implicated in its modulation.

1.5.1 Role of GSK-3β

Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch (Ruel *et al.*, 1993). Glycogen synthase kinase-3 (GSK-3) is a serine-threonine kinase. Molecular cloning identified two isoforms of GSK-3 encoded by distinct genes GSK-3α and GSK-3β (Woodgett, 1990). The difference in size between the 51kD GSK-3α and 47kD GSK-3β is due to a glycine-rich extension at the N-terminus of GSK-3α (Figure 1.15). Structurally, they are similar with 98% homology in their kinase domain and 36% identity in the last 76 C-terminal residues (Ali *et al.*, 2001) .

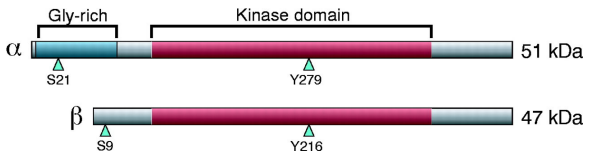


Figure 1.15: Schematic Diagram of the GSK-3 Isoforms (Doble and Woodgett, 2003)

Despite being similar, structurally, the two isoforms have been shown to have different expression patterns, substrate preferences and cellular functions. GSK-3α, but not GSK-3β, was shown to be necessary in cardiomyocyte survival during zebrafish cardiogenesis (Lee *et al.*, 2007). GSK-3β -null mice reportedly die during embryogenesis as a result of liver degeneration (Yao *et al.*, 2002), whereas GSK3α knock-out mice are viable and display improved glucose tolerance in response to glucose load and elevated hepatic glycogen storage and insulin sensitivity.

GSK-3β is unique in that many of its substrates require phosphorylation at a residue four amino acids C-terminal to the GSK-3β phosphorylation site. This is performed by a priming kinase such as casein kinase II, protein kinase A or DYRK (Liu *et al.*, 2004) (Woods *et al.*, 2001). Not all substrates require this priming and an example of this is the APC-β-catenin complex in the Wnt pathway (Woodgett, 1990).

GSK-3β was first identified as a regulator of glycogen synthesis, due to its phosphorylation and deactivation of glycogen synthase (Hall *et al.*, 2001). It has since been shown to have a broad range of substrates and its dysregulation has been associated with a wide variety of diseases, including Alzheimers, bi-polar disease and cardiovascular disease. Therefore, it is an enzyme that requires strict regulation and this is carried out through a number of mechanisms. Unlike most serine-threonine kinases, GSK-3β is constitutively expressed. It is regulated through phosphorylation at two sites. Tyrosine phosphorylation at site 216 is optimal, but not essential for its activity (Bax *et al.*, 2002). It has been shown to increase activity 200-fold (DePaoli-Roach, 1994) and has been shown to be an autophosphorylation event (References *et al.*, 2004).

GSK-3β is negatively regulated through phosphorylation at serine 9.

This is may be mediated by a number of stimuli, including insulin (Pearl and Barford, 2002). Insulin stimulation results in phosphorylation through a phosphoinositide 3-kinase (PI-3 kinase)-dependent mechanism. This pathway results in activation of Akt, which binds to and directly phosphorylates both GSK-3α and GSK-3β at ser21 and ser9 respectively (Cross *et al.*, 1995). Phosphorylation may also occur though a MAPK pathway (Forde and Dale, 2007). Growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) may regulate the phosphorylation of GSK-3β through both the MAPK and PI-3kinase pathway (Shaw and Cohen, 1999).

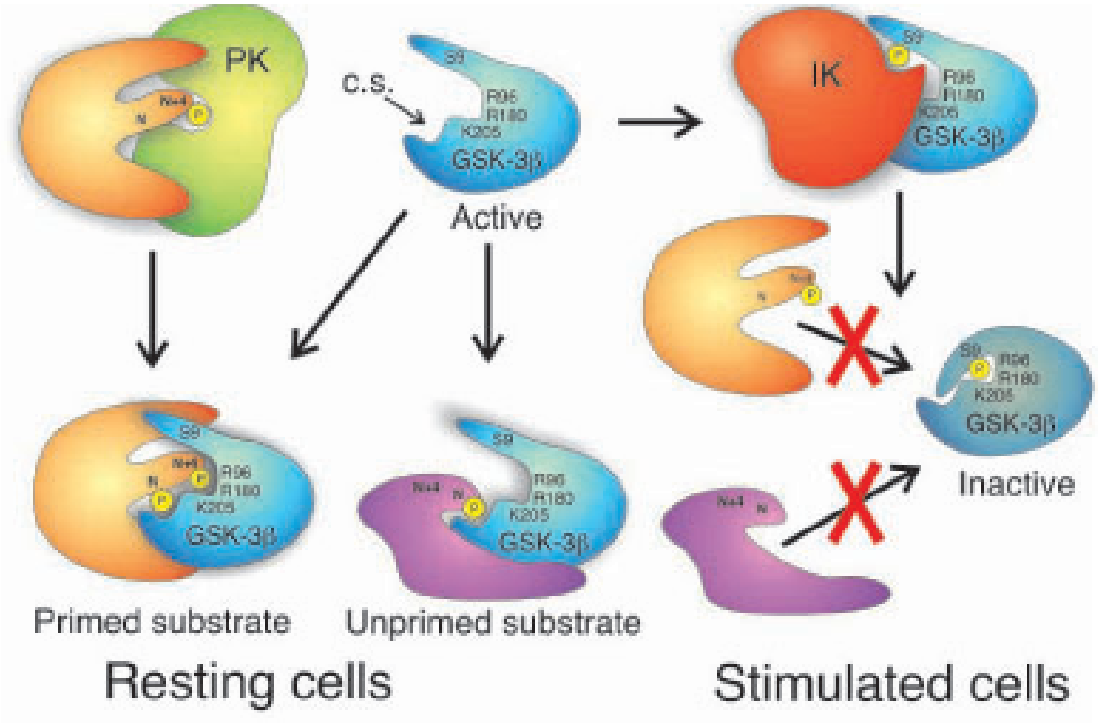


Figure 1.16: Negative Regulation of the GSK-3β through ser9 phosphorylation (Doble and Woodgett, 2003)

The mechanism of GSK-3β regulation is described in Figure 1.16.

Phosphorylation of serine 9 on GSK-3β serves as a form of pseudosubstrate. Both primed and unprimed substrates may be phosphorylated by GSK-3β in its resting state. A priming kinase (PK) directs the binding of a serine or threonine to the catalytic site (CS). Binding of a phosphate to serine 9 on GSK-3β, by an inactivating kinase (IK), causes the N-terminal end to fold over the active or catalytic site (CS) of the enzyme, preventing binding and phosphorylation of the substrate (Doble and Woodgett, 2003).

Regulation of GSK-3β is also controlled though its subcellular location. Akt has been shown to localise in the mitochondria following insulin stimulation, resulting in rapid phosphorylation of mitochondrial GSK-3β (Bijur and Jope, 2003b). Apoptotic stimuli can induce nuclear accumulation of GSK-3β (Bijur and Jope, 2001). Nuclear GSK-3β is in a relatively greater activation state then cytosolic GSK-3β (Bijur and Jope, 2003a) and is able to regulate a number of transcription factors, such as p53, that support cell survival (Watcharasit *et al.*, 2003). Its ability to associate with other proteins in subcellular compartments is also important in selectively directing its actions towards specific substrates and GSK-3β has a number of binding partners including Tau, presenilin, p53 and Notch 2 (Jope and Johnson, 2004). This binding pattern is of importance in the Wnt pathway. In the absence of Wnt, GSK-3β is active in a multi-protein complex containing the scaffold protein axin, and APC (Figure 1.17) . This active GSK-3β phosphorylates the transcription factor β-catenin, targeting β- catenin for proteosomal degradation . In the presence of Wnt, Dishevelled is activated and results in inhibition of GSK-3β activity within the complex (Luo *et al.*, 2007). Interestingly, this inhibition of GSK-3β activity does not involve phosphorylation of the

serine 9 residue. Free GSK-3β may be phosphorylated at serine 9, by stimuli such as insulin, but enzyme bound to the complex may not (Ding *et al.*, 2000). Inhibition of activity occurs through the disruption of the interaction of axin with GSK-3β , thereby dissociating β-catenin from the complex and preventing its phosphorylation (Frame and Cohen, 2001).

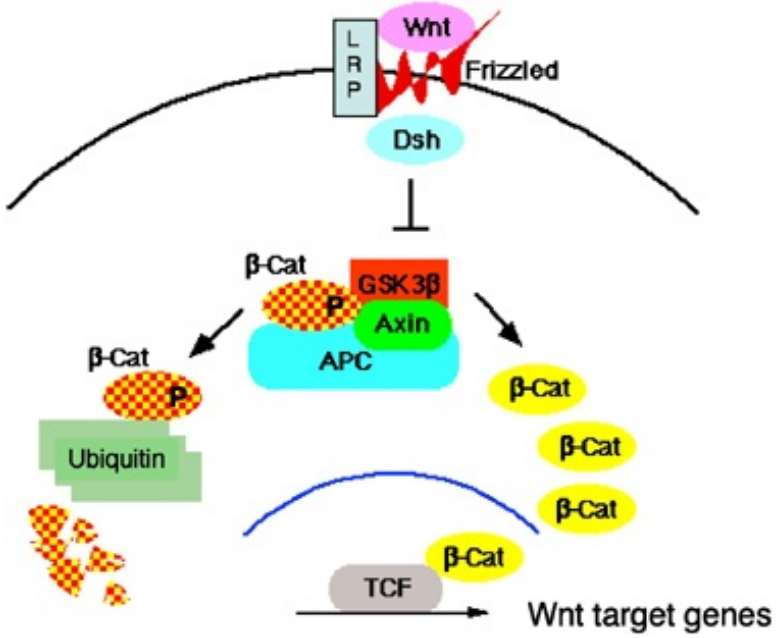


Figure 1.17: Role of GSK-3β in Wnt signalling (Taketo, 2006)

GSK-3β has a diverse range of substrates, involved in many biological processes. This includes metabolism, where GSK-3β is involved in inhibition of glycogen synthesis, through glycogen synthase and inhibition of protein synthesis through eIF2b (Kockeritz *et al.*, 2006). GSK-3β is also involved in cell division. Cyclin D1 has been shown to be phosphorylated by GSK-3β, triggering rapid cyclin D1 degradation and facilitating entry into the DNA synthetic phase of the cell cycle (Diehl *et al.*, 1998). GSK-3β is very important in development and embryogenesis, through Wnt signalling and its target β-catenin (Li *et al.*,

2006), and also regulates cytoskeletal proteins via Tau and APC (Frame and Cohen, 2001). A number of transcription factors have been identified as substrates of GSK-3β, and these include those involved in proliferation, differentiation and apoptosis. Generally, its effect is inhibitory targeting the transcription factor for degradation. Phosphorylation of the pro-proliferative transcription factor, c-jun, interferes with its binding to DNA and hence represses c-jun-directed transcription (Ginger *et al.*, 2000). However, GSK-3β can also stimulate transcription, for example through phosphorylation of the cell survival transcription factor, NF$\kappa\beta$ (Ougolkov *et al.*, 2005).

GSK-3β has been implicated in a number of diseases including cancer, Alzheimers, bi-polar disease, diabetes and cardiovascular disease. In Alzheimer's disease, hyperphosphorylation of tau results in the formation of neurofibrillary tangles, leading to neurodegeneration and there is much evidence to demonstrate that GSK3β is involved in this hyperphosphorylation (Hooper *et al.*, 2008). Lithium chloride, an inhibitor of GSK3β, has been used for many years as a treatment for bi-polar disease and, in animal models, GSK3β manipulation has been shown to have an effect on manic symptoms (Rowe *et al.*, 2007). Insulin resistance in diabetic patients can lead to GSK3β hyperactivity in some peripheral tissues, and inhibition of GSK3β may be able to contribute to controlling glucose levels in diabetic subjects (Jope *et al.*, 2007). The associated increase in GSK-3β may also have implications for cardiovascular disease. There is a potential role for GSK-3β in glucosamine-induced endoplasmic reticulum stress. The resulting lipid accumulation, inflammation and apoptosis may pay a causative role in accelerated atherosclerosis (Robertson *et al.*, 2006). Recent evidence, both

in vitro and in vivo, suggests that GSK-3β is a negative regulator of cardiac hypertrophy. Inhibition of GSK-3β has been shown to reduce myocardial cell death following ischaemia and reperfusion (Murphy and Steenbergen, 2005). Transgenic mice, which overexpress a mutant active form of GSK-3β (GSK-3βs9A) inhibit hypertrophy in response to aortic banding and isoproterenol stimulation, both of which are inducers of hypertrophy (Hardt and Sadoshima, 2002).

1.5.2 Crosstalk with other Pathways

The Notch signalling Pathway is one of a number of developmental cell-cell signalling pathways that include Wnt, TGF-β and Hedgehog (Hh) Barolo (2002). Crosstalk between these pathways is known to occur during embryogenesis, to maintain tight control on all cellular processes. It is thought that this interaction may be recapitulated in adult cells during the pathogenesis of disease. Indeed, it is known that the Hh ligand, sonic hedgehog (Shh) is known to up-regulate the expression of Notch target genes during arterial differentiation and vascular endothelial growth factor (VEGF) acts downstream of Shh and upstream of the Notch pathway in zebrafish. Loss of Vegf or Shh resulted in loss of arterial identity in zebrafish embryos, while exogenous expression of these factors caused ectopic expression of arterial markers. Microinjection of VEGF mRNA into embryos lacking Shh activity rescued arterial differentiation. Finally, activation of the Notch pathway in the absence of VEGF signalling could rescue arterial marker gene expression (Lawson *et al.*, 2002). This crosstalk between pathways is of interest as another mechanism by which Notch is regulated in adult cells and as a possible cause of altered phenotype in vascular disease.

Wnt/Wingless Pathway

A Wnt protein is the ligand to Wnt/Wingless signalling, so called as Wingless is the Drosophila orthologue of Wnt. The receptors of Wnt signalling include Frizzled and LRP protein families. The effector of Wnt signalling in the nucleus is β-catenin (Hayward *et al.*, 2008). As discussed previously, in the absence of Wnt, active GSK-3β phosphorylates the transcription factor β-catenin, targeting β- catenin for proteosomal degradation (Figure 1.17). On binding of a Wnt ligand, Dishevelled is activated and disrupts the interaction of axin with GSK-3β. This prevents the phosphorylation of β-catenin, allowing transcription to occur (Frame and Cohen, 2001).

Wnt signalling is involved with the prepatterning required for lateral inhibition to occur, through the creation of equivalence groups through positional information. Notch signalling then acts on these groups to restrict the neural potential to one or two cells through lateral inihibition. Interactions between Wnt and Notch signalling were first uncovered in the context of the development and patterning of the wing of Drosophila (Hayward *et al.*, 2008). Crosstalk between Notch and Wingless(Wg) signalling is known to occur through Dishevelled. Dishevelled (Dsh) is a cytoplasmic protein that binds to the carboxy terminus of the Notch receptor (Axelrod *et al.*, 1996). Notch is antagonized by elements of the Wingless (Wg)-signalling cascade to allow alternative cell fate choices, resulting in a repression of neural fate, by a Notch signalling distinct from that involved in lateral inhibition and CBF-1 activity. Loss of sensory organs associated with this phenotype results from a constitutive activation of a Deltex-dependent Notch-signalling event. This activity, mediated by Deltex (Dx), represses neural fate and is antagonized by

elements of the Wingless(Wg)-signalling cascade to allow alternative cell fate choices (Ramain *et al.*, 2001).

Hedgehog signalling Pathway

Hedgehog (Hh) genes are a class of 19-kDa morphogens, of which there are three human homologues: sonic Hh (Shh), Desert Hh and Indian Hh. Hedgehog signalling functions through binding of the Hh ligand to its transmembrane receptor, Patched 1 or Patched 2. The patched receptor normally inhibits downstream signalling through interaction with a second transmembrane protein, Smoothened. Binding of Hh ligand to Patched removes its repressive effect on Smoothened and allows signalling and activation of its target genes, the Gli transcription factors 1-3, to progress (Figure 1.18). The downstream targets of the Gli gene products include both Patched and Gli (Mullor *et al.*, 2002).

Similar to Notch, post-translational modification of Hh is important for its signalling. Autolytic cleavage of the hedgehog precursor protein to generate an N-terminal active polypeptide occurs and a C-terminal fragment is responsible for catalzying the autolytic cleavage. Cholesterol moiety is thought to mediate Hh binding to the membrane. Addition of a palmitoyl group to the N-terminus of the mature active protein is necessary for signalling to occur (Weed *et al.*, 1997). Hh ligands may be tethered to the plasma membrane of the signalling cell to effect short range signalling, or they may be released from the signalling cell in a diffusible form to act as a long-range signal. The release of the ligand is regulated by a protein called Dispatched (Burke *et al.*, 1999)

Ptc1 is a 140 kDa transmembrane protein expressed through the vasculature in both juvenile and adult mice (Pola *et al.*, 2001). Ptc

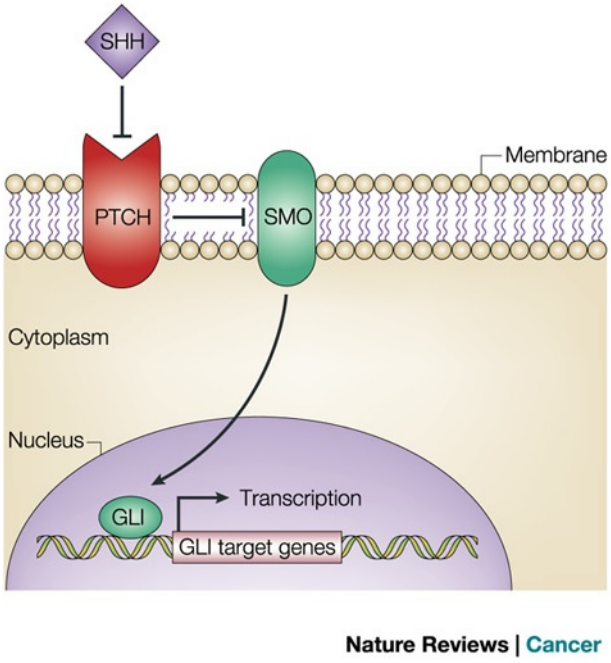


Figure 1.18: Canonical Hedgehog signalling (Owens and Watt, 2003).Binding of Sonic Hedgehog (Shh) ligand to Patched (PTCH) receptor relieves PTCH repression of Smoothened (SMO). SMO translocates to the nucleus, where it stabilises Gli, allowing transcription of its target genes.

regulates the activity of Smoothened (Smo), a G protein-coupled receptor-like protein essential for Hh signal transduction. Ptc may control Smo activity by regulating an intracellular trafficking process dependent upon the integrity of a Ptc sterol-sensing domain (Strutt *et al.*, 2001). In Drosophila, Smo signals to an intracellular microtubule-attached multi-protein complex containing Costal2 (Cos2), the protein kinase Fused (Fu) and the transcription factor Cubitus interruptus (Ci). In the absence of Smo, this complex regulates the cleavage of full-length Ci to a truncated repressor protein, Ci75, in a process that is dependent on the proteasome and priming phosphorylations by Protein kinase A (PKA) (Ogden *et al.*, 2008) Smo signalling results in the phosphorylation of Fu and Cos2 in Drosophila. This causes the complex to detach from the microtubules. Full length Ci/Gli is stabilised, and is no longer subject to phosphorylation by PKA. It is then able to translocate to the nucleus where it acts as transcriptional activator. Binding of Hh to Ptc blocks Ptc-mediated Smo inhibition, allowing Smo to signal to the intracellular components to attenuate Ci cleavage (Nybakken and Perrimon, 2002).

There is also evidence of a Gli-independent Hh pathway through COUP-TFII (Krishnan *et al.*, 1997). COUP-TFII is also involved in suppression of Notch signalling during the regulation of vein identity (You *et al.*, 2005)

VEGF Pathway

The vascular endothelial growth factor (VEGF) pathway involves binding of a diffusible VEGF ligand to a cell receptor tryrosine kinase. There are five forms of VEGF ligand in mammals - VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PLGF). VEGF-A, also known as

VEGF, is the most well-characterised, with expression observed in many cell types, including vascular smooth muscle cells (Holmes *et al.*, 2007). Six splice-variant isoforms have been detected, which differ in their binding to heparin and thereby their association with the extracellular matrix (Cross *et al.*, 2003). $VEGF_{165}$ is the most abundant and biologically active form. It is expressed as a 46kDA homodimer composed of two 23kDA units, which has the ability to bind to the VEGF receptor VEGFR-1 but mainly binds to VEGFR-2 (Ho and Kuo, 2007). VEGF plays a pivitol role in both vasculogenesis and angiogenesis, through its involvement in cell proliferation, survival and migration. It signals through many pathways, including the Ras-RAf-MEK-ERK and MAPK pathways in proliferation, is activated by PI-3 kinase/Akt pathway to promote survival and also interacts with FAK, PI3Kinase/Akt and MAPK signalling to regulate migration (Zachary and Gliki, 2001).

VEGF gene expression is upregulated by hypoxia and also by transforming growth factor-β, angiotensin II and basic fibroblast growth factor in VSMCs (Ferrara and Bunting, 1996). Additionally, VEGF is upregulated in rat VSMCs following cyclic strain (Shyu *et al.*, 2001). A study by Inoue, et al. (1998) identified VEGF expression in human coronary atherosclerotic lesions (Inoue *et al.*, 1998). Also, expression of VEGF is upregulated in mechanically injured arteries removed from healthy rats (Tsurumi *et al.*, 1997). Several agonists, such as thrombin, have been shown to release VEGF in VSMC, which may help repair of damaged endothelium following injury (Bassus *et al.*, 2001).

The Notch and VEGF pathway are known to crosstalk in the modulation of arteriogenesis and angiogenesis in zebrafish (Lawson *et al.*, 2002). Similarly, in humans, Notch1 and Dll4 are regulated by vascular

endothelial growth factor in human arterial endothelial cells (Liu *et al.*, 2003) The VEGF-induced specific signalling is mediated through VEGF receptors 1 and 2 and is transmitted via the phosphatidylinositol 3-kinase/Akt pathway but is independent of mitogen-activated protein kinase and Src tyrosine kinase (Wu and Bresnick, 2007). The crosstalk between the Notch and VEGF pathway in VSMCs has yet to be explored and may contribute to the changes in cell fate apparent in atherosclerosis, the arterial response to injury and other vascular disease.

GSK-3β and Vascular Disease

In the absence of Wnt signalling, β-catenin has been shown to be phosphorylated by GSK-3β binding, thereby marking β-catenin for proteosomal degradation. This can be seen in a similar manner in Hh signalling in Drosophila. Shaggy, the Drosophila form of GSK-3β, again in the absence of ligand binding, phosphorylates Ci, the Drosophila form of Gli resulting in its degradation. As discussed previously, GSK-3β has also been shown to be a possible modulator of Notch signalling in a number of cell types, through phosphorylation of the active receptor. It is becoming apparent that GSK-3β is a multi-faceted enzyme, critical in many signalling events. It is tempting, therefore, to speculate that GSK-3β may be involved in the recapitulation of signalling pathways in adult vascular cells, resulting in altered cell fate decisions, leading to vascular disease. Studies have shown that the Hh and Notch pathway are important in these altered cell fates and therefore these signalling pathways are of particular interest in identifying GSK-3β as a player in vascular diseases such as arteriosclerosis, atherosclerosis, stroke and heart failure.

1.6 Summary and Rationale

Cell growth, the balance between proliferation and apoptosis, is important in both vascular development, and maintenance of homeostasis within the adult vasculature. Dysregulation of either cell proliferation or apoptosis, however, is a common response to vascular injury, contributing to the progression of vascular disease states, such as hypertension and atherosclerosis.

The Notch signalling pathway has previously been implicated in several aspects of vascular development, including arterio-venous differentiation, angiogenic processes, and in the regulation of developmental VSMC fate decisions (Proweller *et al.*, 2005). In addition, genetic disorders such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) and Alagille Syndrome demonstrate a crucial role of the Notch pathway in vascular development. Notch has been shown to be pro-proliferative in vascular smooth muscle cells (Morrow *et al.*, 2005a) and to be modulated by both biomechanical stimuli *in vitro* and following vascular injury *in vivo* (Morrow *et al.*, 2005a), (Wang *et al.*, 2002).

Notch signalling is regulated on many different levels, including post-translational modification of the Notch receptor. Phosphorylated forms of Notch have been identified in the nucleus (Foltz and Nye, 2001) and GSK-3β has been suggested as a possible regulator responsible for NotchIC phosphorylation (Espinosa *et al.*, 2003). Crosstalk with the Hh signalling pathway has also been implicated in the highly complex regulation of Notch signalling. Indeed, Shh is known to up-regulate the expression of Notch target genes during arterial differentiation and vascular endothelial growth factor (VEGF) acts downstream of Shh and

upstream of the Notch pathway (Lawson *et al.*, 2002).

The principal aims of this study, therefore, are to establish the presence and activity of GSK-3β within VSMCs and determine the effect, if any, of this kinase on Notch signalling in VSMCs in both a static and cyclic strain environment. We aim to establish a role for Hh signalling in the regulation of Notch in VSMCs and determine the role of GSK3β in this signalling crosstalk. We also aim to establish a role for GSK-3β in the Notch regulation of VSMC growth. In addition, *in vivo* models of altered biomechanical force are used as an *in vivo* correlation of the *in vitro* findings of alterations in GSK3β and Notch signalling pathway expression due to changes in cyclic strain. This study aims to provide increased understanding into the regulation of cell growth in VSMC, both under physiological and pathological conditions. It aims to provide new insights into the molecular mechanisms involved in conditions such as hypertension and atherosclerosis and thereby, aid the development of potential therapeutic targets in the field of cardiovascular disease.

The findings of this research project has been divided into three results chapters with the following objectives:

Chapter 3:

The aim of this chapter is to examine the role of GSK-3β in Notch signalling in RVSMCs.

Chapter 4:

The aim of this chapter is to examine the role of GSK-3β in the regulation of Notch at the level of crosstalk with another developmental pathway, the Hh pathway and to identify the role of GSK-3β in Notch-dependent changes in RVSMC growth.

Chapter 5:

The aim of this chapter is to identify a role for GSK-3β in the Notch response to cyclic strain and to further elucidate the mechanism involved.

Chapter 2

Materials and Methods

2.1 Materials

All reagents used in this study were of the highest purity commerically available and were of cell culture standard when applicable. See appendix for full list of materials.

2.2 Enabling Technologies

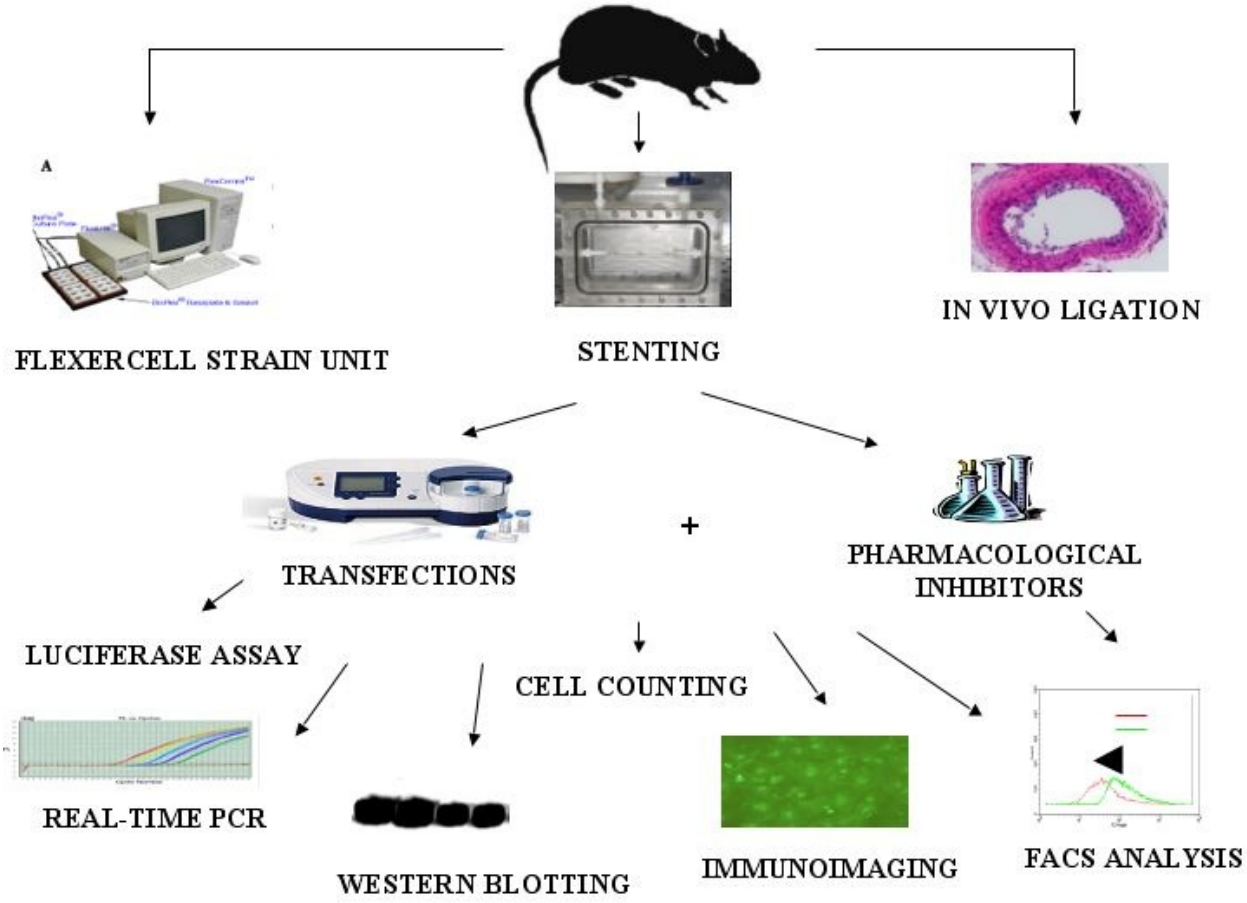


Figure 2.1: Summary of Methods.

2.2.1 Cell Culture

All cell culture techniques were carried out in a clean and sterile environment using a Bio air 2000 MAC laminar flow cabinet. Cells were visualized using an Olympus CK30 phase contrast microscope. The cell line used in this study were RVSMCs, purchased from Cell Applications Inc.(CA, USA) Cat No. R-354-05. The cells were grown in RPMI-1640, supplemented with 10% foetal calf serum (FCS), and 1% penicillin/streptomycin (P/S), and were maintained in a 37°C humidified atmosphere of 5%CO_2/95% air in a Hera water jacketed cell culture incubator. The cells were cultured in 175cm^2 , 75cm^2 , 25cm^2 and six-well plates and were routinely fed every 3-4 days. Passages 5-15 were used for experimentation and sub-culturing of cells was carried out at 80-90% confluency by trypsinisation. Cells were washed in sterile phosphate-buffered saline and incubated in 2x Trypsin/Ethlyenediamine Tetracetic Acid (EDTA), diluted from the 10x stock solultion with PBS for 5 min at 37°C. A volume of 1 ml of 1x Trysin/EDTA was used per 25 cm^2 tissue culture flask area. An equal volume of RPMI-1640 media containg 10% foetal calf serum (FCS), and 1% penicillin/streptomycin (P/S) was then added to the cells to stop the trypsination process. The cell suspension was centrifuged at 300 g for 5 min. Cells were seeded at a density of 1 x 10^5 cells/cm^2.

2.2.2 Cryogenic Cell Storage and Recovery of Cells.

The RVSMCs were maintained long-term in liquid nitrogen in a cryofreezer unit. Cells to be frozen were trypsinised from the flask, spun down in serum-containing media for 5 min at 300 g, and the pellet was

resuspended in 20% (v/v) FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% (v/v). 1ml of suspension was then transferred to sterile cryovials and frozen at 80°C freezer at a rate of 1°C/minute using a Nalgene cryo freezing container. Cells were recovered from longterm storage by rapid thawing at 37°C and resuspension in 5ml of growth medium followed by centrifugation at 300 x g for 5 min. The resultant cell pellet was resuspended in fresh medium and transferred to a culture flasks. The following day the media was removed, the cells were washed in PBS and fresh culture media added.

2.2.3 Cyclic Strain

For cyclic strain studies, RVSMCs were seeded into 6-well Bioflex plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately 1 x 10^5 cells/well. When the cells had reached 80% confluency, the cells were exposed to cyclic strain. Bioflex™ plates contain a pronectin-coated silicon membrane bottom that enables precise deformation of cultured cells by microprocessor-controlled vacuum (Gilbert *et al.*, 1994) (Figure 2.2). A Flexercell Tension Plus™ FX-4000T™ system (Flexcell International Corp., Hillsborough, NC) was employed to apply a physiological level of cyclic strain to each plate (0-15% strain, 60 cycles/min, 0-24 h) providing equibiaxial tension using the Hearbeat™ Simulation protocol. Control cells remained unstrained. Following strain, the cells were washed twice in I x PBS, and either protein or RNA samples were isolated as described in sections respectively.

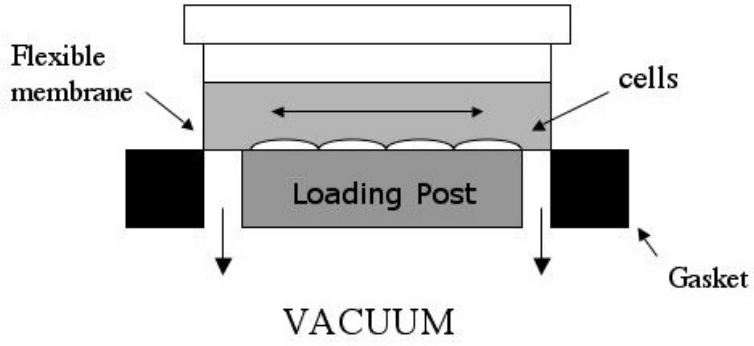


Figure 2.2: Flexercell Tension Plus ™ Induction of Cyclic Strain

2.2.4 Stented Sylgard® Mock Coronary Artery

A study on a stented mock coronary artery was carried out in collaboration with Dr. Alberto Colombo (School of Engineering, DCU). Samples of bovine aortic vascular smooth muscle cells (BASMCs) on a perfused stented mock coronary artery were prepared by Dr. Colombo, using the procedure outlined below. Mock coronary arteries (MCAs) were manufactured from transparent Sylgard ® 184, a silicon elastomer. Following coating with fibronectin,. BASMCs were seeded onto the MCA. A bare metal stent (BMS) (provided by Medtronic Ave) was deployed inside the MCA by means of a Basix 25 angioplasty inflation syringe (Merit Medical Systems, South Jordan, Utah) and expanded by a 9 mm angioblasty balloon catheter. The stented MCA was then placed into a culture chamber containing 100ml of RPMI 1640 media supplemented with 10% FBS and primocin antibiotic (100μg/ml) (Amaxa, MD, USA). The culture chamber consisted of a biocompatible Plexiglas® open box with an inlet and outlet for medium perfusion of the MCA (Figure 2.3).

The culture chamber was then attached to a CellMax® bioreactor

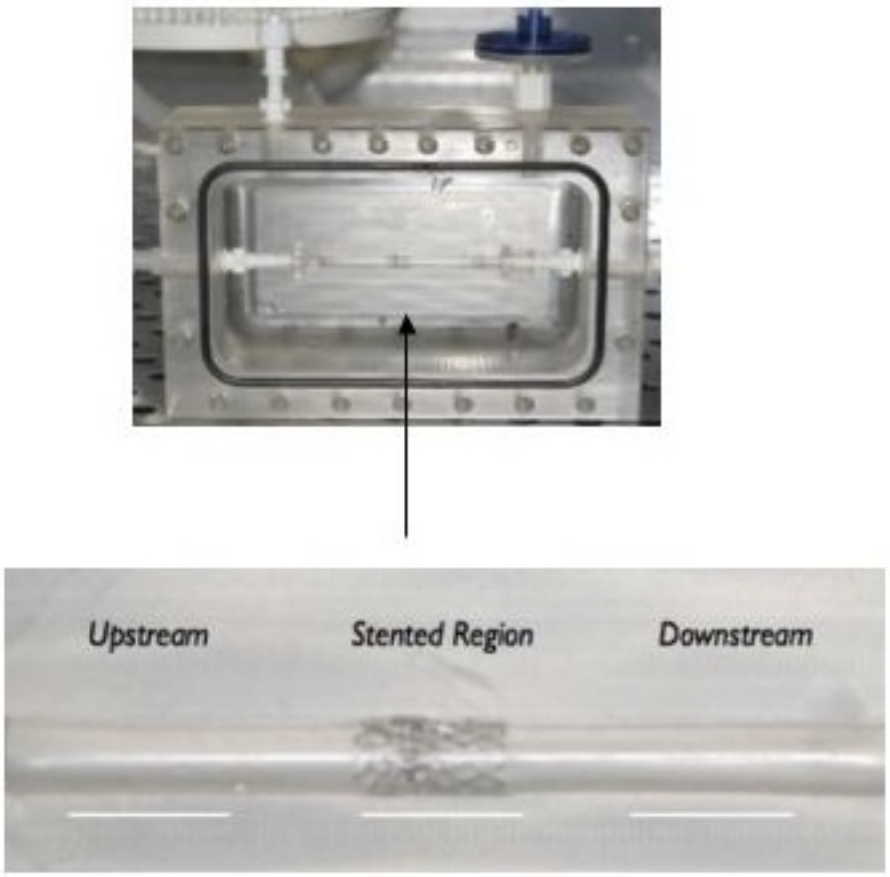


Figure 2.3: Mock Stented Artery in Culture Chamber.

tubing system (Figure 2.4). The BASMCs were exposed to pulsatile flow for 7 days, following which the mock coronary artery was removed and analysed. See appendix 4 for a detailed protocol.

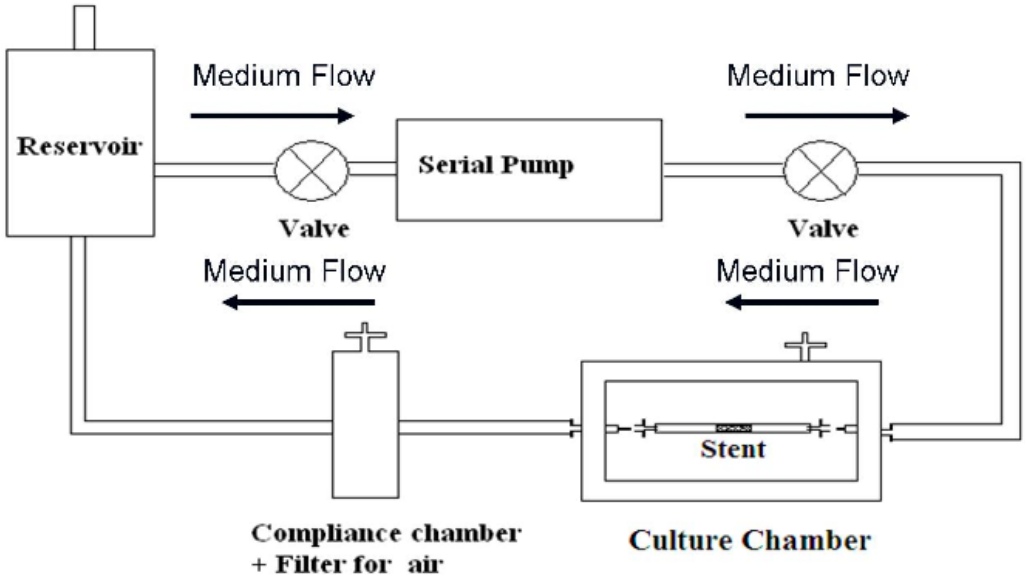


Figure 2.4: Schematic of Stented MCA Bioreactor System (*Courtesy of Alberto Columbo*)

2.2.5 Carotid Artery Ligation

The left carotid arteries of male C57B16/J mice (Charles River Laboratories, Massachusetts, USA) were ligated, with the right carotid artery acting as a high flow control. Anesthesia was induced in animals pre-medicated with atropine sulphate (0.04 mg/kg, intra-muscular) through administration of pentobarbital sodium (50 mg/kg, intra-peritoneal) and halothane (inhalation). Following induction of anesthesia, the animal was positioned on a clean operating table, with a

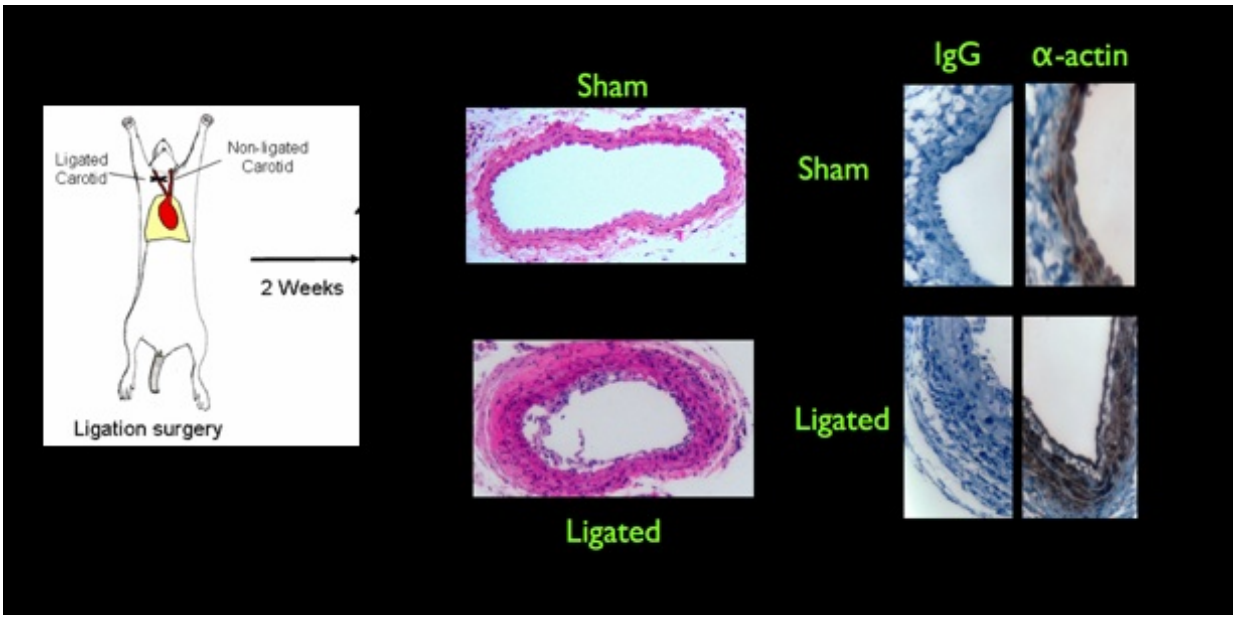


Figure 2.5: Flow-Induced Remodelling in a mouse carotid ligation model. The left panel indicates the ligation site. The middle and right panel show hamotoxylin/eosin and α-actin staining in a remodelled ligated carotid artery 14 days post-ligation compared to sham control.

body pad to maintain body temperature. The animal was clipped and the surgical site prepped using betadine solution and alcohol. A midline cervical incision was made, and, with the aid of a dissecting microscope, the right and left common carotid arteries were isolated. A transonic flowprobe was used to measure carotid blood flow in both the left and right arteries. The left common carotid artery was ligated near the carotid bifurcation using a 6-0 silk suture. The incision was then closed using 4-0 coated Vicryl, running suture pattern. The animal was then allowed to recover, whilst being monitered. Sham operated animals were subjected to the same surgical techniques, with the exception of the carotid artery ligation. Vessels were harvested from sham operated and ligated animals at 3 days and 14 days post-ligation for RNA isolation (4 vessels per preparation), and tissue imaging (Figure 2.5). Terminal surgery was carried out by halothane inhalation and cervical dislocation, followed by harvesting of the carotid arteries.

2.2.6 Human Arteriosclerotic Tissue

Human arteriosclerotic cDNA and its matched non-arteriosclerotic cDNA was purchased from Biochain (Hayward, CA, USA).

2.3 DNA Manipulations

2.3.1 Expression and Reporter Plasmids

Table 2.1: Expression and Reporter Plasmids

Plasmid	Source	Description
pcDNA3HA HACTHUGSK3β pcDNA3GSK3βCTA9GOOD	Dr.Jim Woodgett, Samuel Lenfeld Research Institute, Toronto, Canada.	HAVTHUGSSK3β expresses the wild type form of the plasmid. pcDNA3GSK3βCTA9GOOD expresses a mutant form of GSK3β, where the serine from position 9 has been replaced by an alanine, forming a constitutively active form of the enzyme.
pcDNA3GSK3βK85M	Dr.Eileen Redmond, Dept. of Surgery, University of Rochester, Rochester, New York.	pcDNA3GSK3βK85M expresses a mutant form of GSK3β, where the lysine at position 85 has been replaced by a methionine, forming a dominant negative form of the enzyme.
pGa50-7 pGa98-1-6	Dr. Bettina Kempes, GSF-Institute of Clinical Molecular Biology, Neuherberg, Germany.	THe pGA98-1-6 reporter construct was generated using a 50-bp oligonucleotide harboring both CBF-1 binding sites of the EBV TP1 promoter, which was then ligated as a hexamer into plasmid pGA50-7 (Minoguchi et al., 1997).
pPGKpuro	Dr. Peter Laird, University of Southern California, Kerk School of	Puromycin resistance plasmid (Tucker et al., 1996).

Plasmid	Source	Description
	Medicine, Los Angeles, California, USA.	
pCMVLacZ	Dr. Dermot Walls, Dublin City University, Ireland.	Expresses the gene encoding β-galactosidase.

2.3.2 Transformations

DH5α chemically competent cells were purchased from Invitrogen. A volume of 50μl of the competent cells were placed in a sterile eppendorf. Following quantification of plasmid DNA, 50ng of DNA was added to the cells and the contents of the tube were mixed gently. The tube was then placed on ice for 30 min, before being heat shocked at 42^{o}C for 90 s. The tube was then placed on ice again for 2 min. 1ml of sterile SOC medium (20% tryptone, 5% yeast extract, 0.5% NaCl, 1% 0.25M KCl, 2% 1 M glucose) was then added to the tube, and the tube was incubated at 37^{o}C for 1 h. The tube was then centrifuged for 2 min at 100 x g and the pellet was then resuspended in 100μl of SOC medium. The culture was then plated out on LB agar plates containing 100μg/ml ampicillin and incubated overnight at 37^{o}C. Non-transformed competent cells were also plated out as a negative control. Only transformed colonies contain the ampicillin resistance gene, and therefore grow on the ampicillin-containing agar plates. A single colony, therefore, was transferred to a 50ml sterile tube containing 5mls of LB broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl). This primary culture was then

incubated at 37°C with agitation of 200 rpm for 8h. 400μl of the culture was transferred to a 250ml sterile conical flask containing 50mls broth and incubated overnight at 37°C with agitation of 200 x g. DNA minipreparation were then carried out on this secondary culture as described in Section 2.3.3. The remainder of the 5ml primary culture was used for glycerol stocks.

2.3.3 QIAGEN^{TM}PLASMID DNA PURIFICATION PROTOCOL

Plasmid DNA was purified using the QIAGEN-tip HiSpeed® system from Promega, according to the manufacturer's instructions for plasmid purification of animal cells. DNA was then quantified by spectrophotometric analysis as described in section 2.3.4.

2.3.4 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. The sample was read at wavelengths of 260 and 280 nm, and the concentration of the DNA in the sample was calculated out as follows;

Abs_{260nm} x dilution factor x 50 = concentration of DNA (μg/ml)

The purity of the DNA was determined by calculating the ratio of absorbance at 260 nm to 280 nm, the value of which should be greater

than 1.6. All samples were tested in triplicate and were kept on ice at all times during the experiment. DNA samples were then stored at 20°C for use in transient transfections.

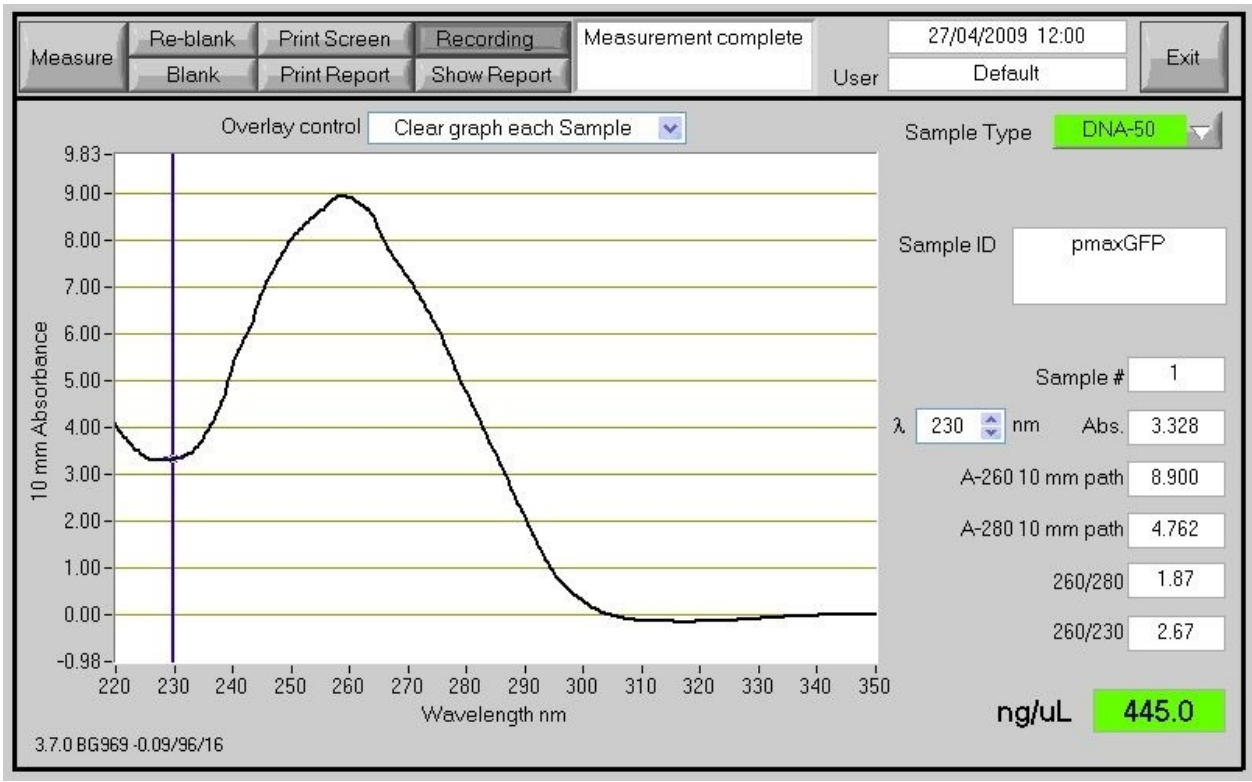


Figure 2.6: Sample Nanodrop Readout for Plasmid Quantification

2.3.5 Transient Transfections

Nucleofection™ is a transfection method based on the physical method of electroporation. Nucleofection™ uses a combination of optimized electrical parameters, generated by a special device called Nucleofector®, with cell-type specific reagents. Rat aortic smooth muscle cells were transiently transfected using a Basic Nucleofector® Kit for Primary Smooth Muscle Cells (SMC) from Amaxa. Cells were transfected

with plasmid DNA at no greater then 80% confluency. Cells were trypsinised as previously described in section 3.1.1 and counted to determine the cell density. A volume containing 1 x 10^6 cells was used per transfection. The cells were centrifuged at 300 x g for 5 min, The pellet was resuspended in room temperature Basic Smooth Muscle Nucleofactor Solution to a final concentration of 1 x 10^6 cells/100μl. 100μl of cell suspension was mixed with a total of 3μg DNA. The nucleofection sample was then transferred to a amaxa cuvette. The cuvette was inserted into the cuvette holder of the Nucleofector and the appropriate Nucleofector program was selected. After the programme was finished, the cells were transferred from the cuvette, into an eppendorf tube and 500μl of pre-warmed culture medium containing serum and supplements was added. The eppendorf tube was then incubated at 37^{o}C, until all transfections were completed. The samples were then transferred from the eppendorf tubes into 6-well plates containing 1ml of culture medium with serum and supplements (3 wells/sample). The cells were then incubated at 37^{o}C and the medium changed 16-18h post nucleofection.

Cells were routinely co-transfected with either a β-gal or green fluorescent protein (GFP) encoding plasmid as a means to determine approximate levels of transfection.

2.3.6 Small-interfering RNA Technolology

Small-interfering RNAs (siRNAs) are 20-25 nucleotide-long double-stranded RNA molecules, which have the ability to interfere with the expression of a specific gene through the RNA interference (RNAi) pathway. RNAi is a protecting mechanism against invasion by foreign genes and has been demonstrated in diverse eukaryotes such as insects,

plants, fungi and vertebrates. RNAi is the mechanism of sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNAs (dsRNA) homologous to the gene being suppressed (Scherr *et al.*, 2003). In the cell, long dsRNAs are cleaved into short 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), unwinding in the process. Activated RISC then binds to complementary transcript by base pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing (Figure 2.7).

An siRNA sequence against VEGF-A was designed as follows. A sequence was selected in the open reading frame of the cDNA which was at least 75-100 bp downstream of the start codon. This region was chosen as untranslated regions near the start codon may be richer in regulatory protein binding sites, which may interfere with binding of the siRNP endonuclease complex. An AA dimer was located and the next 19 nucleotides was recorded, such that the G/C content of the AA-N19 base was greater than 30% and less than 70%. Following this, the 21 base sequence was subjected to a blast search to ensure that only one gene is targeted. The siRNA sequence was then purchased from MWG-BIotech.

A commercially designed siRNA sequence against GSK-3β was purchased from Applied Biosystems. The siRNA sequences were transfected into RVSMCs as described in the previous section and a commercially available non-specific control siRNA sequence (MWG-Biotech) was transfected into control cells. Knockdown of target proteins was verified by western blotting.

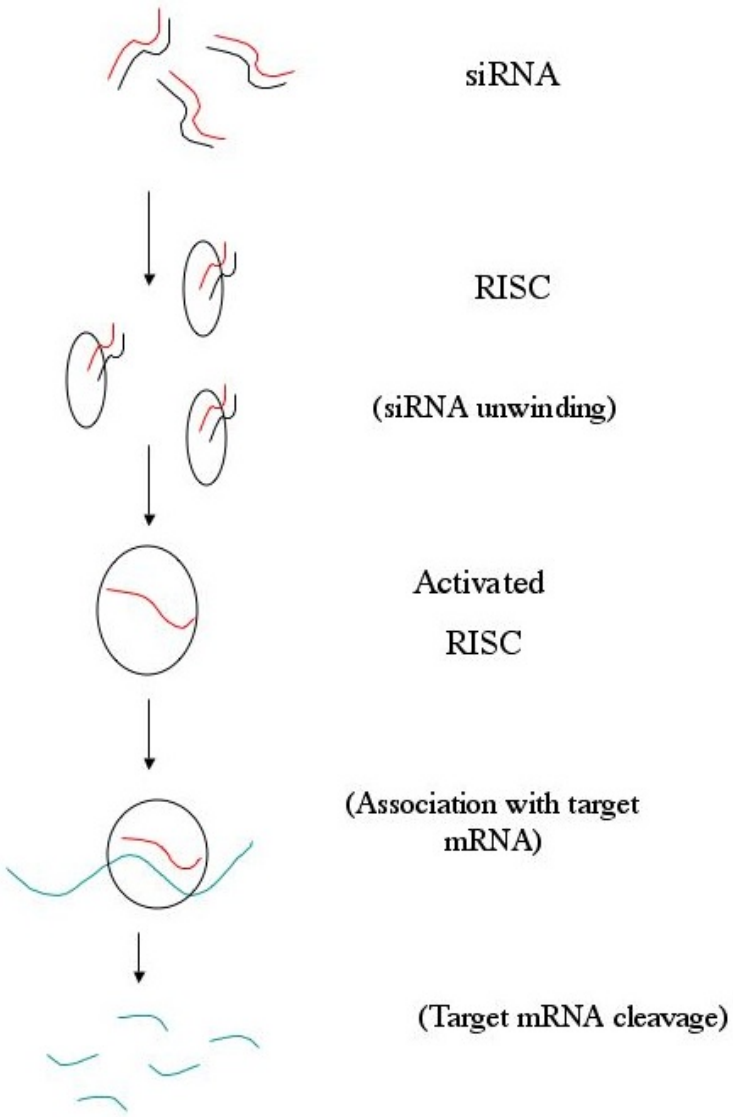


Figure 2.7: The Mechanism of small-interfering RNA (siRNA).

Table 2.2: siRNA Sequences

Target Gene	Sequence of siRNA Duplex	GC Content %	Mol. Weight (g/mol)
GSK3β	GG CCA CAG GAA GUC AGUUAtt	48	13340
VEGF	AA GUU CAU GGA CGU CUA CCA G	52	13220
Control	GGA GAU UUA CGA AAC AAU ATT	28.6	13255

2.3.7 β-galactosidase Assay

A Lac Z plasmid encoding β-galactosidase was used to monitor transfection levels. Increased levels of β-galactosidase activity was attributed to successful transfection of the gene of interest. β-galactosidase activity was analysed using a β-galactosidase assay kit (Stratagene) Following transfection and cell lysis, 20μl sample was added to a well of a 96-well plate.130μl of 1 x CPRG was added to the sample. The reaction was incubated for 30 min up to 72 h at 37^{o}C until the sample turned dark red. The reaction was subsequently stopped with 80 μl of Stop Solution (Stratagene) and optical density read at 570-595nm. Suitable positive and negative controls were included in this assay.

2.3.8 Puromycin Selection

Alternative to transfection with a Lac Z gene, cells were co-transfected with pPGK-puromycin plasmid, which offers puromycin resistance to any transfected cells. Following overnight recovery untransfected cells were selected out with treatment of cells in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 ug streptomycin, and 2μg/ml

puromycin for 48 h.

2.4 Analytical Methods

2.4.1 Luciferase Assay

To analyse transactivation of luciferase tagged reporter genes, cells were harvested 24 h post transfection. The cells were washed twice in 1 x PBS. 100 μl of 1x reproter lysis buffer (Promega) was added to each well and the cells were harvested by scraping. The samples were transferred to eppendorf tubes and were freeze thawed once. The lysates were centrifuged at 270 x g for 2 min. A volume of 40μl of sample was then added with 50μl of luciferase assay buffer (Promega) to a well of a white 96-well plate (Thermofisher Scientific) at room temperature. Light emission was measured over a period of 60 s after a lag period of 10 s by a luminometer (Labsystems Luminoskan). This light emission is a photon of light produced due to promoter activation on a luciferase tagged plasmid. The promoter activation causes the generation of the enzyme luciferase, which catalyses the conversion of D-luciferin to oxyluciferin thereby producing a photon of light.

2.4.2 SDS-PAGE and Western Blot Analysis

Preparation of Whole Cell Lysates

Cells were washed in 1 x PBS and then 100μl/cm^2 1X RIPA lysis buffer (20mM Tris, 150mM NaCl; 1mM Na_2EDTA; 1mM EGTA; 1% Triton X-100 (v/v); 2.5mM sodium pyrophosphate; 1mM β-glycerophosphate; 1mM sodium orthovanadate; 1μg/ml leupeptin) supplemented with

protease-phosphatase inhibitor cocktails (1/100 dilution of stock, Sigma Aldrich) was added to the cells. The cells were then scraped into the lysis buffer using a cell scraper and transferred to an eppendorf. The lysates were spun for 1hr at 4°C and centrifuged at 15, 700 x g for 20min. Samples were stored at -20°C for short-term storage or -80°C for long-term storage.

Bicinchoninic Acid (BCA) Protein Microassay

The bicinchoninic acid protein microassay utilizes the biuret reaction, the reduction of Cu^{++} to the cuprous cation (Cu^{+}) by protein under alkaline conditions, with the selective colourimetric detection of Cu^{+}) using a reagent containing bicinchoninic acid. Bicinchoninic acid, sodium salt, is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ion (Cu^{+}) in an alkaline environment.This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations over a broad working range of 20-2000 μg/ml. The two separate reagents used were supplied in the commercially available assay kit (Pierce Chemicals): A, an alkaline bicarbonate solution and B, a copper sulphate solution. A working solution was prepared by mixing 1 part reagent B with 50 parts reagent A. On a microtitre plate 200μl of the working solution was added to 10 μl of the whole cell lysate or bovine serum albumin (BSA) protein standard. The plate was then incubated at 37°C for 30 min. The absorbance of each well was then read at 560 nm using a Tecan Spectra plate reader. All samples and standards were tested in triplicate. Quantitation was carried out by interpolation from a BSA standard curve (0-2 mg/ml).

Western Blotting

SDS-PAGE was performed as described by Laemmli using 10% polyacrylamide gels (Laemmli *et al.,* 1970). 10% resolving and 5% stacking gels were prepared as follows:

Resolving Gel:

2.5	Buffer A (1.5M Tris pH8.8)
2.5	40% acrylamide stock
4.7	distilled water
100μl	10% (w/v) SDS
50μl	10% (w/v) ammonium persulphate
10μl	TEMED

Stacking Gel:

0.75ml	Buffer B(0.5M Tris pH6.8)
0.75ml	40%acrylamide stock
3.25ml	distilled water
50μl	10%(w/v) SDS
25μl	10%(w/v) ammonium persulphate
7.5μl	TEMED

For analysis of cell lysate protein concentration was determined by BCA assay and a equal amounts of protein were resolved on the gel. Samples were mixed with 4X loading buffer (8% SDS, 20% β-mercatoethanol, 40% glycerol, Brilliant Blue R in 0.32M Tris pH6.8) and boiled at 95°C for 5 min, then immediately placed on ice. The gel was electrophoresed in reservoir buffer (0.025M Tris pH 8.3; 0.192M Glycine; 0.1% (w/v) SDS) at 100V 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 15 min in cold transfer buffer (0.025M Tris pH8.3; 0.192M Glycine; 15% v/v methanol). Nitrocelluose membrane and 12 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 for 80 min at 125V in an ATTO semi-dry transfer system. Following transfer membranes were soaked in Ponceau S solution to confirm transfer of protein to the membrane and also to normalize for variations in protein loading. Ponceau stains all transfered bands of proteins red (Figure 2.8). Membranes were blocked for a minimum of 1h in blocking solution [5% BSA- PBS-1%Tween].

Membranes were then incubated overnight at 4°C with the appropriate dilution of primary antibody in blocking solution (Table 2.3). The blots were then vigorously washed in three changes of PBST and then incubated for 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 West Pico Supersignal reagent (Pierce Chemicals). Briefly, an equal volume of solution A and B were mixed and the blot was incubated for 5 min at room temperature. Blots were exposed to autoradiographic film (Amersham Hyperfilm ECL) to visualize bands present on the blot and developed (Amersham Hyperprocessor Automatic Developer) (Figure 2.9). Bands of interest were identified by use of molecular weight markers. Exposure times varied depending on the antibody being used but were typically between 1-5 min.

Figure 2.8: Sample Ponceau Stain.

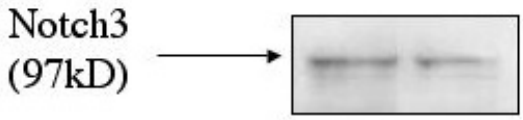


Figure 2.9: Sample Western Blot: Notch3 IC in RVSMCs

Antibody Dilutions

Table 2.3: Antibody Dilutions

Primary Antibody	Dilution	Secondary Antibody	Dilution
Anti Notch 1 (Upstate)	1:1000	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti Notch 3 (Upstate)	1:1000	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti HRT 1 (Santa Cruz)	1:500	HRP-Conjugated Anti-goat IgG (Sigma)	1:1000
Anti HRT 2 (Santa Cruz)	1:500	HRP-Conjugated Anti-goat IgG (Sigma)	1:1000
Anti HRT 3 (Santa Cruz)	1:500	HRP-Conjugated Anti-goat IgG (Sigma)	1:1000
Anti Patched (Santa Cruz)	1:500	HRP-Conjugated Anti-goat IgG (Sigma)	1:1000
Anti VEGF (Oncogene)	1:500	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti GSK3β (Alexis)	1:1000	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti phospho-GSK3β (Cell Signal)	1:1000	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti p38 (Cell Signal)	1:1000	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti phospho-p38 (Calbiochem)	1:1000	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti p44/42	1:1000	HRP-Conjugated Anti-rabbit IgG	1:1000

Primary Antibody	Dilution	Secondary Antibody	Dilution
(Cell Signal)		(Amersham Biosciences)	
Anti phospho-p44/42	1:1000	HRP-Conjugated Anti-rabbit IgG	1:1000
(Cell Signal)		(Amersham Biosciences)	
Anti HA-HRP	1:1000	(NA)	(NA)
(Roche)			
Anti α-SMC actin	1:1000	HRP-Conjugated Anti-mouse IgG	1:1000
(Sigma)		(Amersham Biosciences)	

2.4.3 Polymerase Chain Reaction(PCR)

Preparation of Total RNA

Total RNA was isolated from RVSMCs using Trizol® reagent according to the method of Chomczynski and Sacchi (1987). The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, maintains the integrity of RNA while disrupting cells and dissolving cell components. Growth media was removed and cells were washed with PBS twice. Cells were then lysed directly by adding trizol reagent to the flask, 1 ml per 10 cm^2. The lysate was transferred to a sterile falcone tube and incubated for 5 min at 15-30^{o}C to permit the complete dissociation of nucleoprotein complexes. Chloroform was added at a concentration of 0.2 ml of chloroform per 1 ml of trizol, the tube was then shaken vigorously for 15 s and then spun down at 12,000 x g for 15 min at 2 to 8^{o}C. The resulting aqueous phase was then transferred to a fresh tube. RNA was then precipitated by mixing with isopropyl alcohol, 0.5 ml per 1 ml of trizol. The samples were then incubated at 15^{o}C to 30^{o}C for 10 min and spun down at 12,000 x g for 10 min at 2 to 8^{o}C. The RNA was

then visible as a gel like pellet on the side and bottom of the tube. The supernatant was then removed and the pellet washed with at least 1ml of 75% ethanol per 1 ml of trizol used. The sample was then mixed by vortexing and spun down at 7,500 x g for 5 min at 2 to 8°C. The supernatant was removed and the pellet washed again in ethanol. After washing the pellet was air-dried and the RNA re-suspended in 30-50μl of RNase free water and incubated at 60°C for 10 min. All total RNA preparations were stored at -80°C.

Quantification of Total RNA in Samples

To determine the amount of total RNA in samples, the sample was analysed using a Nanodop 1000 Spectrophotomoter (Thermo Scientific) blanked with RNase free water. 1.5μl of sample was measured at wavelengths of 260 and 280 nm, and the concentration of the RNA in the sample was determined as follows;

$$\text{Abs}_{260nm} \text{ x dilution factor x } 40 = \text{concentration of RNA } (\mu\text{g/ml})$$

The purity of the RNA was determined by calculating the ratio of absorbance at 260nm to 280nm. A ratio of 1.9 to 2.0 was indicative of a highly purified preparation of RNA. A ratio lower than this was indicative of protein contamination. Absorbance at 230 nm reflected contamination of the sample by phenol, while absorbance at 325 nm suggests contamination by particulates. All samples were tested in triplicate and were kept on ice at all times during the experiment. RNA samples were then stored at -80°C.

Design of PCR Primer Sets

A number of web based programs, Primer 3 Output and NCBI/BLAST were utilized to design the primer sets used in this study (Table 2.4). The Primer 3 program picks primers from the given sequence, and the BLAST program allows multiple sequence alignment, which allows primers to be designed from highly conserved areas. Primers were designed with 50% GC content so the annealing temperature for all sets was 55-60°C.

Table 2.4: Primer Sequences

Target Gene	Primer Sequence	Annealing Temp. (°C)	Product Size (bp)
GSK3β	for 5′ GGATCTGCCATCGAGACATT 3′	60	169
(rat)	rev 5′ GTGGCTCCAAAGATCAGCTC 3′		
HRT 1	for 5′ CTGGACGAGACCATCGAGG 3′	55	181
(rat)	rev 5′ GCAGCATTTTCAGGTGATCCAC 3′		
HRT 2	for 5′ CTGCACACAGCTTCCCTCTGTC 3′	55	154
(rat)	rev 5′ CTCCAACTTCTGTCCCCCAGGG 3′		
HRT 3	for 5′ CGCAGAGGGATCATAGAGAAAC 3′	55	133
(rat)	rev 5′ CAGGGCTCGGGCATCAAAG 3′		
Patched 1	for 5′ GCTGGAGGAGAACAAGCAAC 3′	60	164
(rat)	rev 5′ CCAGGAGTTTGTAAGCGAGG 3′		
Gli 2	for 5′ CGCCTGGAGAACTTGAAGAC 3′	60	168
(rat)	rev 5′ TTCTCATTGGAGTGAGTGCG 3′		
VEGF	for 5′ AATGATGAAGCCCTGGAGTG 3′	60	192
(rat)	rev 5′ GCATTGCTGACAATCTTGAC 3′		
GAPDH	for 5′ TGCTGACTATGTCGTGGAGT 3′	60	176

Target Gene	Primer Sequence	Annealing Temp. Temp. (°C)	Product Size (bp)
(rat)	rev 5′ GCATTGCTGACAATCTTGAC 3′		

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

All total RNA samples were prepared by the Trizol method as previously described in Section 2.6.1. RNA preparations were then quantified by absorbance spectroscopy as described in Section 2.6.2. Reverse transcriptase was carried out using an iScript^{TM} cDNA Synthesis Kit according to the manufacturer's instructions. Briefly, 2μg of RNA was added to 1μl of reverse transcriptase enzyme and 4μl of 5x buffer in a PCR tube. RNase and DNase free H_2O was then added to make a total of 20μl sample volume. The tubes were centrifuged briefly, and placed in a PCR Sprint thermocycler (Thermo Hybaid) and the following programme was run: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min.

The samples were then stored at -20°C and used for real-time PCR as described in Section 2.6.5.

Real-Time PCR

Quantitative PCR was carried out using a Real time Rotor-GeneRG-3000^{TM} lightcycler (Corbett Research). The principle of real time amplification detection is that the amount of fluorescence is proportional to the concentration of product in a reaction. Higher fluorescence indicates a higher concentration of a product. Each PCR reaction was set up as follows:

SYBR-Green	12.5μl
RNAse free water	8.5μl
cDNA	2.0μl
Forward primer(10μM)	1.0μl
Reverse primer(10μM)	1.0μl

Each sample was assayed in triplicate, and the program used for the different primer sets was as follows;

Denaturing Phase	95^{o}C - 20s
Annealing Phase	55-60^{o}C - 30s (55 cycles)
Elongation Phase	72^{o}C - 30s

The Comparative Ct method was used for quantitative analysis (Figure 2.11) while Melt Curve analysis was carried out for qualitative analysis.

Agarose Gel Electrophoresis

All DNA gel electrophoresis was carried out using a GibcoBRL Horizon 20.25 Gel Electrophoresis Apparatus. Before use the gel box was cleaned with ethanol and the gel cast was set up as described in the manufacturers instruction manual. A 2.5% agarose gel stock was made up by dissolving 2.5 g of agarose in 100 ml of 1x Tris Acetate EDTA (TAE) (40 mM Tris-Acetate, 1 mM EDTA). The agarose was dissolved by heating in a microwave (700 mHz) at full power for 5 min. The agarose was then poured into the cast, the comb put into place and the gel allowed to set. Once set the comb was removed and the apparatus filled with 1x TAE

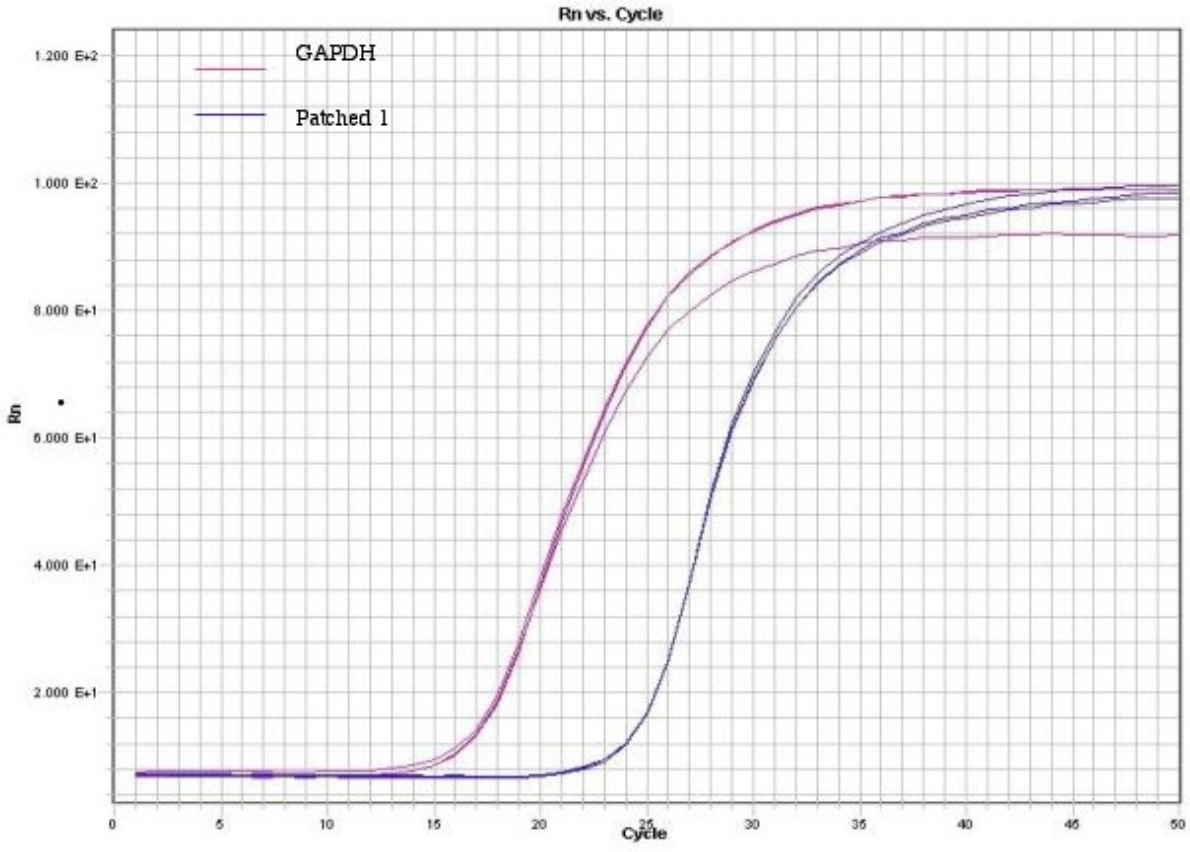


Figure 2.10: Sample Amplification Curves: Patched 1 and GAPDH in RVSMCs

RQ Manager 1.2 RQ Study Results

StudyName ShhGSKnotch.sdm

Operator Shaunta

Well	PlateID	Sample	Detector	Task	Ct
25		ShhPatched	Shh	Target	20.132397
26		ShhPatched	Shh	Target	20.304611
27		ShhPatched	Shh	Target	20.196156
73		GAPDH	GAPDH	Endogenous C	15.836678
74		GAPDH	GAPDH	Endogenous C	15.800581
75		GAPDH	GAPDH	Endogenous C	15.764856

Summary Data for Study ShhGSKnotch.sdm

Figure 2.11: Sample Readout of Ct Values

buffer. Loading dye was added to the sample (17μ of loading dye to 6μl of sample). 10μl was then loaded to each well in duplicate. The gel was run at 80 V, 110 mA and 150 W until the dye front had migrated the length of the gel. The gel was stained in a 2mg/L ethidium bromide staining bath for 10-15 min, and then placed on an Ultra Violet Products UV transilluminator for visualization. A picture was taken using a Kodak DC290 digital camera. The gel was then disposed of in the appropriate EtBr waste container.

2.4.4 Immunocytochemistry

Cells were washed in PBS and fixed with 0.3% formaldehyde for 15 min at room temperature. Cells were washed twice in PBS and permeabilised with 0.025% Triton-X-PBS. Cells were washed twice in PBS and blocked

in 5% BSA- PBS-1% Tween for 1 h at room temperature and incubated overnight with the appropriate primary antibody at 4°C. Cells were washed twice for 15 min in PBS-1%Tween and incubated in the appropriate secondary antibody for 30mins in the dark. Following, two 15 min washes in PBS, cells were visualised using the Olympus DP-50 fluorescent microscope (excitation 460-490 nm, emission 515-565 nm) (Figure 2.12).

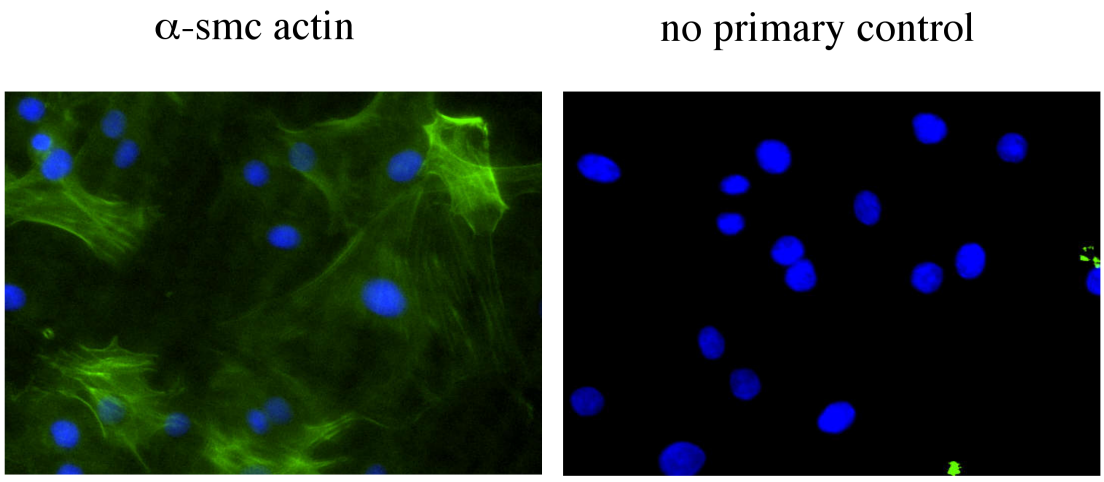


Figure 2.12: Sample Image using Immunocytochemistry: α-smc actin in RVSMCs.

2.4.5 Cell Count

To analyze RVSMC proliferation, cells were seeded equally at a density of 1 x 10^3 cells per well after serum deprivation for 48 h. Cells were then counted using a Sigma Brightline Haemacytometer after 5 days where the average of 3 wells was observed. Furthermore, in parallel experiments protein lysates were extracted and proliferating cell nuclear antigen

(pCNA) expression was determined by western blot analysis as described in Section 2.5.1.

2.4.6 Flow Cytometry

Flow cytometry is a method for counting, and sorting microscopic particles suspended in a stream of fluid. FACS™ (Fluorescent Activated Cell Sorting) is a specialised type of flow cytometry that measures the amount of light emitted by fluorescent molecules associated with individual cells. Lasers are used to excite the fluorescent molecules, which are excited at one range of wavelengths and emit at a second range. Filters in front of each of a series of detectors restrict the light that reaches the detector to only a small range of wavelengths (Tung *et al.*, 2007). Examples of the fluorescent molecules that can be detected are Annexin V, which is described in Section 2.7.3 and is used to measure the number of apoptotic cells in a cell suspension and carboxy-fluorescein diacetate succinimidyl ester (CFDA,SE), which can be used to measure proliferation.

Proliferation

Carboxy-fluorescein diacetate succinimidyl ester (CFDA,SE) is a colorless and nonfluorescent dye which passively diffuses into cells. Its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well-retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away. The dye -

protein adducts that form in labeled cells are retained by the cells throughout development, meiosis, and in vivo tracing.The label is inherited by daughter cells after cell division or cell fusion, and is not transferred to adjacent cells in a population (Hodgkin, 1996).

For proliferation analysis, RASMCs were seeded into 6-well plates and allowed to grow for 48 h. Cells were washed once with PBS and 1 ml of 5 μM CFDA,SE, prepared in PBS, was added to each well for 5 min at 37^{o}C. Following incubation, CFDA,SE was replaced with fresh media and the cells were allowed to recover for 6 h before 48 h quiescence. Cells were then treated and harvested afer 5 days by trypsinization/centrifugation and washed twice with 1 ml ice-cold PBS (containing 0.1% BSA). Cells were then placed on ice pending flow cytometry analysis. The concentration of the dye in the cells, which is inversely proportional to the rate of proliferation, was measured by flow cytometry (Becton Dickinson FACSCaliber) at an excitation peak of 492nm and emission peak of 517nm. This is demonstrated in Figure 2.13, where the control cells (red) have a lower concentration of dye and therefore, a higher proliferative rate then the treated cells (blue).

Apoptosis

For apoptosis analysis, cells were washed once in PBS, harvested by trypsinization, pelleted by centrifugation (300 x g for 5 min), washed in 1 ml ice-cold PBS (containing 0.1% BSA), and resuspended by gentle pipetting. Cells were again pelleted by centrifugation and re-suspended in 200μl of 1x Annexin-binding buffer. Propidium Iodide (0.4 μl from 100 μg/ml working solution) and 1 μl AlexaFluor 488 Annexin V were added to the cell suspension and incubated at room temperature for 15 min.

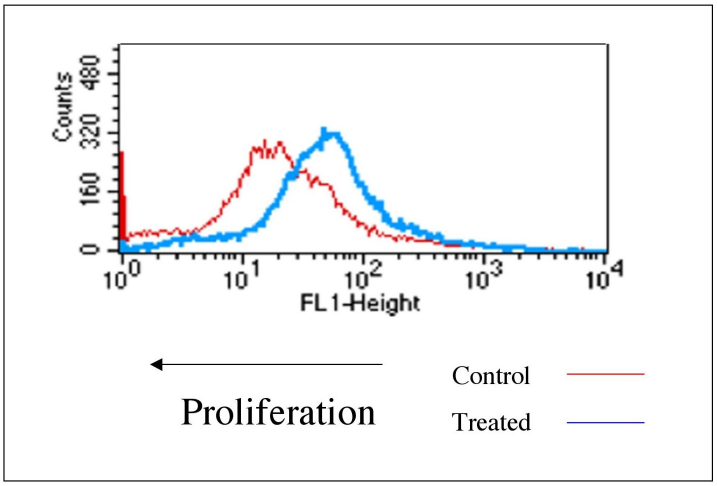
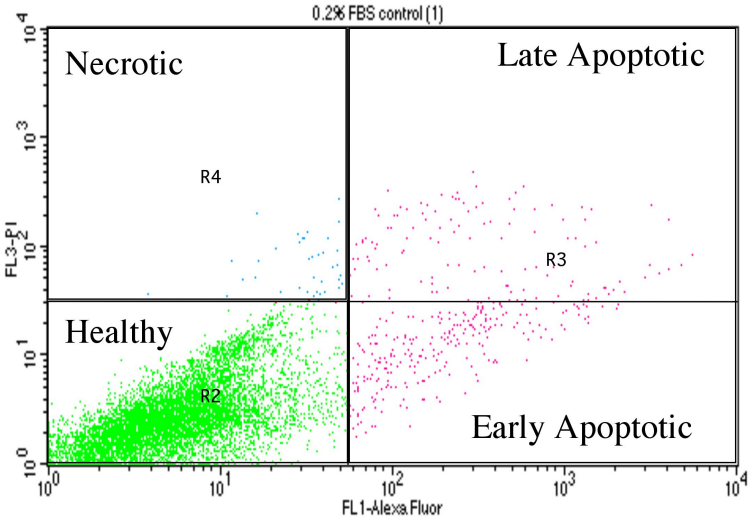


Figure 2.13: Sample FACS Proliferative Graph

Cells were then placed on ice pending flow cytometry analysis. Apoptotic cells are then counted and quantified by flow cytometry (Becton Dickinson FACS Caliber), measuring the fluorescence emission at 530nm and >575nm. Cells were divided into healthy, early apoptotic, late apoptotic and necrotic and their numbers quantified as percentage of total cell population (Figure 2.14).

2.5 Data Analysis

Results are expressed as mean ± s.e.m. Comparison between control versus treated cells were made by i) Students unpaired t test and ii) One-way Anova with statistical significance established at $p<0.05$. Statistical analysis was carried out using the GraphPad Prism 5 ® for Mac OS X application.



Gate: G1 Gated Events: 8497

Total Events: 13023 Quad Location: 55, 31

Quad	Events	% Gated	% Total
UL	41	0.48	0.31
UR	123	1.45	0.94
LL	8085	95.15	62.08
LR	248	2.92	1.90

Figure 2.14: Sample FACS Apoptosis Readout. UL=upper left quadrant, UR=upper right quadrant, LL=lower left quadrant and LR=lower right quadrant.

Chapter 3

Results

3.1 Introduction

Altered cell fate decisions, including proliferation and apoptosis, are critical in many conditions including vascular remodelling, the pathophysiology of atherosclerosis and other cardiovascular disorders. Pathological changes in vessel structure are induced, in part, by signalling pathways that govern SMC growth. The Notch signalling pathway is a highly conserved develomental pathway, which controls multiple cell differentiation processes during embryonic and adult life. Notch 1 and 3 IC receptors control the modulation of VSMC fate in response to growth factor stimulation (Sweeney *et al.*, 2004).

Binding of a Notch ligand to the extracellular domain of full-length Notch receptor causes cleavage of the receptor, and translocation of the cytoplasmic Notch intracellular (Notch IC) domain to the nucleus. This Notch IC is the active form of the receptor, which initiates transcription of the downstream target genes (Gordon *et al.*, 2008).

GSK-3β is a constitutively active serine-threonine kinase, which is

involved in a large number of cellular processes. It has been implicated in a number of disease states including diabetes, Alzheimers disease, bipolar disease and cancer. GSK-3β has been shown to be able to bind and phosphorylate active Notch1IC *in vitro* (Foltz *et al.*, 2002). This current study, therefore, examined whether GSK-3β modulates Notch signalling in VSMCs.

The aim of this chapter was to examine the role of GSK-3β in controlling Notch signalling in RVSMCs

3.2 Results

3.2.1 GSK-3β and Notch signalling components are present in RVSMCs

Vascular smooth muscle cells derived from the rat aorta were characterised to confirm a pure culture. Vascular smooth muscle cells stain positively for the contractile protein α-smooth muscle cell specific actin (α-smc actin) and display a noncontact-inhibited growth pattern (Figure 3.1 A.). A blue fluorescent stain, 4', 6-diamidino-2-phenylindole (DAPI) was used for nuclear staining.

Previous studies in our lab have shown the presence of Notch 1 and 3 receptors in serum-stimulated adult RVSMCs. This initial study confirmed the presence of the intracellular domains (IC) of Notch receptors 1 and 3 and the Notch downstream targets HRT1, 2 and 3 in RVSMCs (Figure 3.2 A and B). The presence of GSK-3β in RVSMC was examined and was revealed by western blotting, immunocytochemistry and real-time PCR of GSKβ mRNA transcripts (Figure 3.1 B).

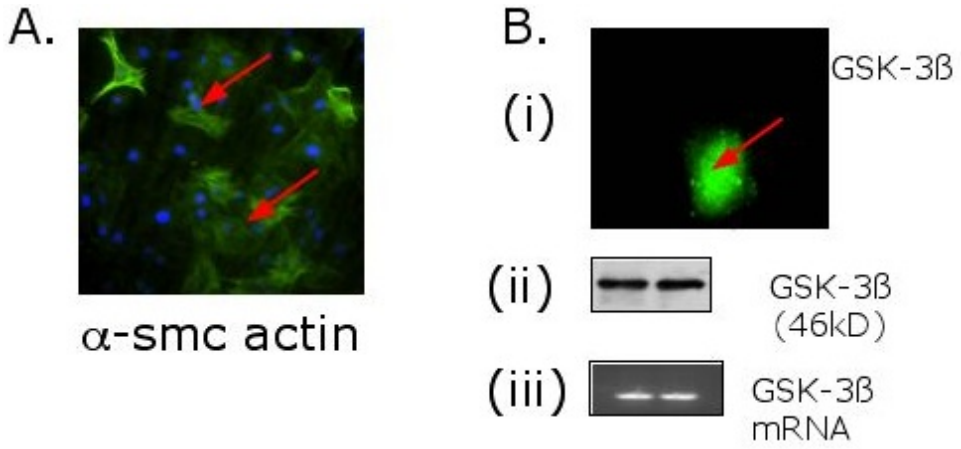


Figure 3.1: Presence of GSK-3β in RVSMCs. A) Immunocytochemistry staining of α-SMC actin (4x Mag.) B) (i) Immunocytochemistry staining of GSK-3β (100x Mag.) (ii) Western blot analysis of GSK-3β (iii) Real-time PCR analysis of GSK-3β. The experiments were performed in triplicate, with images representative of n=3.

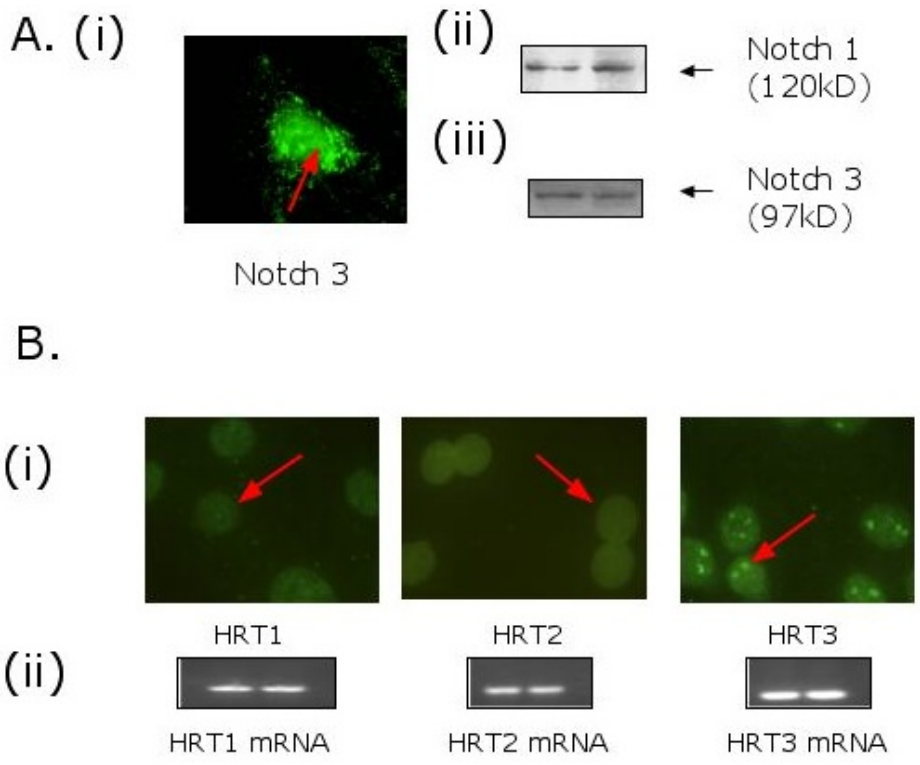


Figure 3.2: Presence of Notch signalling components in RVSMCs. A) (i) Immunocytochemistry analysis of Notch 3 (100x Mag.) (ii) Western blot analysis of Notch1. (ii) Western blot analysis of Notch3. B) (i) Immunocytochemistry staining of HRT 1, HRT 2 and HRT 3 expression (100x Mag.) (ii) Real-Time PCR Analysis of HRT1, HRT2 and HRT3. The experiments were performed in triplicate, with images representative of n=3.

3.2.2 Enforced Expression of GSKβ - Transient transfection of VSMCs

While transient transfection of certain transformed cell lines is highly productive, transfection efficiency is often unacceptably low in primary, such as VSMCs or early passage nonimmortalized cultured cells. This may be due to poor uptake of the vector, poor processing or expression of the vector once taken up by the cell, and inactivation or destruction of the vector by cellular enzymes (Chen *et al.*, 1999).

Transfection of RVSMCs was initially performed using Lipofectamine™ from Invitrogen. Lipofectamine™ is a cationic-lipid transfection reagent, which facilitates formation of a lipid-DNA complex between the reagent and the DNA vector. Successful gene delivery by use of the cationic liposomes involves condensation of the DNA into the genosome and its protection from degradation by intracellular nucleases, adhesion of the DNA-lipid complex onto the cellular surface, genosome internalization, fusion of the internalized DNAcationic liposome complex with the endosome membrane, escape of DNA from the endosome; and entry of DNA into the nucleus followed by gene expression (Zhdanov *et al.*, 2002). By imaging of GFP protein, transfection efficency of RVSMCs was assessed using Lipofectamine™ within a range of concentrations. The maximal efficiency achieved was approximately 30% (see appendix). However, the cost of the reagent at this concentration, and the level of cell toxicity observed was considerable. We then transfected RVSMCs using a GFP plasmid and the Amaxa nucleofector system. This technology uses an empirically derived combination of cell line-specific solutions and nucleofector programmes to electroporate nucleic acid substrates directly into the cell nucleus (Han *et al.*, 2008). Electroporation involves subjecting

the cell membranes to a high-voltage electric field, which results in their temporary breakdown and the formation of pores that are large enough to allow DNA to enter the cytoplasm (Potter, 2003). Optimal transfection efficiency of approximately 70%, with low levels of cytotoxicity, was determined using the programme setting of A-33 (as recommended by the manufacturer for the transfection of SMCs) (Figure 3.3 A). RVSMCs were transfected with three plasmids: an empty vector, pcDNA3, a plasmid expressing the wild type form of GSK-3β (wt.GSK-3β) and a plasmid expressing a mutant form of the enzyme (mut.GSK-3β). The serine on site 9 of the mutant enzyme has been changed to an alanine. This forms an enzyme which cannot be phosphorylated/negatively regulated at serine 9 and therefore is a constitutively active form of GSK-3β. GSK-3β ectopic expression was determined by analysing the levels of HA-tag expression by Western blotting (Figure 3.3 B).

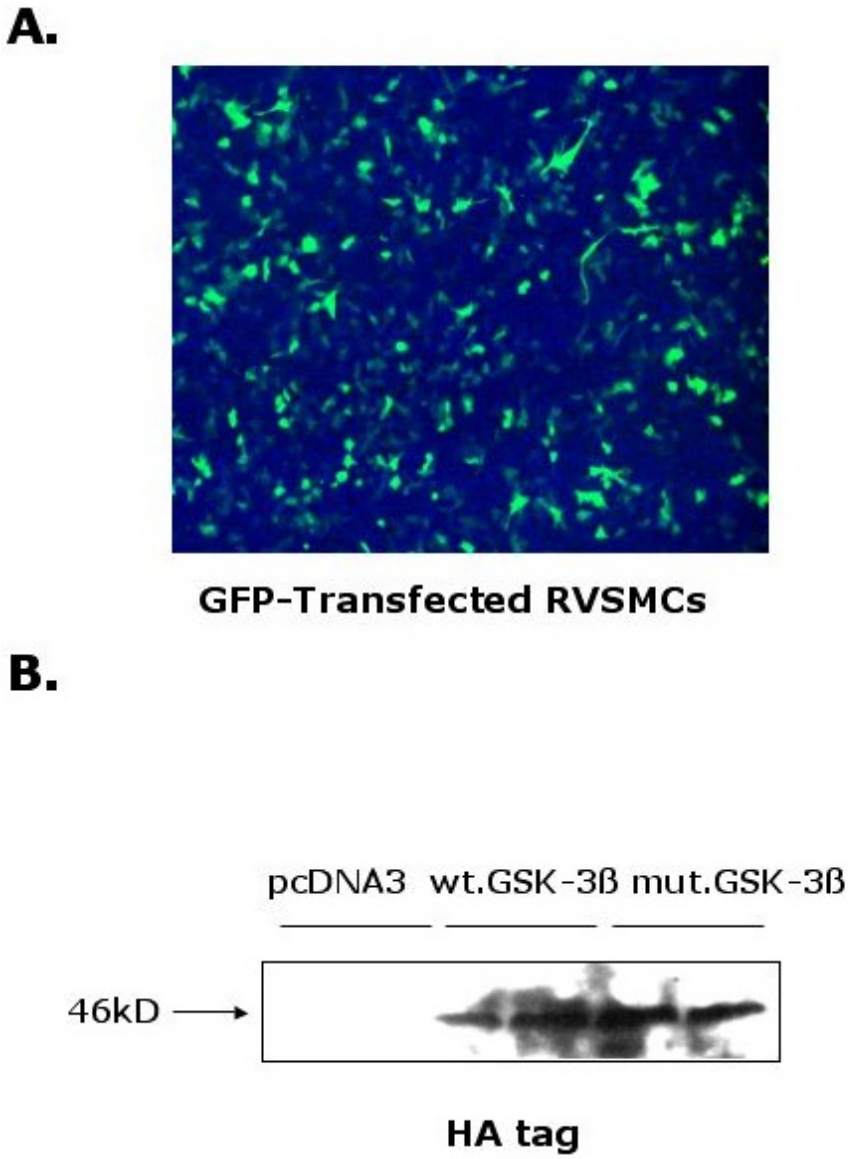


Figure 3.3: Transfection of VSMCs. A) GFP Expression in RVSMCs following transfection by nucleofection of a GFP-expression plasmid. B) Western blot of HA expression in RSMCs following transfection by nucleofection of the empty vector pcDNA3 or wild-type GSK-3β or mutant GSK-3β. The cells were co-transfected with the puromycin resistance plasmid, pGK3puro. Following overnight recovery, cells were incubated in puromycin-containing growth medium (2μg/ml, 72hrs). The experiments were performed in triplicate, with images representative of n=3.

3.2.3 Knockdown of GSK-3β Gene Expression in RVSMCs

Small, hairpin RNAs (shRNAs) can regulate gene expression via elements of the RNAi machinery (Paddison *et al.*, 2002). The shRNA hairpin structure is incorporated into a vector allowing introduction into cells and is then cleaved by the cellular machinery into siRNA. This can induce sequence-specific gene silencing in mammalian cells (as described in Section 2.4.1). RVSMCs were transfected by nucleofection with a Sigma MISSION® shRNA plasmid targeted against GSK-3β. Knockdown of GSK-3β protein of < 0.05 fold was achieved using this method (see appendix 3).

Non-commercially designed siRNA against GSK-3β was designed using a method that was previously successful in the lab (described in Section 2.4.1). RVSMCs were transfected with the designed sequence of siRNA and a knockdown of GSK-3β of < 0.05 fold was achieved (see appendix 3).

RVSMCs were transfected with a GSK-3β dominant-negative expression plasmid (K85M-GSK-3β), where a lysine residue in the catalytic site of the enzyme is replaced by a methionoine, and knockdown of GSK-3β of < 0.05 fold was achieved (see appendix 3).

A commercially designed siRNA sequence targeted against GSK-3β was purchased from Ambion. RVSMCs were transfected with the sequence and a significant knockdown of GSK-3β protein levels was observed (0.473 ± 0.04 fold) (Figure 3.4).

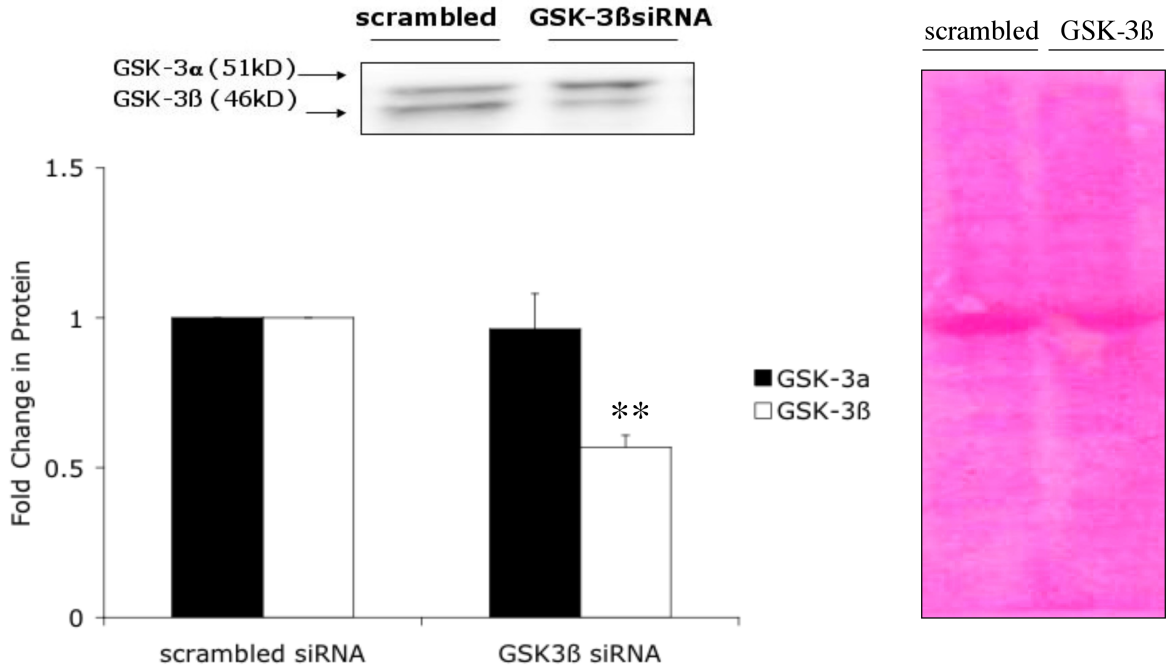


Figure 3.4: GSK-3β Knockdown by siRNA. Western blot of GSK-3β following transfection with Ambion-designed GSK-3β-targeted siRNA. Cells were allowed to recover for 72 h before harvesting of cell lysates. Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate, with images representative of n=3.$**p< 0.01$ as compared to control (students t test).

3.2.4 GSK-3β modulates Notch Receptor Levels

The next stage of this study sought to establish a role for GSK-3β in the modulation of Notch signalling. The effect of ectopic expression, knockdown by siRNA and pharmacological inhibition of GSK-3β on Notch 3IC expression was investigated using SB216763, a potent, selective inhibitor of GSK-3β, which works in an ATP-competitive manner (Cross *et al.*, 2001).

Following ectopic expression of the constitutively active form of GSK-3β, a significant increase in the levels of Notch3 IC was detected by western blotting (2.424 $\pm$ 0.639 fold, n=3) (Figure 3.5). There was a significant decrease of Notch3 IC protein levels, following both siRNA knockdown and pharmacological inhibition of GSK-3β by SB216763 (25μM) (to 0.669 $\pm$ 0.049 fold, and 0.45 $\pm$ 0.16 fold, n=3) (Figure 3.6 and Figure 3.7).

Pharmacological inhibition of GSK-3β reduced the levels of Notch1 IC (0.75 $\pm$ 0.08 fold) (Figure 3.8). The effect of GSK-3β inhibition on Notch receptor transcriptional expression was investigated and it was found that Notch1 mRNA was reduced following treatment with the GSK-3β pharmacological inhibitor (to 0.67 $\pm$ 0.04 fold)(Figure 3.9).

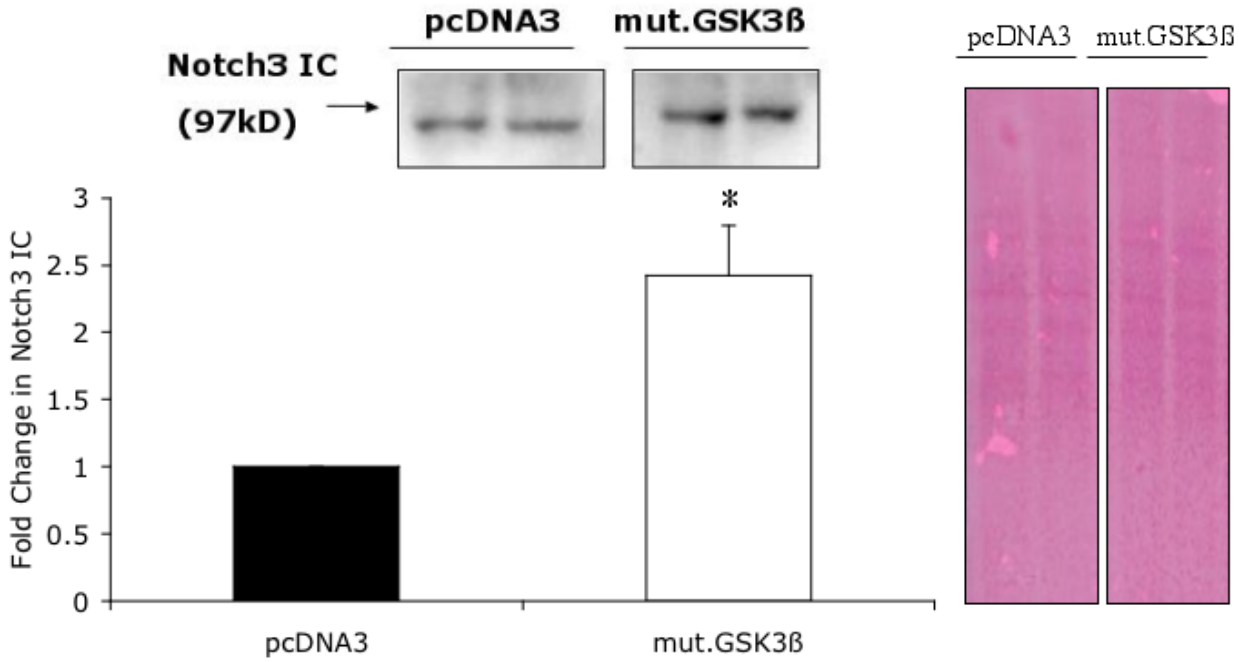


Figure 3.5: Ectopic Expression of GSK-3β increases Notch3 IC levels. Western blot analysis of Notch3 IC following transfection of RVSMCs with the empty vector pcDNA3 or mutant GSK-3β and co-transfection with the puromycin resistance plasmid, pGK3puro. Following overnight recovery, cells were incubated in puromycin-containing growth medium (2μg/ml, 72 h). Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate, with images representative of n=3. $*p< 0.05$ as compared to control (students t test).

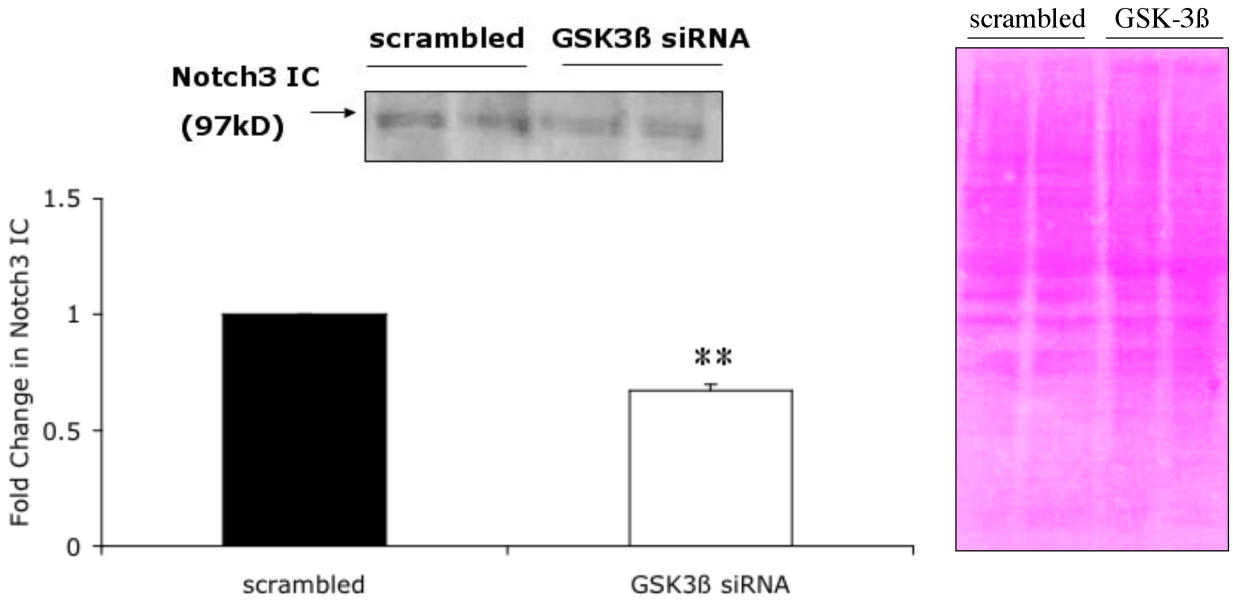


Figure 3.6: GSK-3β Knockdown decreases Notch3 IC levels Western blot analysis of Notch3 IC following transfection of RVSMCs with GSK-3β siRNA. Cells were allowed to recover for 72 h. Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate, with images representative of n=3. $**p< 0.01$ as compared to control (students t test).

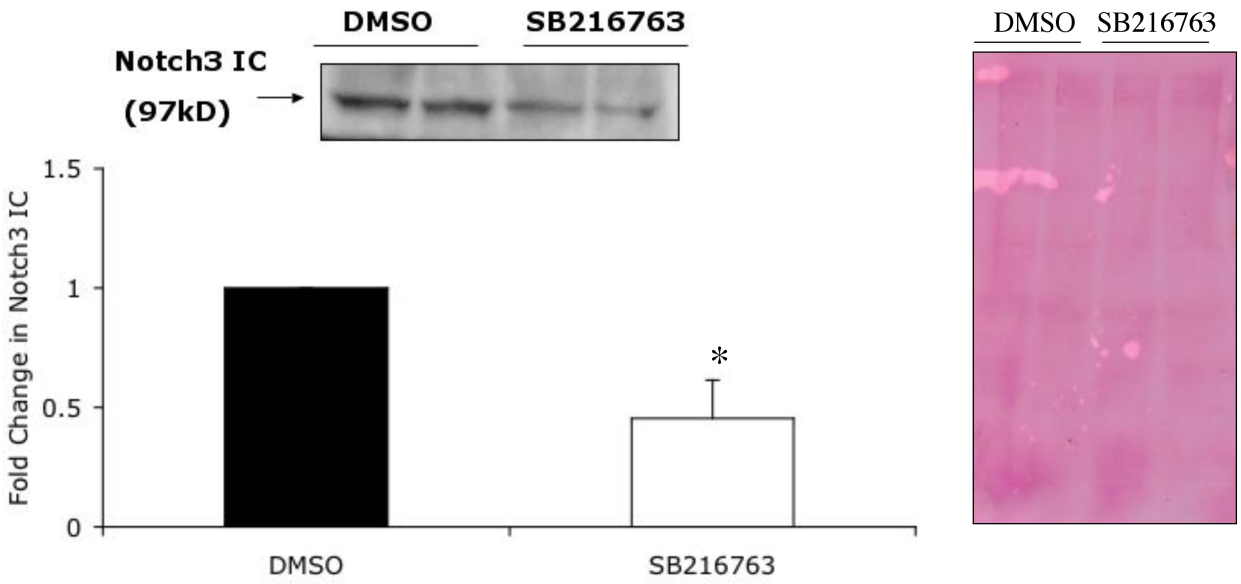


Figure 3.7: Pharmacological inhibition of GSK-3β decreases Notch3 IC levels. Western blot analysis of Notch3 IC following treatment with SB216763 (25μM) for 24 h. Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate, with images representative of n=3. $*p< 0.05$ as compared to control (students t test)

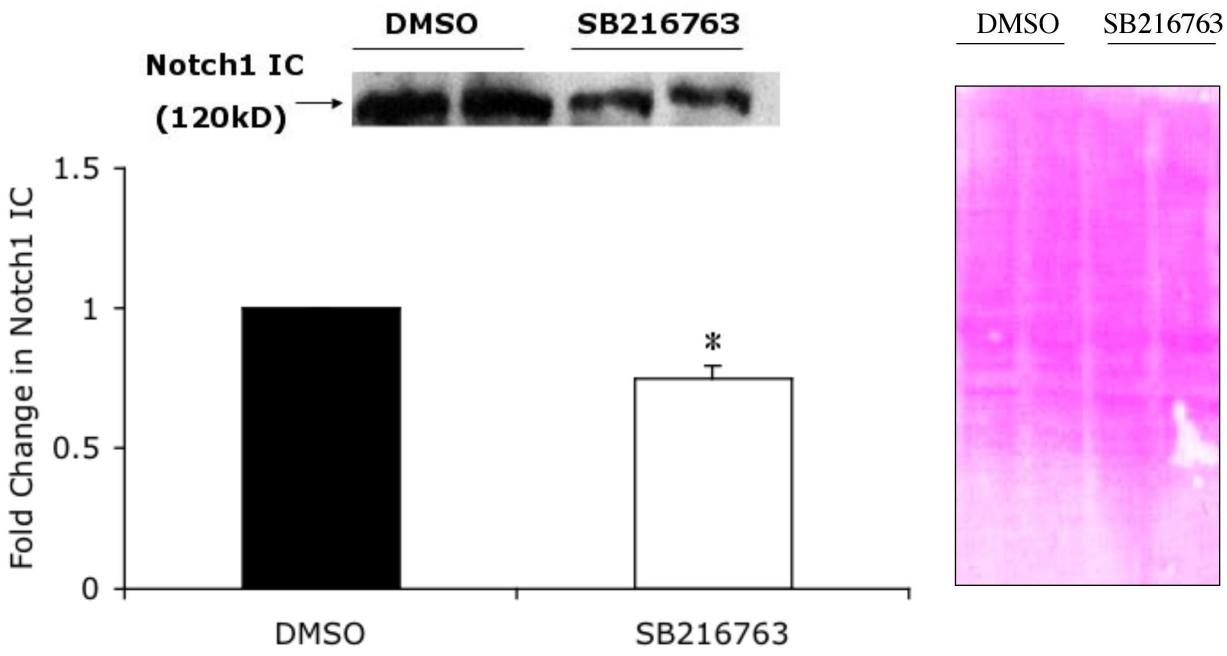


Figure 3.8: GSK-3β modulates Notch1 IC. Western blot analysis of Notch1 IC and following treatment with SB216763 (25μM) for 24 h. Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate, with images representative of n=3. $*p< 0.05$ as compared to control (students t test).

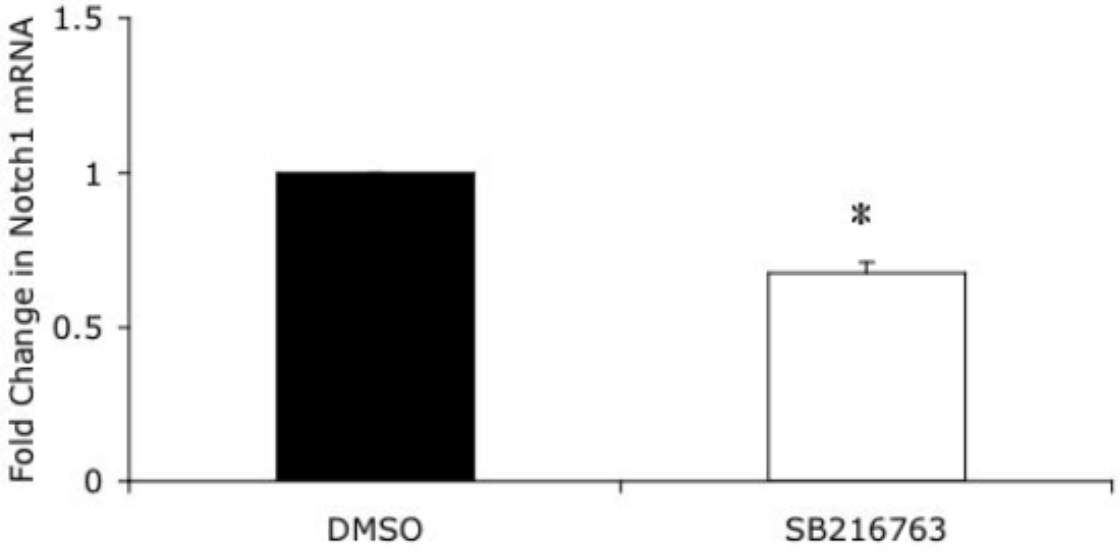


Figure 3.9: GSK-3β modulates Notch1 mRNA Expression. Real-time PCR analysis of Notch1 following treatment with SB216763 (25μM) for 24 h. The experiment was performed in triplicate. $*p< 0.05$ as compared to control (students t test).

3.2.5 GSK-3β modulates Notch Target Genes

Having observed the positive effect of GSK-3β on Notch receptor expression, modulation of Notch target gene expression by GSK-3β was determined. Ectopic expression of GSK-3β resulted in an upregulation of HRT1 and HRT2 mRNA expression (2.883 $\pm$ 0.47 fold and 2.383 $\pm$ 0.597 fold, n=3)(Figure 3.10). siRNA knockdown of GSK-3β was found to reduce the expression of Notch target genes HRT1, HRT2 and HRT3 (0.39 $\pm$0.036 fold and 0.583 $\pm$ 0.087 fold, n=3)(Figure 3.11.), as did pharmacological inhibition of GSK-3β (0.627 $\pm$ 0.096 fold, 0.565 $\pm$ 0.093 fold and 0.452 $\pm$ 0.093, n=3)(Figure 3.12).

The ability of GSK-3β to regulate downstream targets of Notch signalling was then confirmed by protein analysis of HRT3 by western blotting. Ectopic expression of constitutively active GSK-3β increased the levels of HRT3 protein(2.02 $\pm$ 0.226 fold, n=3)(Figure 3.13). siRNA knockdown of GSK-3β and pharmacological inhibition of GSK-3β reduced the levels of HRT3 protein expression (0.592 $\pm$ 0.047 fold and 0.664 $\pm$ 0.146 fold, n=3) (Figure 3.14) and (Figure 3.15).

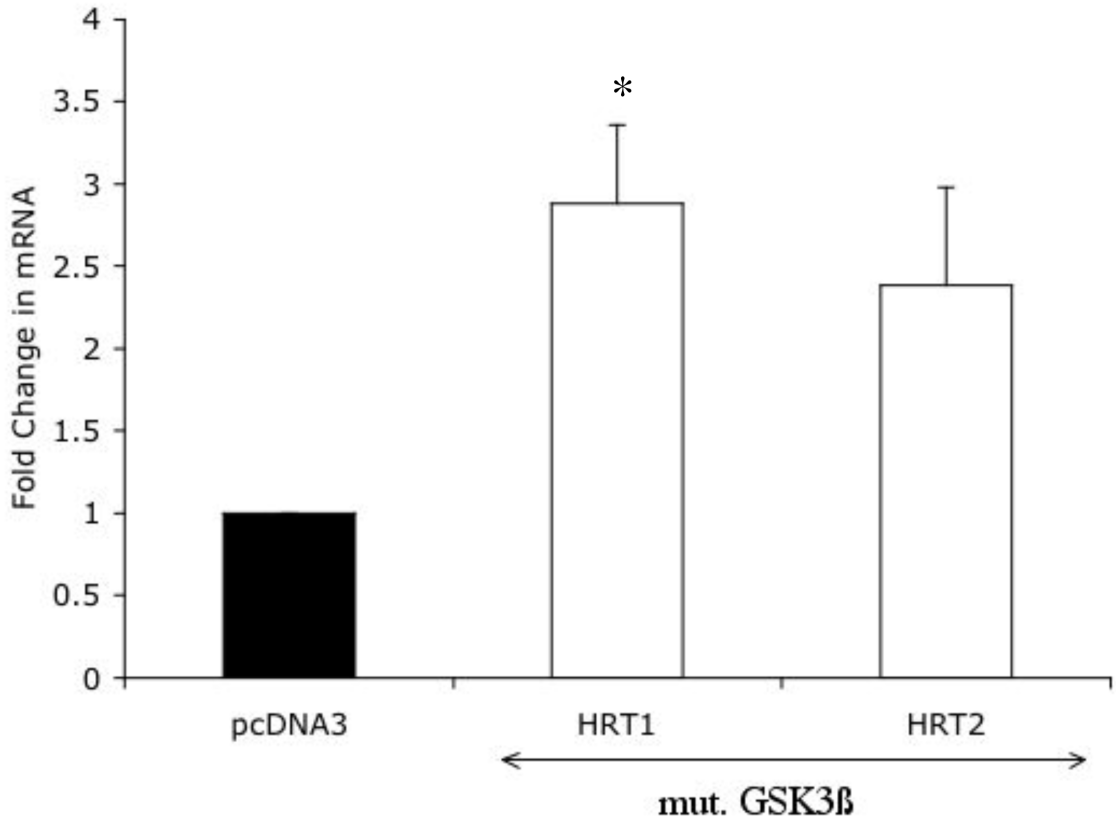


Figure 3.10: Ectopic expression of GSK-3β increases Notch Target Gene mRNA. Real-time PCR analysis of HRT1 and HRT2 mRNA following transfection of RVSMCs with the empty vector pcDNA3 or mutant GSK-3β and co-transfection with the puromycin resistance plasmid, pGK3puro Following overnight recovery, cells were incubated in puromycin-containing growth medium (2μg/ml, 72 h). The experiment was performed in triplicate. $*p < 0.05$, as compared to control (students t test)

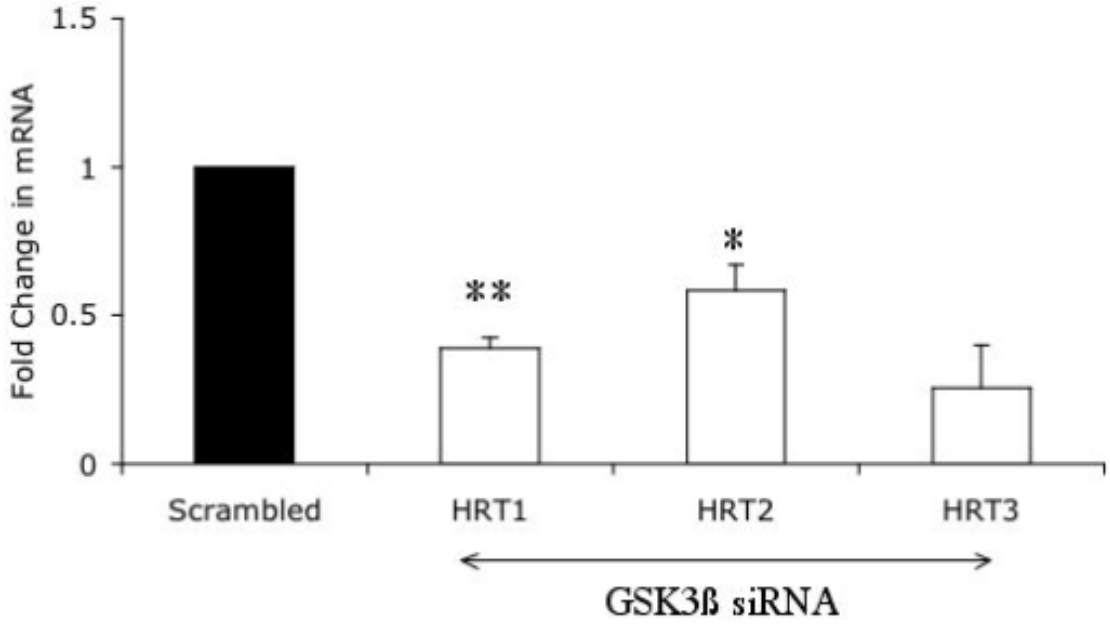


Figure 3.11: GSK-3β knockdown decreases Notch Target Gene mRNA. Real-time PCR analysis of HRT1, HRT2 and HRT3 and mRNA following transfection of RVSMCs with GSK-3β-targeted siRNA. Cells were allowed to recover for 72 h. The experiment was performed in triplicate. $*p< 0.05, **p< 0.01$ as compared to control (students t test).

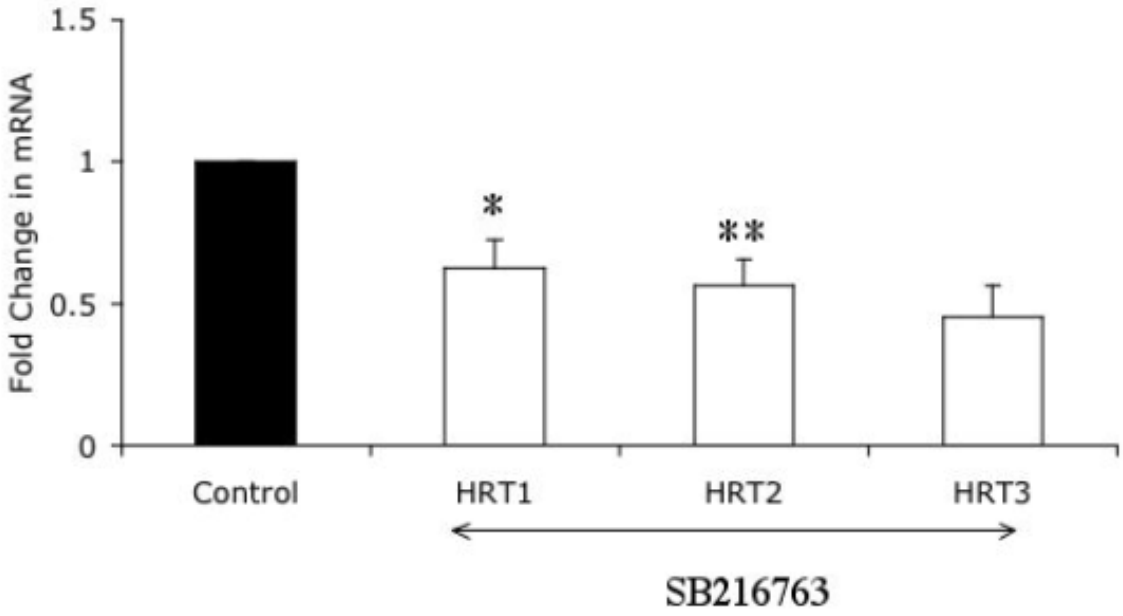


Figure 3.12: Pharmacological inhibition of GSK3β decreases Notch Target Gene mRNA. Real-time PCR analysis of HRT1, HRT2 and HRT3 mRNA following treatment with SB216763 (25μM) for 24 h. The experiment was performed in triplicate. $*p< 0.05$, $**p< 0.01$ as compared to control (students t test).

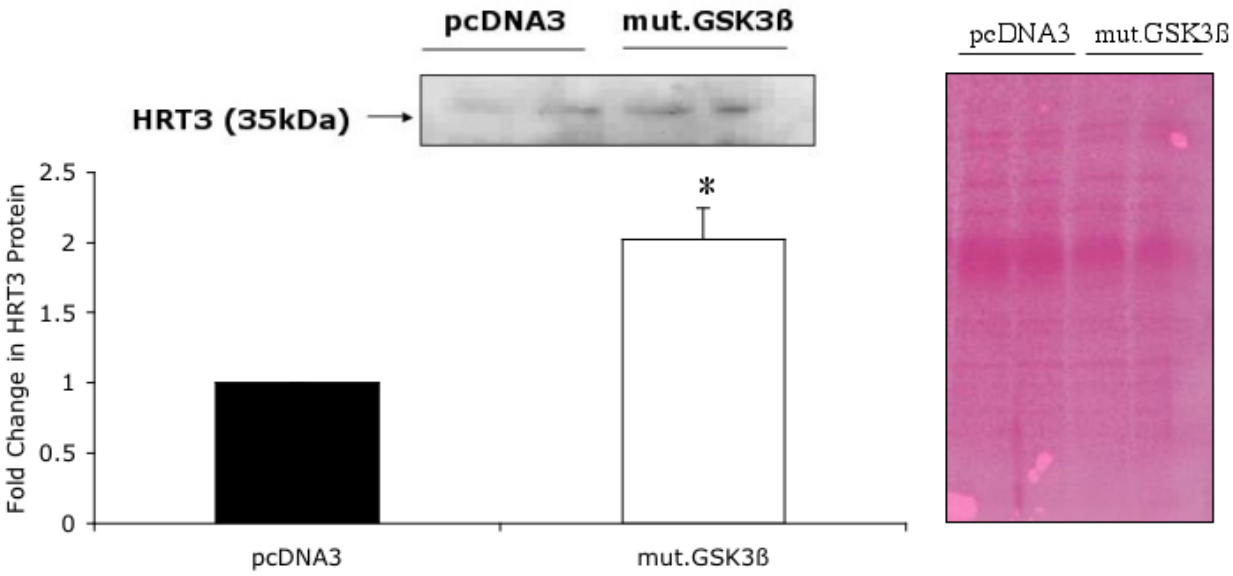


Figure 3.13: Ectopic expression of GSK-3β increases Notch Target Gene Protein Expression. Western blot analysis of HRT3 following transfection of RVSMCs with the empty vector pcDNA3 or mutant GSK-3β. Cells were allowed to recover for 72 h. Expression was normalized to Ponceau levels (right frame). Images representative of n=3. $*p< 0.05$, as compared to control (students t test).

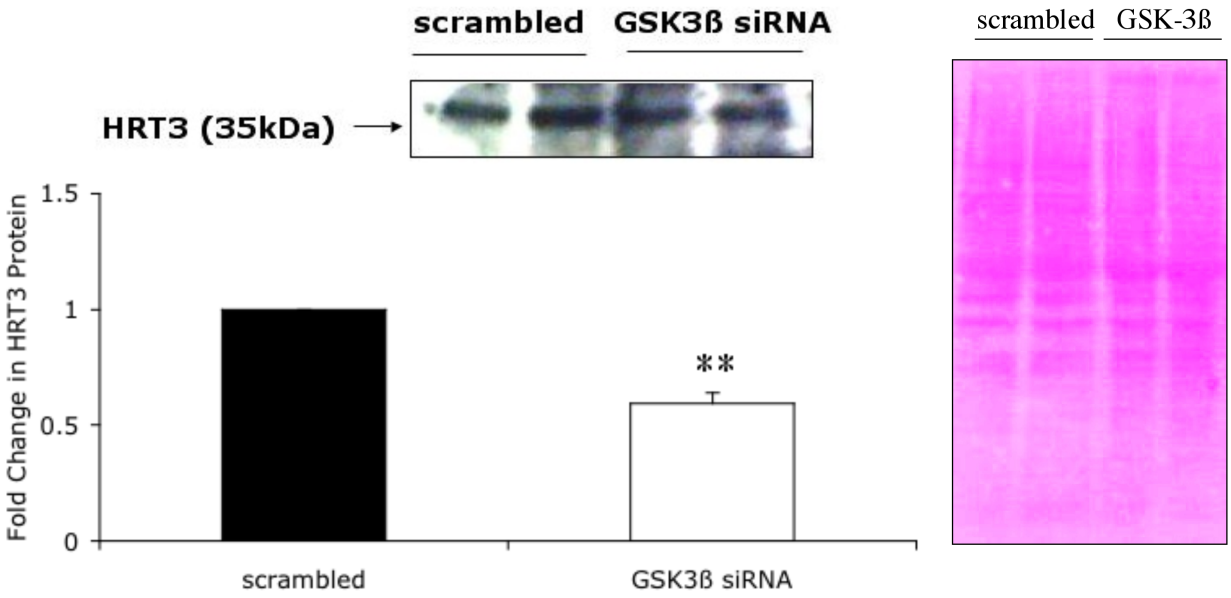


Figure 3.14: GSK-3β knockdown decreases Notch Target Gene Protein Expression. Western blot analysis of HRT3 following transfection of RVSMCs with scrambled or GSK-3β siRNA and 72 h recovery. Images representative of n=3. Expression was normalized to Ponceau levels (right frame) $*p< 0.05$, $**p< 0.01$ as compared to control (students t test).

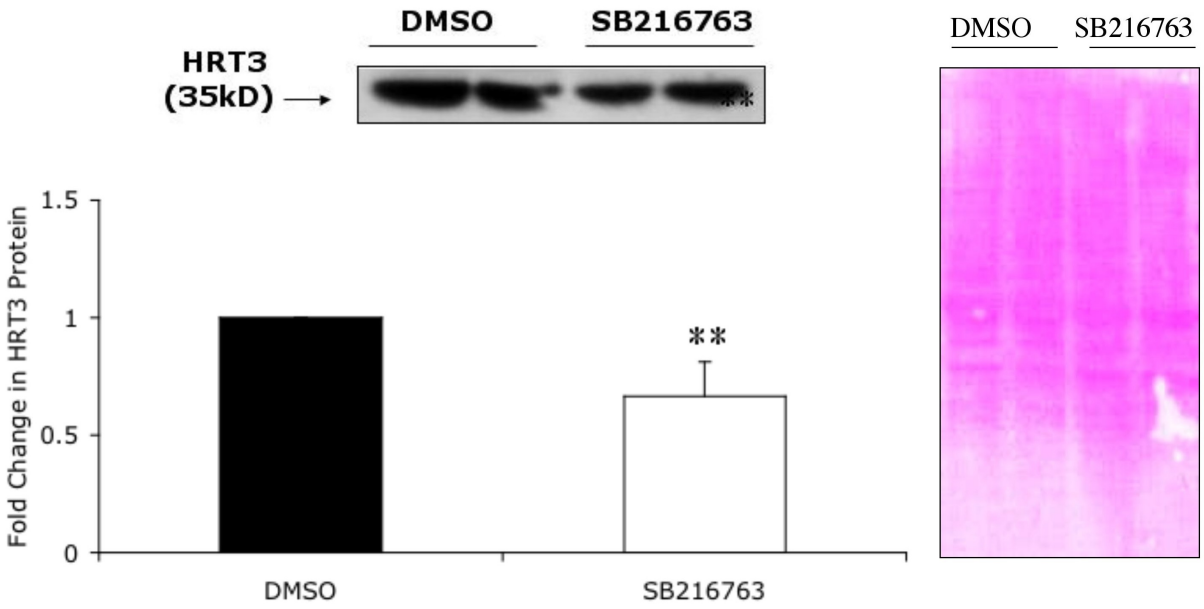


Figure 3.15: Pharmacological inhibition of GSK-3β decreases Notch Target Gene Protein Expression. Western blot analysis of HRT3 following treatment of RVSMCs with SB216763 (25μM) for 24 h. Images representative of n=3. Expression was normalized to Ponceau levels (right frame)$**p< 0.01$ as compared to control (students t test).

3.2.6 GSK3-β modulates CBF-1 promoter activity

Canonical Notch signalling shows Notch receptor binding to a CBF-1 protein complex, allowing the transcriptional activation of Notch target genes. Notch 1 and 3 receptor signalling was previously shown to modulate vascular smooth muscle cell growth via a CBF-1/RBPJ$_\kappa$ dependent pathway (Sweeney *et al.*, 2004). In order to further clarify the role of GSK-3β in Notch signalling, the involvement of GSK-3β in Notch signalling through regulation of a CBF-1-responsive system was investigated. Using an artificial CBF-1-responsive luciferase reporter plasmid, ectopic expression of constitutively active GSK-3β induced a significant increase in CBF-1-regulated promoter transactivation (2.554 $\pm$ 0.359 fold, n=3). This effect was attenuated following pharmacological inhibition of GSK-3β with SB216763. Pharmacological inhibition of GSK-3β also significantly reduced basal levels of CBF-1-responsive promoter activity (0.430 $\pm$ 0.261 fold, n=3)(Figure 3.16).

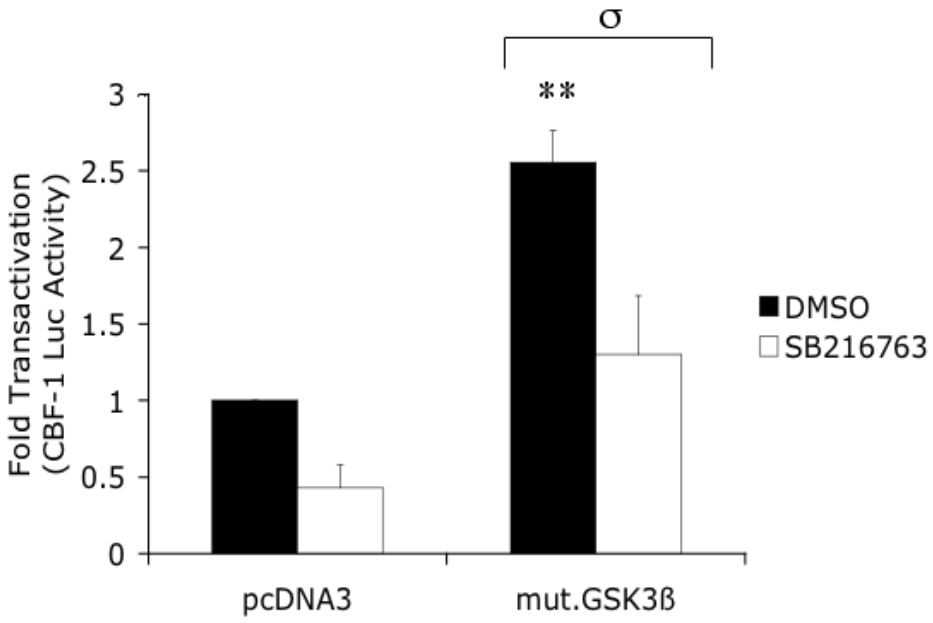


Figure 3.16: GSK-3β modulates CBF-1-regulated promoter activity. Relative luciferase activity analysis of the CBF-regulated promoter following transfection of RVSMCs with the empty vector pcDNA3 or mutant GSK-3β and co-transfection with the CBF-1 promoter reporter plasmid, pGA-981-6.Following transfection, cells were incubated in DMSO- or SB216763 (25μM)-containing media for 48 h. The experiment was performed in triplicate. $**p< 0.01$ as compared to pcDNA3 control. $\sigma< 0.05$ as compared to SB216763 treated control (One-way Anova).

3.3 Discussion

The Notch signalling pathway has previously been implicated in several aspects of vascular development (Proweller *et al.*, 2005) and Notch has been shown to be pro-proliferative and apoptotic in vascular smooth muscle cells (Morrow *et al.*, 2005a) and to be modulated by both biomechanical stimuli *in vitro* and following vascular injury *in vivo* (Morrow *et al.*, 2005a), (Wang *et al.*, 2002).

Strict control of Notch regulation is required to maintain a non-pathological level of signalling. This is achieved through many levels of regulations including cell-type specific expression of ligands. Prior to ligand binding, various mechanisms regulate the receptor, firstly through transcriptional control of full-length Notch receptor (FL-Notch) expression (Seugnet *et al.*, 1997). Post-translational modifications, including fringe-dependent glycosylation of the EGF-repeats on the full-length receptor, and ubiquitination leading to endocytosis positively modulates signalling by the receptor (Bruckner *et al.*, 2000). For ligand binding to occur, a furin-based cleavage of FL-Notch is required and allows presentation of a heterodimeric receptor at the membrane of the cell (Logeat *et al.*, 1998). Association of heteromeric Notch protein is calcium dependent (Rand *et al.*, 2000). Following binding of the ligand to the receptor, endocytosis of the ligand must occur. This is thought to pull on the Notch EC domain, causing a conformational change that allows exposure of the S2 site for cleavage of the receptor by TACE/ADAM proteases. This is quickly followed by presenilin-dependent γ-cleavage at the transmembrane site on the receptor and release and translocation of the active Notch receptor to the nucleus (Kovall, 2008). Within the nucleus, following activation, phosphorylation and ubiquitination of the

Notch IC is required to promote degredation of the Notch IC in the proteosome, allowing rapid turnover of Notch IC (Baron *et al.*, 2002).

An important form of regulation is cell-type specific expression of Notch receptors. In higher vertebrates multiple Notch homologs have been identified. Notch1 and Notch2 are expressed in a number of tissues. Notch1 is expressed broadly in most tissues, including brain, liver, heart, lung, kidney, intestine, bone marrow, skeletal muscle, spinal cord, eye and thymus while, Notch2 is expressed predominantly in the brain, liver, kidney and stomach, which partially overlaps the expression pattern of Notch1 (Wu and Bresnick, 2007). There is little evidence of Notch2 activity in adult vascular smooth muscle. However, inactivation of Notch2 in cardiac neural crest cells (precursors for vascular smooth muscle cells in greater arteries) results in abnormally narrow aortas and pulmonary arteries due to a decrease in smooth muscle proliferation (Varadkar *et al.*, 2008). Notch 4 transcripts are primarily restricted to endothelial cells in embryonic and adult life (Uyttendaele *et al.*, 1996). Notch 3 is predominantly expressed in adult arterial smooth muscle cells in humans (Joutel *et al.*, 2000). CADASIL is caused by mutations in the Notch receptor, Notch 3, gene and is the most common form of inherited stroke and vascular dementia in humans (Gridley, 2007) and is characterised by systemic VSMC degeneration (Wang *et al.*, 2008).

It is possible to conceive that structural differences or varied expression profiles of the receptors may lead to differential modes of regulation. Notch1 and 2 have the broadest expression pattern, and the most diverse biological context and also have the highest homology to each other, while Notch3 and Notch4 are structurally diverged from Notch1 and Notch2 , both in the extracellular and intracellular domains.

Notch4 has fewer epidermal growth factor (EGF)-like repeats and a shorter intracellular domain than other mouse Notch homologues (Uyttendaele *et al.*, 1996).

The presence of Notch 1 and Notch 3 receptors has previously been identified in VSMCs, and this was again confirmed in this study (Figure 3.2). It has also previously been shown that HES and HRT are direct downstream target genes of Notch 1 and 3 signalling in VSMC (Sweeney *et al.*, 2004).

Based on their previously studied expression in VSMCs, and their potential involvement in vascular disease, the decision was made to examine further the mechanisms involved in the regulation of Notch1 and Notch3. Because of their structural differences, it is very possible that they are differentially regulated. Notch3 specifically lacks the equivalent of EGF-repeat 21 and lacks an EGF-repeat-sized region comprising parts of EGF-repeats 2 and 3 (Lardelli *et al.*, 1994). This may have an impact on the specificity or extent of ligand binding. Subtle differences have been reported in the transmembrane domain of Notch3 and this may differentially regulate Notch3 intramembranous cleavage, as well as its recruitment to the membrane or its eventual relationship with other membrane-tethered and/or recruited proteins (Bellavia *et al.*, 2008). The RAM (RBP-jkappa-associated molecule), which interprets CBS (CSL binding site) proximity and orientation has 41% amino acid identity between Notch1 and Notch3 (Beatus *et al.*, 2001). Similarly there is only 21% amino acid identity between Notch1 and Notch3 in the C-terminal region containing the transactiviation and PEST domain (Beatus *et al.*, 2001). The PEST domain contains a peptide sequence which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T). This sequence

is associated with proteins that have a short intracellular half-life and it is hypothesized that the PEST sequence acts as a signal peptide for protein degradation (Rogers *et al.*, 1986). The half-life of Notch IC is relatively short due to the action of ubiquitin ligase, Sel-10 (Wu *et al.*, 2001) and Notch proteins are degraded by the 26S proteosome in the cell.(Wu *et al.*, 2001).

GSK-3β is a serine/threonine kinase that functions in diverse cellular processes including proliferation, differentiation, migration and survival (Luo, 2009). Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch (Ruel *et al.*, 1993) and GSK-3β has previously been shown to have the ability to bind to and phosphorylate Notch1 within the C-terminal PEST domain (Espinosa *et al.*, 2003) It therefore stands out as a potential modulator of Notch receptor levels in VSMCs. Based on structural variations in the PEST domain of the Notch IC, it is possible that GSK-3β modulation of Notch is receptor specific. There also, may be a cell-type specificity to the GSK-3β effect on Notch IC. This study sought to elucidate the effect, if any, of GSK-3β on Notch1 and Notch3 in VSMCs.

It was, therefore, investigated if there was such an involvement in VSMCs, thereby providing a mechanism for regulation of Notch signalling in VSMCs and a potential therapeutic target to treat vascular disease. This was achieved through manipulation of GSK-3β activity in RVSMCs, by ectopic expression of GSK-3β using a constitutively-active GSK-3β expression plasmid, pharmacological inhibition with a well-studied specific inhibitor, SB216763, and knockdown of GSK-3β gene expression using siRNA technology.

Knockdown of GSK-3β protein by siRNA technology was

demonstrated for the first time in VSMCs. Gene-silencing effectiveness depends very much on the target sequence positions (sites) selected from the target gene. Different siRNAs synthesized for various positions induce different levels of gene-silencing. The selection of the target sequence is critical to the effectiveness of the siRNA and we need useful criteria for gene-silencing efficacy when we are designing siRNA sequences. Several factors relate to gene-silencing efficacy. They are, for example, based on binding energy, GC content, sequence features, hairpin formation potential, secondary features of mRNA and weighted sums of sequence motifs and patterns (Takasaki *et al.*, 2006). The positional nucleotide characteristics for siRNA designs seem to be the most important factor for identifying the effective siRNA. Our design was based on the position relative to the start codon (which avoided regulatory protein binding sites which may interfere with binding of the siRNP endonuclease complex) GC content and was blasted to confirm no non-specific targets. However, commercially available siRNA, utilises computational models that account for the secondary features of mRNA and weighted sums of sequence motifs and patterns (Tilesi *et al.*, 2009). Ambion uses design algorithm improvements, bioinformatic filtering to predict and eliminate potentially non-effective siRNAs and incorporate novel chemical modifications demonstrated to improve siRNA specificity. These features may account for the improved level of GSK-3β knockdown observed in RVSMCs compared to self-designed and commercially purchased shRNA.

This study demonstrates for the first time that GSK-3β has the ability to modulate Notch receptor levels in VSMCs. It is the first time that modulation of Notch3 IC levels by GSK-3β has been demonstrated and

the first time that modulation of Notch1 IC levels by GSK-3β in VSMCs has been demonstrated.

Both Notch1 and Notch3 IC receptor levels were significantly increased by GSK-3β activity (Figure 3.5, Figure 3.6, Figure 3.7 , Figure 3.8 and Figure 3.9) suggesting that the structural differences in the C-terminal domain of Notch1 and Notch3 does not impact on the modulation of the active Notch receptor by GSK-3β. A recent study shows that smooth muscle Notch1 mediates neointimal formation after vascular injury (Li *et al.*, 2009), suggesting that regulation by GSK-3β of Notch1 IC levels may have considerable significance, in regards to vascular disease and warrants further investigation.

A likely mechanism for regulation of Notch IC levels by GSK-3β is through enhancement of stability in the nucleus. Notch proteins undergo inactivation by proteasome-mediated degradation and are regulated by components of the ubiquitin ligase cascade. Foltz et al. (2002) demonstrate that activated GSK-3β reduced the quantity of Notch1IC that was degraded by the proteasome, suggesting that GSK3β activity alters the degradation of Notch1IC by the proteasome. It is possible that this occurs through direct phosphorylation of the Notch IC. Foltz et al. (2002) also demonstrate that Notch1 is able to bind and phosphorylate NotchIC in vitro. However, conserved consensus sites for numerous serine/threonine kinases are distributed within the intracellular domain of Notch and therefore, GSK3β could have indirect effect on Notch IC phosphorylation through regulation of other kinases (Foltz *et al.*, 2002). Another possible mechanism is inhibition of antagonists which promote degredation such as Sel-10. Mammalian Sel-10 is itself ubiquinated and degraded by the proteosome (Oberg *et al.*, 2001) and it is easy to speculate

that this event is GSK-3β-dependent. Interaction of Sel-10 with nuclear Notch requires a phosphorylation event (Gupta-Rossi *et al.*, 2001). In studies by Espinosa et al. (2003) and Jin et al.(2009), Notch IC phosphorylation by GSK-3β reduced the activity of the Notch IC (Espinosa *et al.*, 2003), (Jin *et al.*, 2009) . It is possible that, in this instance, GSK-3β mediated the phosphorylation events required for Sel-10 ubiquitination. Fryer et al. identified cyclin-dependent kinase 8 (CDK8) as a kinase that phosphorylates Notch IC in the PEST domain in the nucleus, and was a requirement for Sel-10 phosphorylation (Fryer *et al.*, 2004). Based on the studies discussed above, this is also possible candidate for modulation by GSK-3β.

The mammalian protein Itch has been shown to be able to ubiquitinate Notch and to bind to the N-terminal portion of the intracellular region of the molecule. Itch regulates PEST-independent degredation of cytoplasmic Notch proteins and is required, in the absence of ligand, after the early steps of Notch endocytosis to target it to the lysosomes where it is degraded (Fryer *et al.*, 2004). Furthermore Itch/AIP4 catalyzes Notch polyubiquitination through unusual K29-linked chains. (Chastagner *et al.*, 2008). Notch is associated with Itch/AIP4 in cells but their interaction requires a post-translational modification, or a bridging factor that remains to be identified, suggesting that Itch is another candidate for modulation by GSK-3β.

It may be the case that GSK-3β has the ability to interact with Notch IC on multiple phosphorylation sites or indeed on multiple levels of regulation and this may account for its apparent ability to activate and repress Notch signalling. Another possible mechanism is that GSK-3β prevents cleavage of Notch IC by regulating γ-secretase or presenilins.

γ-secretase is also required for the release of the Notch IC (Mumm and Kopan, 2000) in a presenilin-dependent manner. γ-Secretase cleavage can occur at the cell surface or in endosomal compartments, but cleavage at the membrane favors the production of a more stable form of NICD (Tagami *et al.*, 2008). GSK3β activity has previously been shown to modify the localization and function of presenilin 1 (Uemura *et al.*, 2007). Presenilin 1, a causative gene product of familial Alzheimer disease, has been reported to be localized mainly in the endoplasmic reticulum and Golgi membranes, but endogenous Presenilin 1 also localizes at the plasma membrane as a biologically active molecule. The serine residues (serine 353 and 357) on presenilin 1 can be phosphorylated by GSK-3β and GSK-3β-mediated phosphorylation of presenilin 1 down-regulates its cell-surface expression (Uemura *et al.*, 2007). It has, therefore, been hypothesised that GSK-3β may be involved with cleavage of Notch IC. However, a therapeutic level of lithium chloride (LiCl), an inhibitor of GSK-3β, while reducing $\alpha\beta$ formation by interfering with APP cleavage at the γ-secretase step, did not affect Notch ICD formation from Notch (Phiel *et al.*, 2003). Further research is required to establish the role, if any, for GSK-3β in cleavage of the Notch receptor.

While regulation of Notch IC by GSK-3β through post-translational modifications or cleavage events has been investigated, other mechanisms cannot be overlooked. Our previous study shows that cyclic strain transcriptionally regulates Notch1 levels and strain may be one of a number of stimuli that regulate Notch signalling at this level (Morrow *et al.*, 2005b). Here, a role for GSK-3β in the transcriptional regulation of Notch1 is identified, suggesting that GSK-3β may mediate the effect of cyclic strain on Notch IC. GSK-3β is a multi-tasking protein involved in

many aspects of cell signalling and GSK3β acts within the Wnt/wingless (Wnt/wg) signalling cascade by altering the stability of β-catenin. Foltz et al. (2002) suggest that Notch IC function may be modulated by Wnt/wg signalling through its negative effect upon GSK-3β activity mediated by Disheveled. Crosstalk between signalling pathways is another mechanism which warrants investigation when addressing the role of GSK-3β in Notch signalling in VSMCs.

While Notch receptor levels were clearly shown to be modulated in this study, this was no indicator of the effect of GSK-3β on Notch target signalling. It could be argued that GSK-3β disrupts nuclear translocation of the receptor and that the observed increase in Notch IC levels due to GSK-3β activity, is due to retained cytoplasmic Notch IC that has avoided degradation by avoiding phosphorylation in the nucleus. This would result in a decrease in signalling activity of the active receptors in the nucleus and a decrease in downstream target gene transcriptional activation. A nuclear localizing signal (NLS) was identified in the cytoplasmic sequence of Notch homologs (Stifani *et al.*, 1992) and nuclear localisation is essential for notch signalling. This sequence motif consists of a stretch of approximately 60 residue that harbours a sequence motif conforming to the definition of a casein kinase II (CKII) site/cdc2 kinase (cdc2) site/nuclear localisation sequence. Interestingly, CK II (also called GSK-5) is a priming kinase for GSK-3β (Woodgett and Cohen, 1984) and may assist in modulation of Notch IC by GSK-3β. However, while a study has shown that Akt inhibits proper nuclear localisation of NICD, a dominant negative plasmid of GSK-3β (DN-GSK-3$beta$) has no effect on the subcellular distribution of NICD (Song *et al.*, 2008). Also, the study by Foltz et al., revealed that GSK3β does not control the nuclear localization

of NotchIC in embryonic fibroblasts and imply that differences in the ability of wild-type and GSK3β null cells to activate the HES1 promoter are not a consequence of changes in the translocation of NotchIC.

To confirm a downstream effect of GSK-3β on Notch signalling in VSMCs, the levels of the HRT (1-3) genes, following manipulation of GSK-3β was examined. Previous studies have focused mainly on the effect of GSK-3β on Hes genes (Jin *et al.*, 2009), (Foltz *et al.*, 2002), (Espinosa *et al.*, 2003). HES factors have been shown to be predominantly involved in neurogenesis and development of sensory organs, while HRT factors play critical roles in the cardiovascular system (Fischer and Gessler, 2007). Our previous studies showed that the HRTs are direct downstream target genes of Notch 1 and 3 receptors in SMCs (Sweeney *et al.*, 2004) and that hrts are regulated in response to arterial injury (Morrow *et al.*, 2009). For this reason, it was decided to focus on the regulation of the HRT family of genes in VSMCs. In this study, GSK-3β modulation of the expression of HRT1, 2, and 3 was investigated and it was found that GSK-3β has a positive effect on the expression of these downstream targets at the mRNA level, which was confirmed at the protein level by positive regulation of HRT3 protein by GSK-3β.

In the nucleus, Notch IC acts to affect transcription of its downstream targets by binding to the nuclear protein CBF-1/RBP-$_{J\kappa}$. All HRT promoters contain multiple RBP-$_{J\kappa}$ binding sites and we have previously identified an essential role for CBF-1/RBP-$_{J\kappa}$ in the regulation of HRTs in VSMCs (Sweeney *et al.*, 2004). To further clarify a role for GSK-3β in Notch signalling, GSK-3β involvement in Notch signalling through regulation of the CBF-1 promoter was examined. Using a luciferase reporter plasmid for the CBF-1 promoter binding sites, it was shown that

GSK-3β positively modulates CBF-1 promoter activity. This further strengthens the evidence that GSK-3β has the ability to regulate Notch signalling in VSMCs.

The effect of GSK-3β on Notch downstream targets with a change in Notch receptor levels has yet to be correlated. This is easily addressed by examining if GSK-3β has the ability to recover the effect on Notch downstream signalling, following inhibition of Notch receptor activity using a γ-secretase inhibitor. Proteolysis of Notch receptor by a γ-secretase at an S3 site is a conserved signalling mechanism and all four Notch receptors require an S3-like cleavage event for CBF-1-mediated Notch signalling (Mumm and Kopan, 2000). By inhibiting this cleavage with a γ-secretase inhibitor, it would be possible to show that the effect of GSK-3β on downstream Notch signalling was dependent on activity of the Notch receptor. This would strengthens the hypothesis that GSK-3β modulates Notch signalling in VSMCs through regulation of Notch IC levels.

3.4 Conclusion

Notch is proving to be a critical modulator of cell fate in vascular disease (Li *et al.*, 2009) and, therefore it is of considerable importance to understand the mechanisms involved in its regulation. In this study, a role for GSK-3β in the modulation of Notch signalling in VSMCs was established. It was demonstrated that GSK-3β has the ability to modulate Notch receptor activity and downstream target gene expression and acts upstream of CBF-1 promoter activity. GSK-3β has previously been shown to directly bind to and phosphorylate Notch1 IC (Foltz *et al.*, 2002) and here, a role for GSK-3β in the transcriptional regulation of Notch1 in VSMCs was identified. This data suggests that GSK-3β may modulate Notch on multiple levels. A number of stimuli regulate Notch signaling and GSK-3β may interact with Notch on a number of levels, as summarised in Figure 3.17. Crosstalk between Notch and the Wnt, VEGF and Hh pathways is essential in the developing vasculature (Goishi and Klagsbrun, 2004) and we have previously shown that cyclic strain inhibits Notch receptor signalling in adult VSMCs (Morrow *et al.*, 2005b). A potential role for GSK-3β in Notch signalling at the level of crosstalk with other developmental pathways and through biomechanical modulation is investigated in the following chapters.

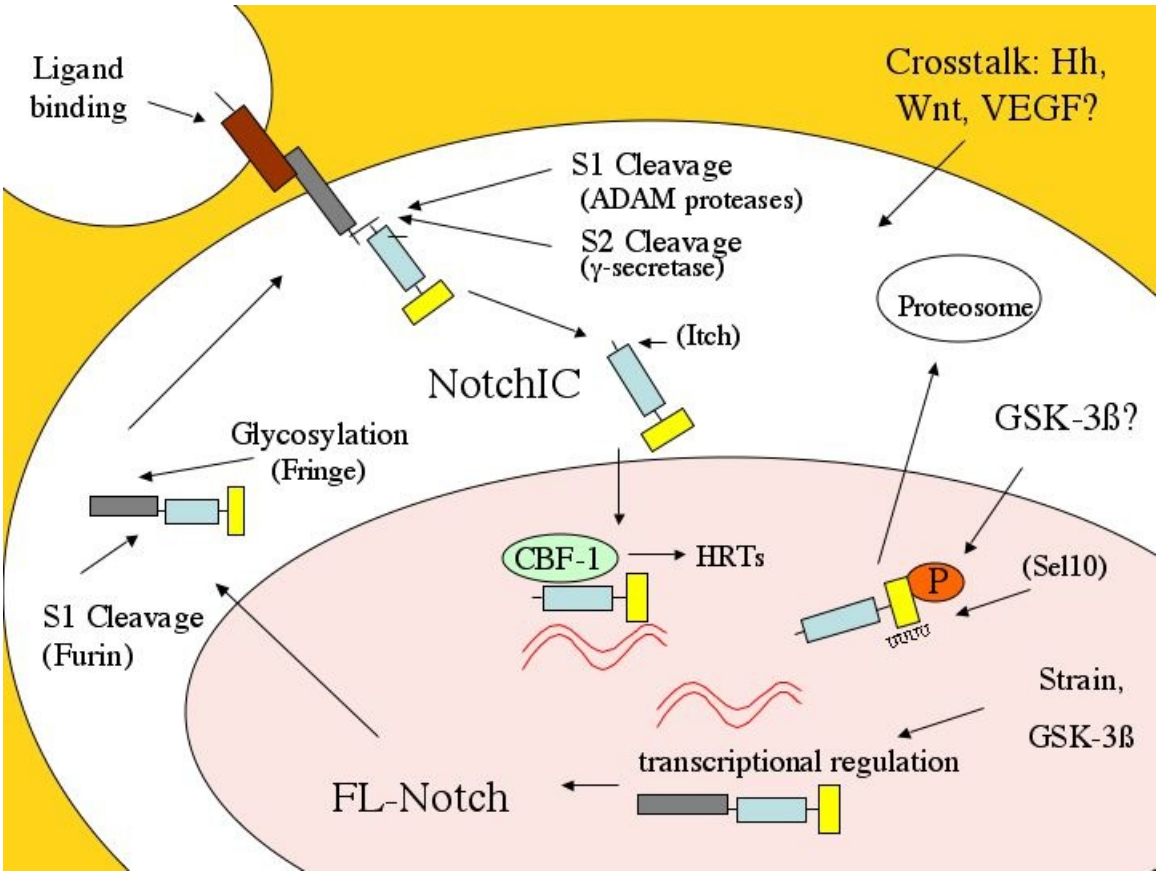


Figure 3.17: Overview of Notch Receptor Regulation in VSMCs. Notch is regulated on a number of levels including cleavage of FL-Notch (full-length Notch) by Furin, cleavage and tracking of receptors (by ADAM proteases, γ-secretase, Itch), post-translation modifications (by Sel-10, GSK-3β) and through transcriptional control of Notch receptor expression (by strain, Hh, Wnt).

Chapter 4

Results

4.1 Introduction

Crosstalk between the developmental pathways of Hh, VEGF and Notch are crucial during vascular morphogenesis and modeling of the embryonic vasculature (Goishi and Klagsbrun, 2004). It has yet to be established if this mechanism of cell fate regulation is conserved in adult cells. This study involved Notch regulation in VSMCs at the level of crosstalk with the Hedgehog Pathway. There are three forms of the ligand, Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian (Hh,) which are expressed in different tissues at different times during development. Shh and Ihh play important roles in embryonic development, and Dhh regulates spermatogenesis (Huangfu and Anderson, 2006). The targets of Hh signalling in vertebrates are Gli 1-3.

It has been recently shown that Shh signalling is present in adult cardiovascular tissues and can be activated *in vivo* to induce robust angiogenesis (Pola *et al.*, 2001). Moreover, Shh and VEGF have been shown to act upstream of Notch during arterial endothelial

differentiation (Lawson *et al.*, 2002). This study therefore examined if Hh components control Notch signalling in VSMCs through VEGF activation.

The Wnt signalling pathway has also been implicated as a modulator of the vasculature through crosstalk with Notch (Phng and Gerhardt, 2009). GSK-3β, a component of the Wnt pathway may, therefore, regulate Notch signalling through crosstalk with other pathways, such as Hh. Having established that GSK-3β has the ability to modulate Notch in VSMCs, this study investigates if there is a role for GSK-3β in Hh signaling in VSMCs.

The Notch signaling pathway is critical for cell fate determination during vascular development and we have previously identified Notch as a positive modulator of VSMC growth (Sweeney *et al.*, 2004). The role of GSK-3β in cell fate decisions is ambiguous as it has been shown to be pro-apoptotic or pro-survival, depending on stimulus or context (Beurel and Jope, 2006). Previous studies have identified GSK-3β as pro-apoptotic in VSMCs in response to stimuli such as hypoxia (Loberg *et al.*, 2002), or balloon injury (Park *et al.*, 2003). However, it was determined that GSK-3β increases Notch signaling in VSMCs in serum-stimulated cycling cells, suggesting a pro-survival role. We investigated, Therefore, the effect of GSK-3β on VSMCs growth under these conditions was investigated.

The aim of this chapter was firstly to determine the role of the Hh and VEGF pathways in the modulation of Notch signaling. Secondly, a role for GSK-3β in regulation of Hh signaling in VSMCs was determined. Thirdly, the effect of GSK-3β on VSMC growth was also examined.

4.2 Results

4.2.1 Notch signalling is regulated by Hedgehog signalling via VEGF

The role of VEGF in Notch signalling in RVSMCs was investigated by real-time PCR, following treatment of RVSMCs with a recombinant form of VEGF-A (25μg, 24h). and found that the Notch target gene expression of HRT1, HRT2 and HRT3 was upregulated following treatment (3.15 $\pm$ 0.22 fold, 5.32 $\pm$ 0.46 fold and 3.04 $\pm$ 0.39 fold, n=3) (Figure 4.1).

RVSMCs were treated with recombinant Shh for 24 h. Notch target gene HRT3 mRNA expression was upregulated following treatment (3.43 $\pm$ 0.39 fold, n=3) (Figure 4.2). Selective knockdown of VEGF-A with a targeted siRNA was confirmed at the protein level (Figure 4.2 A.) HRT3 mRNA expression was downregulated following transfection of VEGF-A targeted siRNA to (0.57 $\pm$ 0.04 fold, n=3). This effect was attenuated (to 0.90 $\pm$ 0.05 fold, n=3) following treatment with recombinant Shh. The effect was further enhanced following co-treatment with recombinant VEGF (to 1.99 $\pm$ 0.45, n=3) (Figure 4.2). These findings indicate a role for Hedgehog signalling in the regulation of Notch through VEGF.

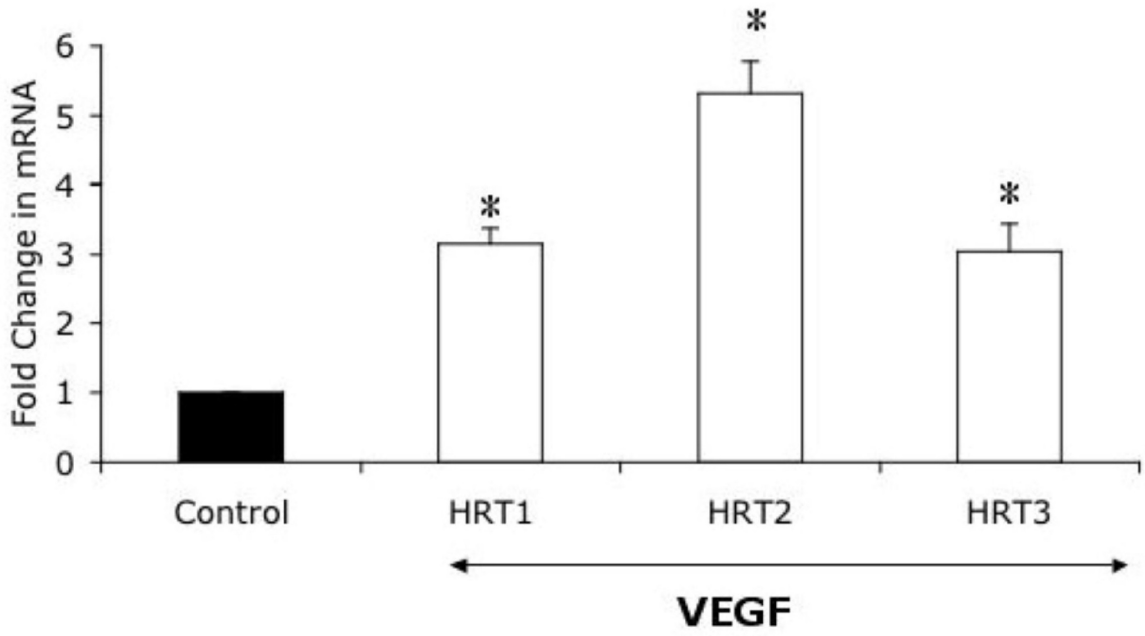


Figure 4.1: Notch signalling is Upregulated by VEGF. Real-time PCR analysis of HRT1, HRT2 and HRT3 following treatment of RVSMCs with 1% BSA or recombinant VEGF (25ng) for 24 h). Expression was normalized to GAPDH levels, and expressed as fold increase over BSA control. The experiments were performed in triplicate, with the graph representative of n=3, $*p < 0.05$ as compared to control (students t test).

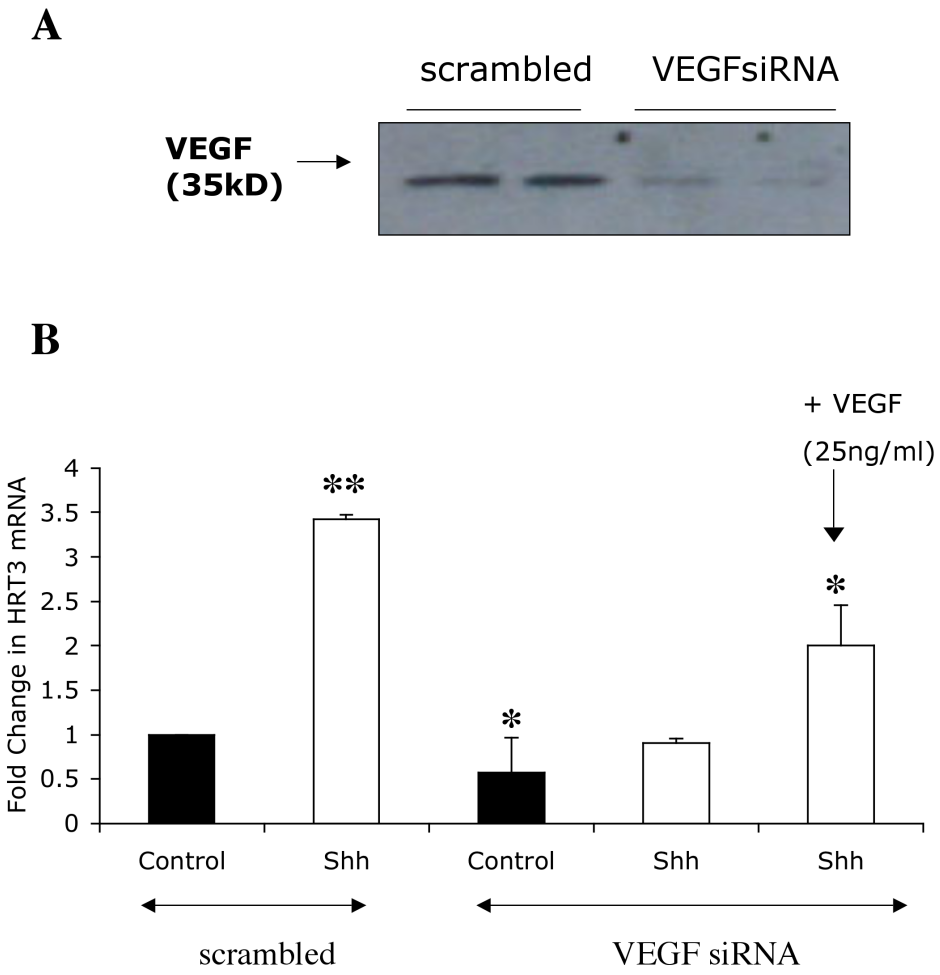


Figure 4.2: Notch signalling is Regulated by Hedgehog signalling via VEGF. A) Western blot analysis of VEGF-A following transfection of RVSMCs with siRNA directed against VEGF or a scrambled control. B) Real-time PCR analysis of HRT3. RVSMCs were transiently transfected with siRNA directed against VEGF or a scrambled control. Following overnight, recovery cells were treated with recombinant Shh (3.5μg) or recombinant VEGF-A (25ng) for 24 h. All data was normalised to GAPDH levels and expressed as fold change over control. The experiments were performed in triplicate, with the graph representative of n=3, $*p< 0.05$, $**p< 0.01$ as compared to control (students t test).

4.2.2 GSK-3β modulates Hh Downstream Targets

In the absence of Hh, a truncated form of Gli, which is generated by proteolytic processing of full-length Gli, represses Hh target genes. In the presence of Hh, Gli is fully converted to the activator form. Gli1 functions as a strong transcriptional activator. However, loss of Gli1 gene function in mice does not appear to result in obviously abnormal phenotypes and therefore, Gli1 is not essential for initial Shh signal transduction in mice. Gli3 is found predominantly in its truncated form, and functions mainly as a transcriptional repressor (Pan *et al.*, 2006). Gli2, is also a strong transcriptional activator and is therefore, an appropriate target for analysing hedgehog signalling. The hedgehog receptor, Patched 1, is also a target gene and therefore a marker for Hh signalling (Mullor *et al.*, 2002).

Gli2 Promoter activity was determined using a Gli2 promoter luciferase-tagged plasmid (pGL3b/8xGli-lcLuc). Ectopic expression of GSK-3β decreased Gli2 promoter activity (0.62 $\pm$ 0.12 fold, n=3). This effect was attenuated following pharmacological inhibition of GSK-3β by SB216763 (25μM) (to 1.29 $\pm$ 0.26 fold, n=3). Pharmacological inhibition of endogenous GSK-3β also increased Gli2 promoter activity (1.53 fold $\pm$ 0.19 fold, n=3)(Figure 4.3). Ectopic expression of GSK-3β decreased Gli2 mRNA expression (Figure 4.4). Conversely, pharmacological inhibition of endogenous GSK-3β increased the mRNA expression levels of Gli2 (2.20 $\pm$ 0.43 fold, n=3) (Figure 4.5). Similarly, Patched1 mRNA expression levels were increased following pharmacological inhibition of GSK-3β (2.52 $\pm$ 0.33 fold, n=3) (Figure 4.7) and decreased following ectopic expression of GSK-3β (to 0.60 $\pm$ 0.13 fold, n=3)(Figure 4.6). Patched 1 protein levels were also increased following pharmacological inhibition

of GSK-3β by SB216764 (3.66 $\pm$ 0.31 fold). (Figure 4.8).

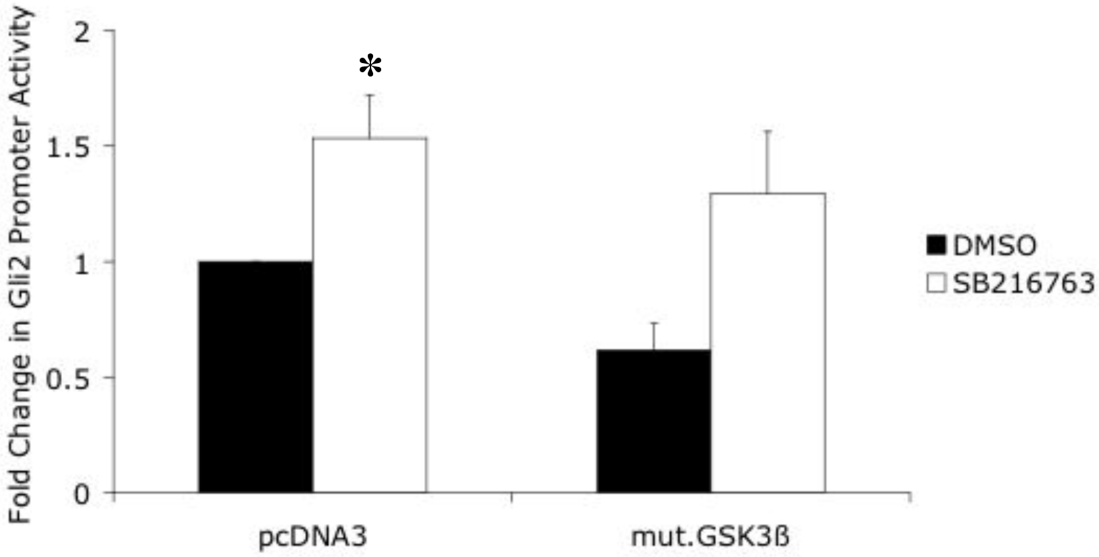


Figure 4.3: GSK-3β regulates Gli2 Promoter Activity. Luciferase activity analysis of the Gli2 promoter activity following transfection of RVSMC with a Gli2 luciferase-tagged reporter plasmid. The cells were co-transfected with the empty vector, pcDNA3, or mut. GSK3β. Additionally, cells were co-transfected with a LacZ plasmid. The cells were treated with DMSO or SB216763 (25μM, 72 h). Protein was isolated and assayed for luciferase activity. Luciferase assays were normalized to β-galactosidase activities and protein levels, n= 3, and expressed as fold increase over control (the value obtained with Gli-promoter transfected cells arbitrarily assigned a value of 1). $*p< 0.05$ as compared to control (Anova).

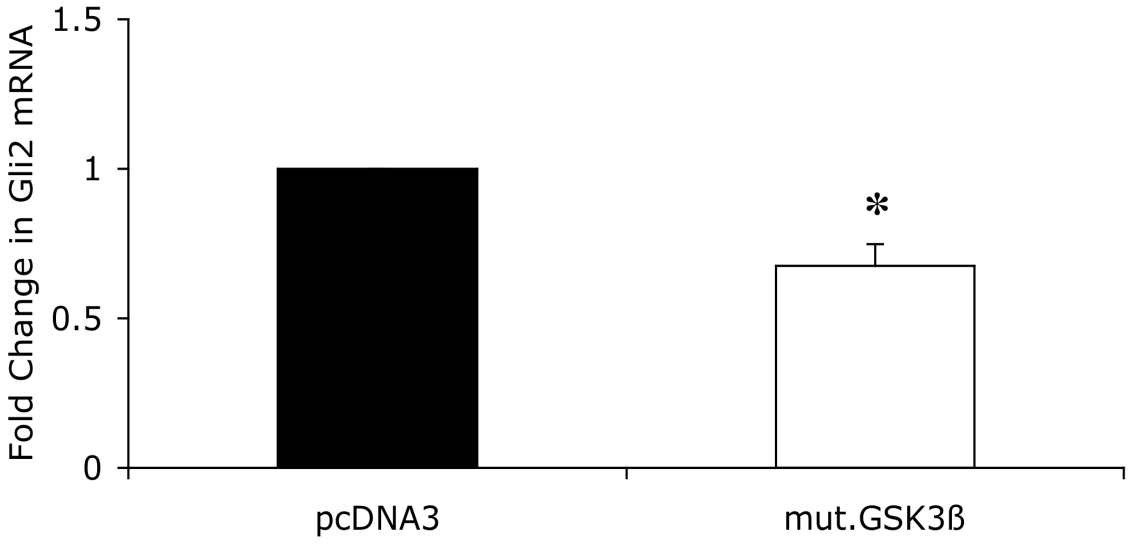


Figure 4.4: Ectopic expression of GSK-3β decreases Gli2 mRNA Expression in RVSMCs. Real-time PCR analysis of Gli2 following transfection of RVSMCs with the empty vector pcDNA3 or mutant GSK-3β and co-transfection with the puromycin resistance plasmid, pGK3puro. The experiment was performed in triplicate. Expression was normalized to GAPDH levels, and expressed as fold increase over control. $*p < 0.05$ as compared to control (students t test).

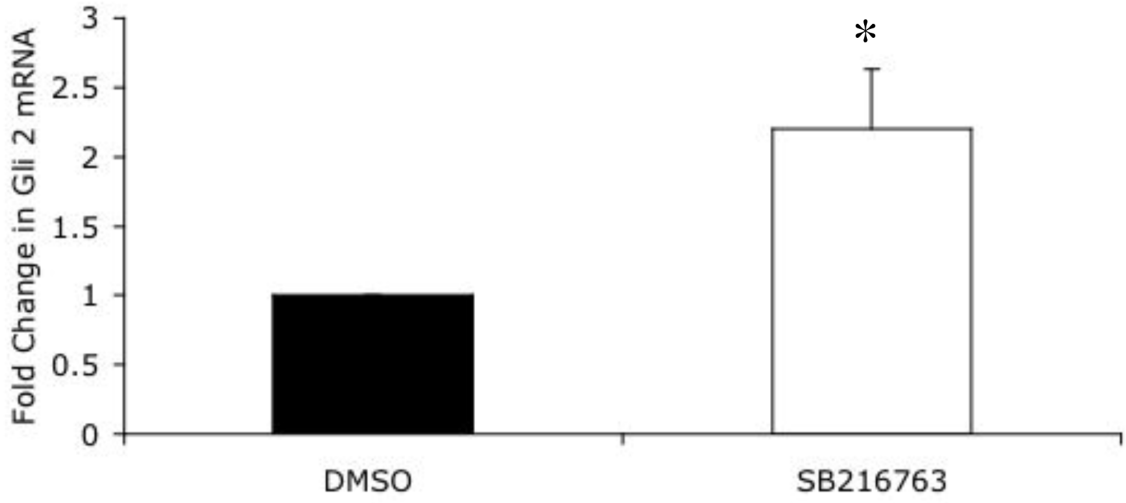


Figure 4.5: Pharmacological inhibition of GSK-3β increases Gli2 mRNA in RVSMCs. Real-time PCR analysis of Gli2 following treatment of RVSMCswith SB216763 (25μM) for 24 h. Expression was normalized to GAPDH levels, and expressed as fold increase over control. The experiment was performed in triplicate. $*p < 0.05$ as compared to control (students t test).

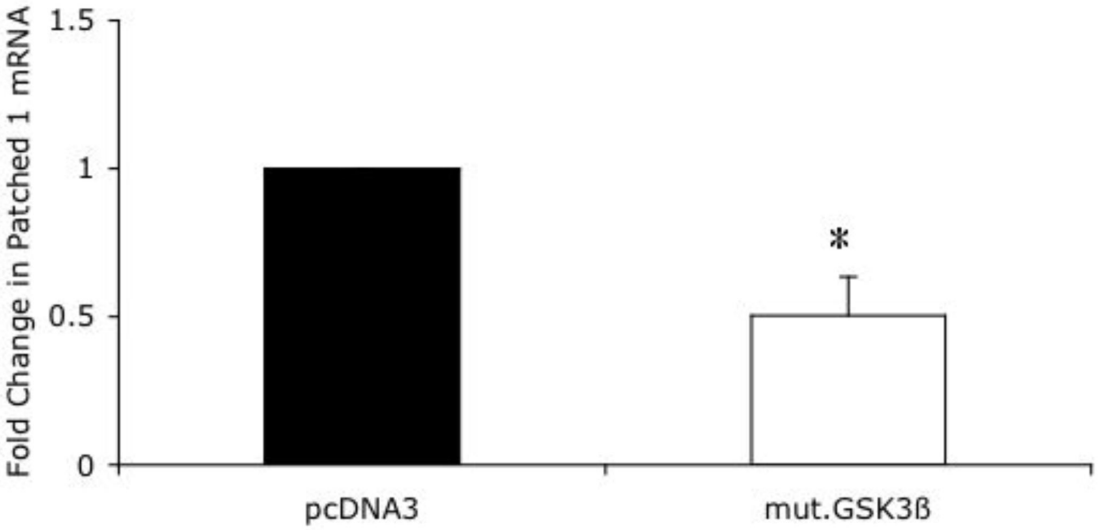


Figure 4.6: Ectopic expression of GSK-3β decreases Patched1 mRNA Expression in RVSMCs. Real-time PCR analysis of Patched1 following transfection of RVSMCs with the empty vector pcDNA3 or mutant GSK-3β and co-transfection with the puromycin resistance plasmid, pGK3puro. Expression was normalized to GAPDH levels, and expressed as fold increase over control.The experiment was performed in triplicate.$*p < 0.05$ as compared to control (students t test).

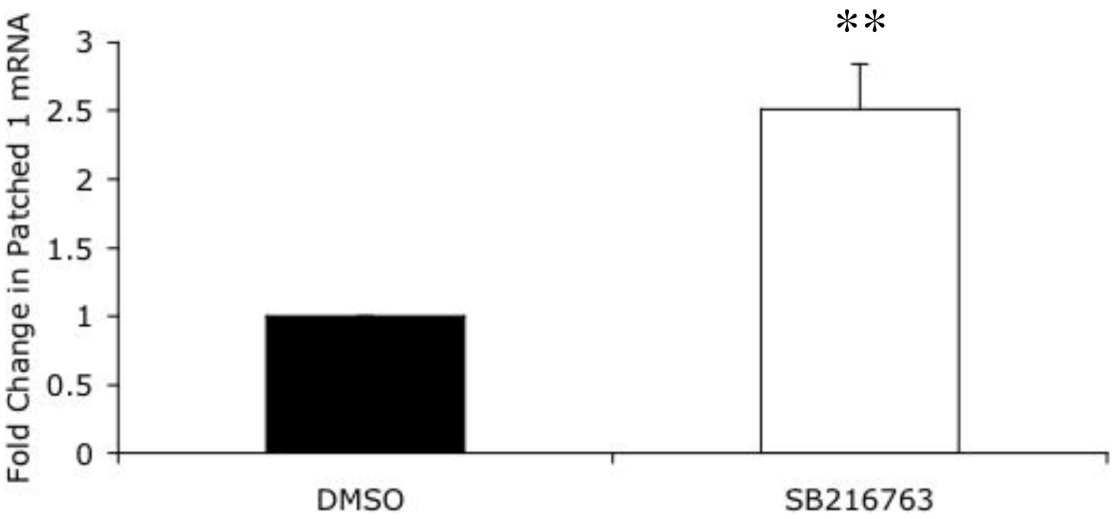


Figure 4.7: Pharmacological inhibition of GSK-3β increases Patched1 mRNA in RVSMCs. Real-time PCR analysis of Patched1 following treatment with SB216763 (25μM) for 24h. Expression was normalized to GAPDH levels, and expressed as fold increase over control.The experiment was performed in triplicate. $**p< 0.01$ as compared to control (students t test).

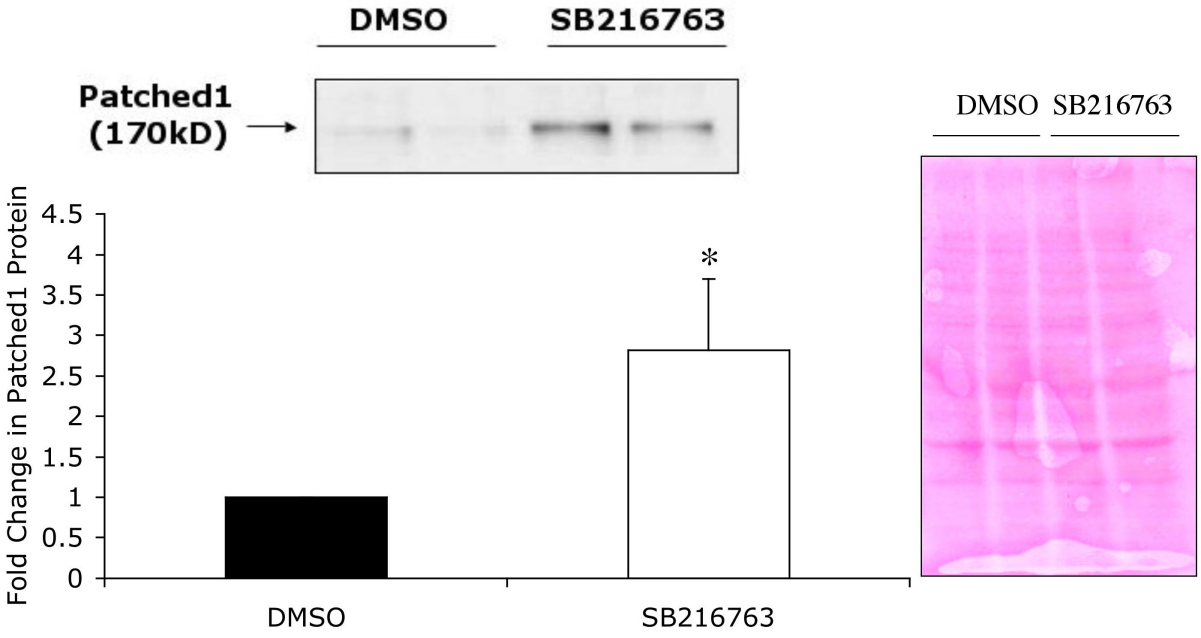


Figure 4.8: Patched1 Protein Expression is Regulated by GSK-3β in RVSMCs. Western blot analysis of RVSMCs following treatment with DMSO or SB216763 (25μM) for 5 days. Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate.

4.2.3 GSK-3β modulates VSMC Proliferation

Previous studies in our lab have shown Notch to be pro-proliferative in RVSMCs (Morrow *et al.*, 2005a). The role of GSK-3β in controlling this cell fate change was therefore examined. Pharmacological inhibition of GSK-3β had an anti-proliferative effect on RVSMCs as measured by FACS CFDA-SE analysis (Figure 4.9). This was confirmed with decreased cell counts (to 0.52 $\pm$ 0.08 fold, n=3) and a reduction in cyclic PCNA expression (to 0.38 $\pm$ 0.09 fold, n=3) following treatment (Figure 4.9 and Figure 4.10). These findings demonstrate that GSK-3β is pro-proliferative in VSMCs. This suggests that GSK-3β may modulate RVSMC cell proliferation through control of putative Notch signalling in these cells.

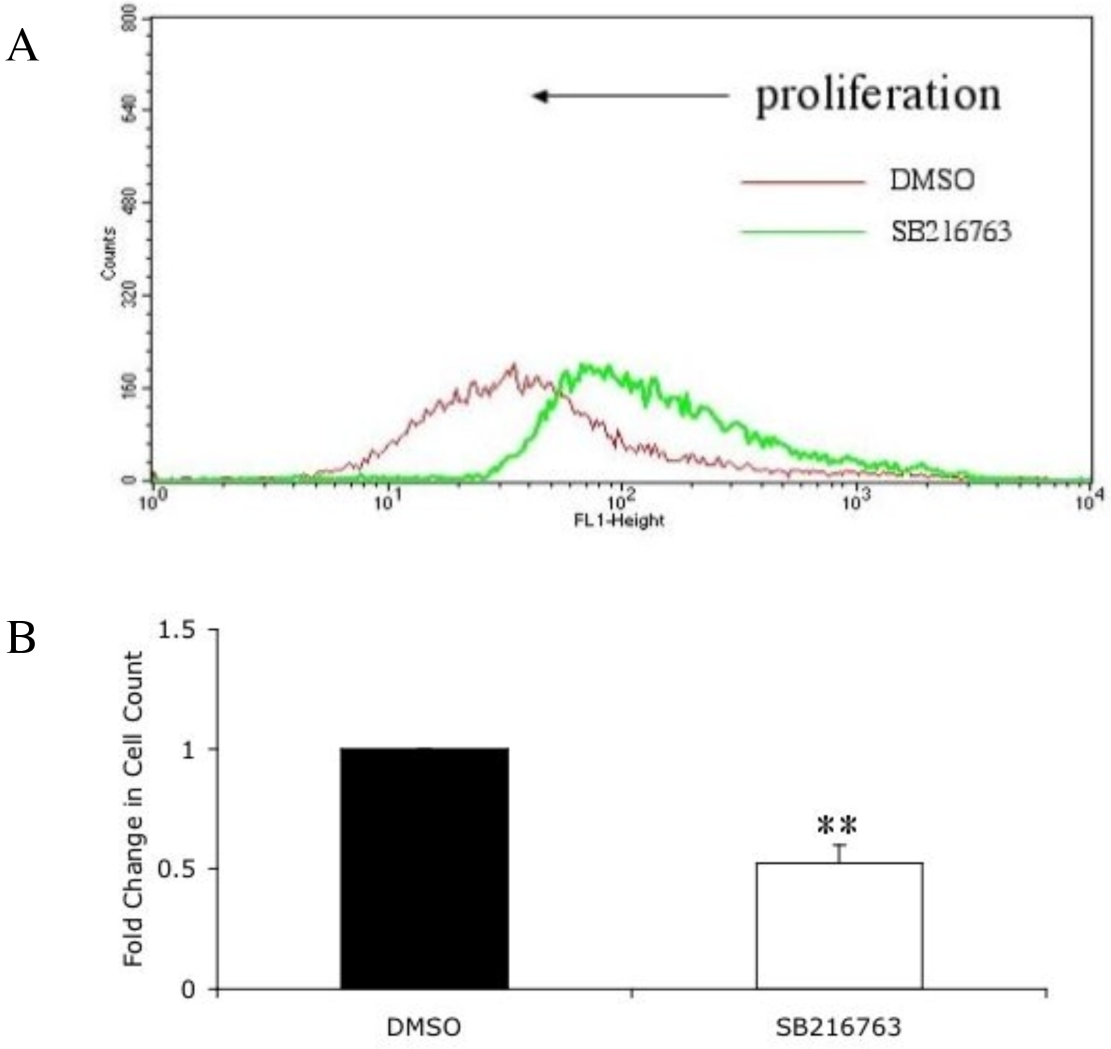


Figure 4.9: Pharmacological Inhibition of GSK-3β Decreases Proliferation in RVSMCs: FACS Analysis and Cell Count. The cells were incubated in CDFA dye for 5 mins, and then quiesced for 48 h. Following treatment with DMSO or SB216763 (25μM, 5 days), cells were A) sorted by FACS analysis or B) counted using a haemocytometer. The experiment was performed in triplicate, with the graph representative of n=3, $**$ p$<$ 0.01 as compared to DMSO control (students t test).

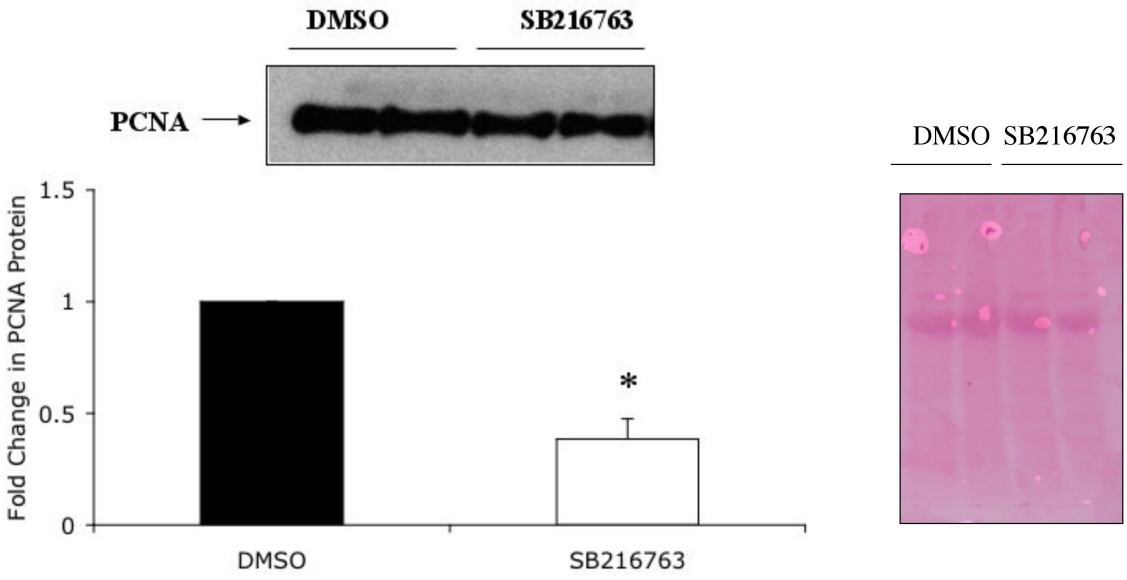


Figure 4.10: Pharmacological Inhibition of GSK-3β Decreases PCNA Expression in RVSMCs: FACS Proliferative Profile. Western blotting of PCNA protein in RVSMCs following treatment with DMSO or SB216763 (25μM, 5 days). Expression was normalized to Ponceau levels (right frame). The experiment was performed in triplicate, with the image representative of n=3, $*$ $p < 0.05$ as compared to DMSO control (students t test).

4.2.4 GSK-3β modulates VSMC Apoptosis

We have also previously shown in our lab that Notch exerts an anti-apoptotic effect on RVSMCs (Morrow *et al.*, 2005a). The effect of GSK-3β on apoptosis in cycling VSMCs was measured by FACS Annexin V analysis, in which apoptotic cells which were phosphatidylserine-positive and propidium iodide-negative were quantified. Ectopic expression of GSK-3β did not induce apoptosis of VSMCs (Figure 4.11). No significant change was observed at its half maximal inhibitory concentration (IC_50) value (34nM)(Figure 4.11). Inhibition of GSK3β using a concentration of SB216763 (25μM) induced apoptosis of GSK-3β (Figure 4.12). Transfection of Notch3 did not recover the increase in apoptosis induced by GSK-3β pharmacological inhibition, suggesting GSK-3β modulates VSMC downstream of Notch signalling (Figure 4.12).

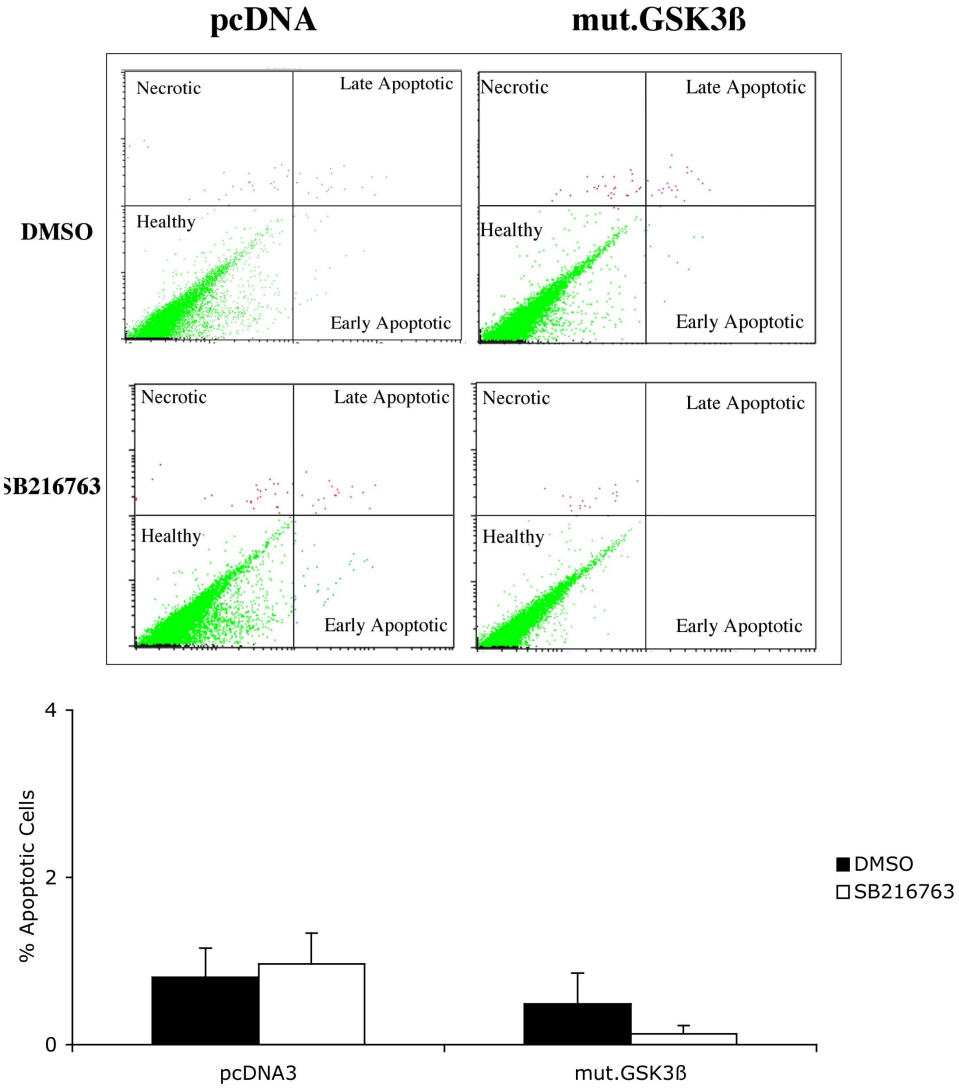


Figure 4.11: Ectopic expression of GSK-3β does not induce apoptosis of VSMCs. FACS Analysis of apoptotic RVSMCs following transfection of empty vector or mut.GSK3β expression plasmid and treatment with normal RPMI media containing 10% FCS and DMSO or SB216763 (34nM, 5 days). The experiment was performed in triplicate, with the graph representative of n=3.

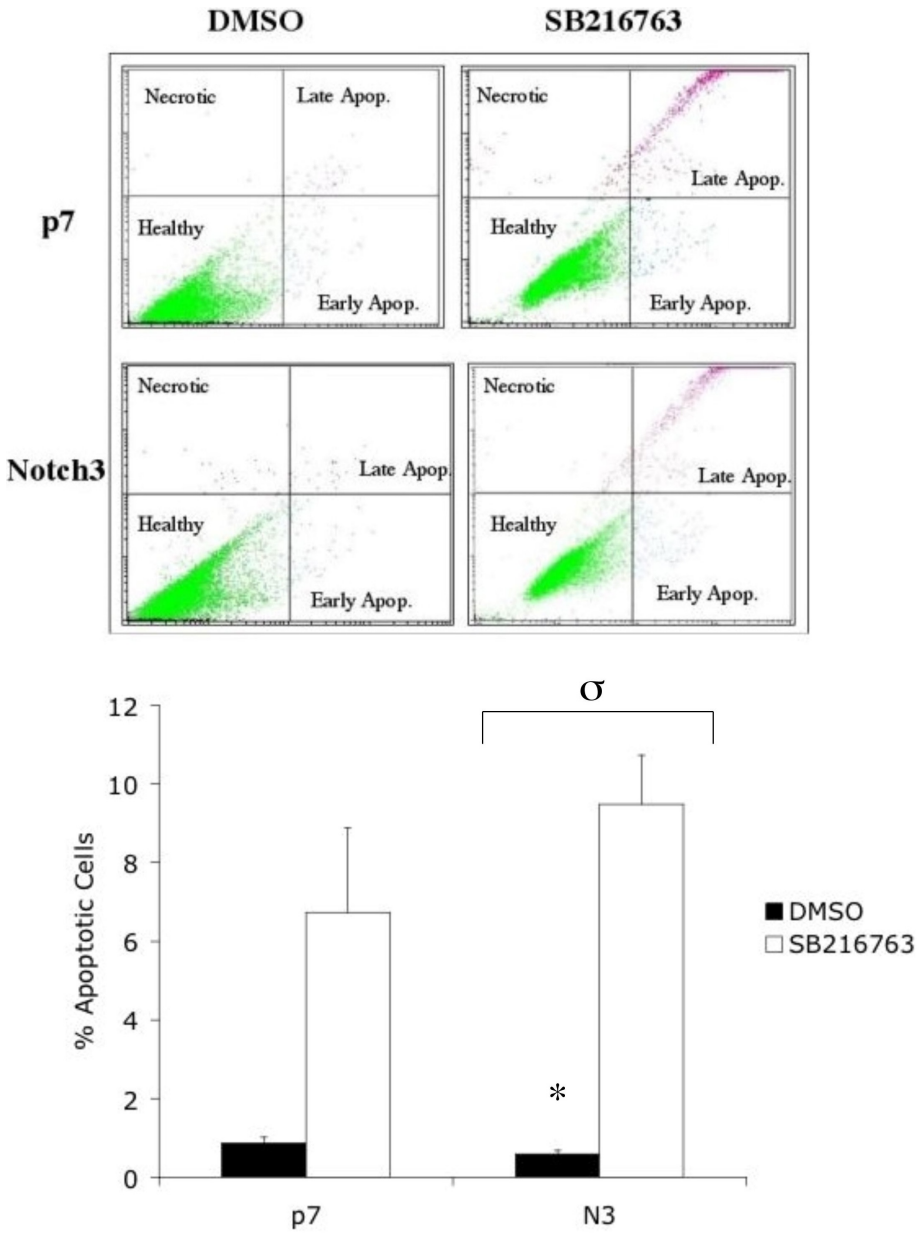


Figure 4.12: GSK-3β modulates VSMC Apoptosis downstream of Notch signalling. (apop.= apoptosis). FACS Analysis of apoptotic RVSMCs following transfection of empty vector or Notch 3 expression plasmid and treatment with normal RPMI media containing 10% FCS and DMSO or SB216763 (25μM, 5 days). The experiment was performed in triplicate, with the graph representative of n=3, $**p< 0.01$ as compared to p7 control. $\sigma< 0.05$ as compared to Notch3-transfected DMSO treated control (One-way Anova).

4.3 Discussion

This study identifies that the Hh-VEGF-Notch axis observed in vascular development is conserved in adult cells, as the Hh pathway was shown to be a positive modulator of Notch signalling and Hh signals via VEGF-A to increase Notch downstream target expression in adults VSMCs. Based on these findings, it was initially postulated that GSK-3β modulates Notch signaling upstream of receptor activity through positive regulation of Hh signaling. However, on further investigation it was determined that GSK-3β, a component of the Wnt pathway, has a negative effect on Hh signaling.

Upon binding of a Hh ligand to the receptor Patched, repression of Smo is relieved, which is able to activate Gli by preventing its proteolysis to a truncated non-active form. It has previously been shown that in the absence of Hh ligands full-length Cubitus interuptus (Ci155), the Drosophila form of Gli, is phosphorylated at three serine sites by GSK-3. Initial priming phosphorylations by PKA and casein kinase1 are also required to initate proteoysis. This occurs by binding of an E3SCF ubiquitination ligase complex of which Slimb is the key substrate recognition component. Ubiquitinated Ci155 is then partially degraded in the proteosome. The N-termini is spared by stable folding of the Ci zinc finger domain coupled to the proteolytic resistance of the adjacent polypeptide region leaving a truncated Ci75 (Smelkinson *et al.*, 2007). The phosphorylation of Ci155 by GSK-3 is a requirement for proteolysis and for Hh pathway silencing (Price and Kalderon, 2002). The kinesin-related protein, Costal-2 (Cos2) facilitates Ci155 phosphorylation by PKA, CK1 and GSK-3. In the presence of Hh signalling, Ci proteolysis is inhibited and Hh targets are activated. Binding of Hh causes partial dissociation of

these protein kinases from Cos2 and may be resposible for the accompanying inhibition of Ci155 (Zhang *et al.*, 2005) .

In vertebrates, Gli1 and Gli3 are similarly degraded. In mouse embryonic fibroblasts, Gli2 was found to be readily degraded by phosphorylation of multiple PKA, CK1 and GSK-3 sites within the Gli2 C-terminal region. This hyperphosphorylation of Gli2 protein created binding sites for βTrCP (the mammalian form of Slimb), which in turn conjugated the multiple ubiquitination molecules onto the Gli2 protein and triggered the proteosome-mediated protein degradation. Both Gli2 processing and degradation were inhibited by Shh, which also induced Gli2 protein transcription activity Pan *et al.* (2006). Interestingly, Gli2 was found to be readily degraded. The vast majority of Gli2 was found to be in the full-length form with only a small fraction existing in the truncated form. Phosphorylation of Gli2 was found to be essential for Gli degredation and Gli2 transcriptional activator function Pan *et al.* (2006). It is very possible that this mechanism is conserved between species. In this study it is demonstrated that GSK-3β has a negative effect on Hh signalling in VSMCs and suggest that this regulation is through phosphorylation of Gli2 by GSK-3β.

Another possible mechanism which we have yet to investigate is the possibility that GSK-3β acts downstream of either Hh or VEGF in the Hh-VEGF-Notch axis and is positively modulated by Hh or VEGF to exert its effect on Notch. However, ectopic expression of wild type GSK3β in pulmonary artery smooth muscle cells (PASMCS) reduced VEGF promoter activity via β-catenin, a direct substrate of GSK-3β activity (Clifford *et al.*, 2008). Secondly, VEGF negatively regulates GSK-3β in ECs, through the Akt pathway (Takeshita *et al.*, 2007). This has

yet to be investigated in SMCs, but conservation of the Hh-VEGF-Notch axis in ECs suggests that this may occur similarly in VSMCs.

It is proposed, therefore, that GSK-3β is not a key player in the upregulation of Notch signalling by Hh in VSMCs. The more likely mediators of regulation of Notch signaling by VEGF in VSMCs are the ligands Delta or Jagged. This is mechanism is observed in ECs where VEGF upregulates Dll4 expression during angiogenesis. (Liu *et al.*, 2003). A role for the forkhead transcription factors, Foxc1 and Foxc2, in the direct activation of Dll4 and HRT2 promoter activity though interaction with VEGF has also been established in ECs (Seo *et al.*, 2006). This study also implicated a role of MAPK in this modulation by Foxc. Investigation of the activity of these components in VSMCs is warranted to further elucidate the mechanisms involved in the modulation of Notch signalling by Hh and VEGF. A summary of these proposals is outlined in Figure 4.13.

GSK-3β has a critical role in many cell processes, particularly cell proliferation and apoptosis. GSK-3β has been shown to be anti-proliferative in many contexts. In VSMCs, VLDLs and endothelin-1 promote proliferation through GSK-3β (Lipskaia *et al.*, 2003), (Tang *et al.*, 2008a). GSK-3β has been shown to induce apoptosis in response to hypoxia, DNA damage, heat shock and growth factor removal (Forde and Dale, 2007). Specifically in VSMCs, enhanced GSK-3β activity mediated hypoxia-induced apoptosis (Loberg *et al.*, 2002) and glucose transporter isoform (GLUT1) inhibits apoptosis via inhibition of GSK-3β (Hall *et al.*, 2001). GSK-3β has been shown to act downstream of the PI3-kinase pathway in regulation of vascular smooth muscle cell apoptosis (Allard *et al.*, 2008). GSK-3β also prevents vascular cell growth

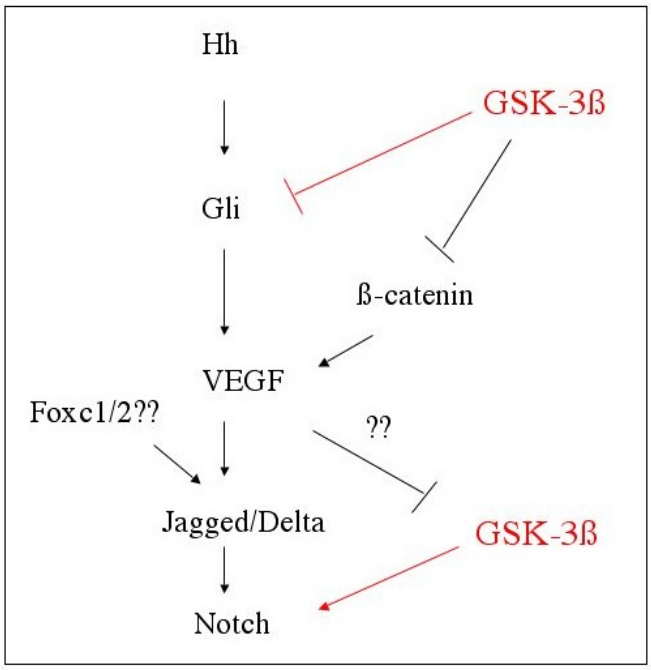


Figure 4.13: Summary of Proposed Hh-VEGF-Notch Axis in VSMCs

following balloon injury in rats (Park *et al.*, 2003).

This study, however, demonstrates a pro-survival role for GSK-3β in VSMCs. It has previously been established that GSK-3β is absolutely essential for survival, as GSK3β knockout mice died during embryonic development (Hoeflich *et al.*, 2000). A novel pathway in which GSK-3β facilitates Nuclear Factor-κB activity, thus preventing tumor necrosis factor-induced hepatocyte apoptosis, due to lack of GSK-3β activity, was identified (Ougolkov *et al.*, 2005).

The pro-apoptotic capacity of GSK-3β is thought to primarily occur through modulation of the tumour suppressor protein p53, and GSK-3β has been shown to directly bind and activate p53 (Watcharasit *et al.*, 2003). p53 has been shown to induces cell cycle arrest or apoptosis through transcription-dependent and independent pathways and

endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice (Mercer *et al.*, 2005). Similarly, GSK-3β has the ability to directly regulate proliferation. Cyclin-D1, a cell-cycle protein has been found to be a direct substrate of GSK-3β (Diehl *et al.*, 1998) and inhibition of GSK-3β induces upregulation of cyclin D1 and DNA synthesis activity in VSMCs (Shin *et al.*, 2003). GSK-3β also inhibits the pro-survival transcription factors β-catenin, c-myc and eIF2B (Beurel and Jope, 2006)

Here, a pro-survival role for GSK-3β in VSMCs through regulation of Notch signalling is proposed. It has previously been shown that Notch is pro-proliferative and anti-apoptotic in VMSCS. (Sweeney *et al.*, 2004). GSK-3β was identified as a positive modulator of Notch signalling in VSMCs and it is demonstrated that GSK-3β positively modulates proliferation and prevents apoptosis of VSMCs. It is also shown that inhibition of GSK-3β induces apoptosis downstream of Notch signalling. Ectopic expression of GSK-3β did not induce apoptosis in cycling VSMCs. Inhibition of GSK-3β at its half maximal inhibitory concentration (IC_50) value (34nM) (Coghlan *et al.*, 2000) also did not induce apoptosis. However, a concentration that significantly reduced Notch receptor level activity (25μM) increased apoptosis in VSMCs in resting cells. Based on these findings, it is proposed that their is a concentration dependent effect of GSK-3β on cell growth on VSMCs. GSK-3β is essential for VSMC survival, possibly through modulation of the Notch signaling pathways but at higher levels of GSK-3β activity and following various stimuli, GSK-3β has the ability to induce apoptosis. An interesting review by Beural and Jope (2006) discusses this paradoxical nature of GSK-3β and suggests that GSK-3β induces the intrinsical/mitochondrial pathway,

while inhibiting extrinsic/death-receptor apoptotic signalling (Beurel and Jope, 2006). Notch has been shown to confer protection against diverse stimuli that trigger both the intrinsic and extrinsic pathways (Sade *et al.*, 2004). It has previously been shown that Notch signalling is anti-apoptotic in VSMCs (Sweeney *et al.*, 2004). This current study demonstrates that Notch signalling is regulated by GSK-3β in VSMCs, and suggests that the pro-survival activity of GSK-3β in VSMCs occurs through modulation of Notch downstream activity.

4.4 Conclusion

GSK-3β contributes to both cell death and survival in many cell types, including VSMCs. We identifed GSK-3β as a negative regulator of the pro-proliferative Hh signalling pathway, while acting as a pro-survival kinase, through the pro-proliferative Notch pathway. Taken together, it is evident that maintenance of appropriate levels of GSK3 activity is crucial, because either too little or too much GSK3 activity can promote cell death in certain conditions (Doble and Woodgett, 2003). Notch signalling has been implicated in the vascular response to injury and the neointimal formation observed in many pathological vascular conditions (Li *et al.*, 2009). It was previously demonstrated that Notch signalling is involved in the changes in cell fate that occur with altered biomechanical stretch (Morrow *et al.*, 2005b). In the following chapter, the role of GSK-3β in the biomechanically-induced changes in Notch signalling in VSMCs was investigated.

Chapter 5

Results

5.1 Introduction

Blood vessels are permanently subjected to the haemodynamic forces of pulse pressure and shear stress. The pulsatile nature of blood flow results in a cyclic mechanical strain in the vessel walls. Altered cyclic strain in which there is a decrease or increase in physiological levels of cyclic strain has been shown to be pathogenic, resulting in changes in VSMC growth, progressing to vascular remodelling and the onset of vascular disease (Morrow *et al.*, 2005a).

Cyclic circumferential strain is caused by a transmural force acting perpendicularly to the vessel wall. Vascular smooth muscle cells convert these mechanical signals into a biochemical response through mechanosensors including integrins, G-protein and G protein-coupled receptors, tyrosine kinase receptors and ion channels. The intracellular pathways of PI3-kinase and mitogen-activated protein kinases (MAPK) are also activated in response to mechanical stimuli (Li and Xu, 2000). The strain-induced change in VSMC growth has been in shown to be

dependent, in part, on Notch signalling (Morrow *et al.*, 2005a). In this study, the mechanism involved in the Notch response to cyclic strain was further elucidated. GSK-3β was previously shown to have the ability to regulate Notch under static conditions, and therefore, it is possible that it may be involved in the strain-induced change in Notch signalling.

The aim of this chapter was to examine the specific role of uniform equibiaxial cyclic strain on GSK-3β in an in vitro model of RVSMCs. The relationship between GSK-3β and MAPK signaling in VSMCs was investigated. The modulation of GSK-3β and components of the Notch signalling pathway in an in vitro model of a stented artery, in an in vivo model of altered mechanical strain and in human arteriosclerotic tissue was also examined.

5.2 Results

5.2.1 Cyclic strain modulates Notch Signalling and GSK-3β in VSMCs

Our lab has demonstrated previously that Notch signalling in RVSMC is downregulated following biomechanical strain (Morrow *et al.*, 2005b). It was confirmed that the commercial cell line used in this study is haemodynamically responsive to mechanical stimulation by analysing the levels of transfected Notch1IC-GFP following exposure to strain. Notch 1 IC-GFP levels were significantly reduced following exposure to 10% cyclic strain for 24 h when compared to static controls (Figure 5.1). GSK-3β is primarily regulated through inhibitory phosphorylation on its serine 9 residue. Therefore, the levels of serine 9 phosphorylation of GSK-3β following exposure to 10% cyclic strain for 24 h were analysed. Phosphorylation of GSK-3β increased with cyclic strain, therefore indicating that GSK-3β activity decreased with cyclic strain. (Figure 5.2 and Figure 5.3 A). The level of GSK-3β mRNA expression following exposure to 10% cyclic strain for 24 h was analysed (Figure 5.2). GSK-3β mRNA expression increased following exposure to 10% cyclic strain for 24 h(1.40 ± 0.109 fold).

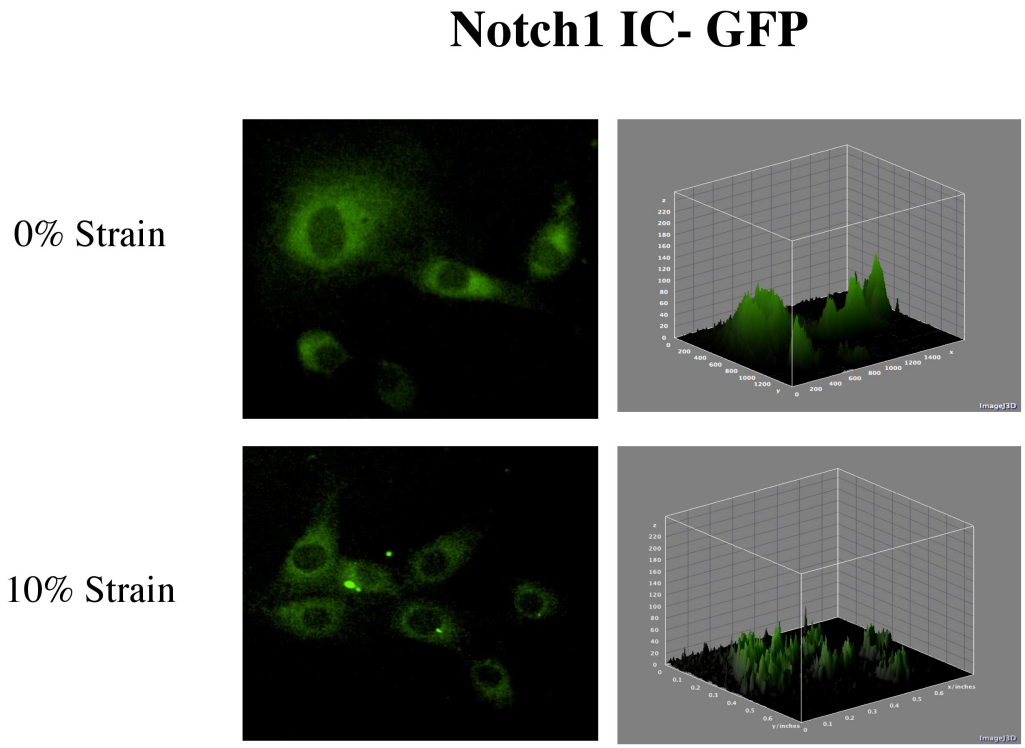


Figure 5.1: Cyclic strain modulates Notch signalling in VSMCs. GFP Expression in RVSMCs following transfection of a Notch1IC-GFP expression plasmid in RVSMCs. RVSMCs were exposed to 10% biaxial cyclic strain for 24 hrs and compared to static controls (left frames). The images were quantified using Image J (right frames). The experiment was performed in triplicate, with the image representative of n=3

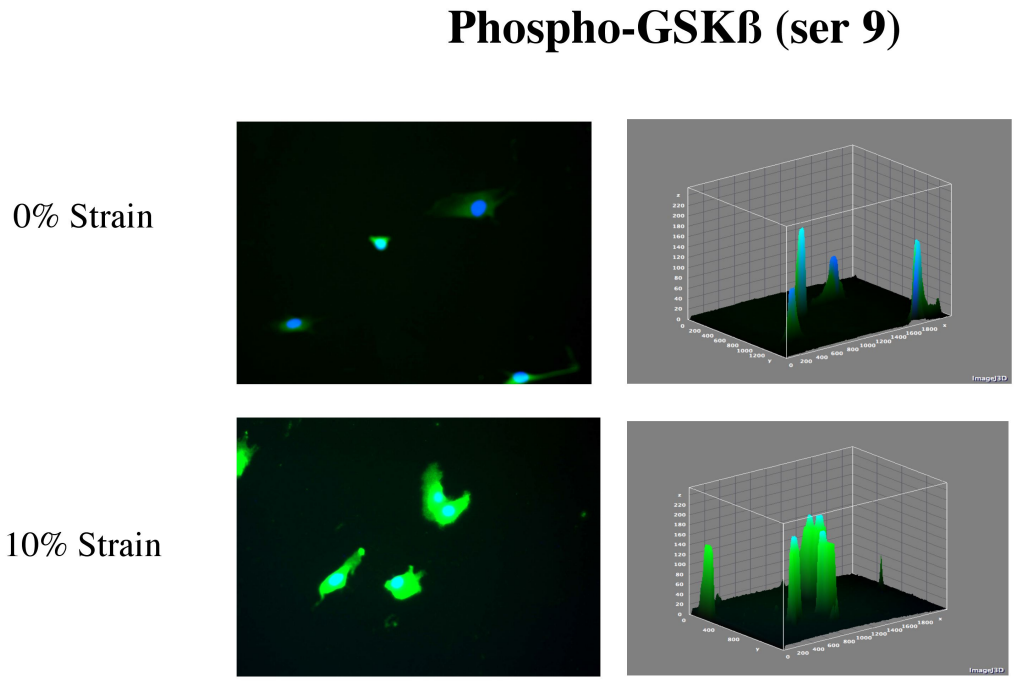


Figure 5.2: Cyclic strain modulates GSK-3β activity in VSMCs. Imaging of phospho-GSK-3β Expression following exposure of RVSMCs to 10% biaxial cyclic strain for 24 hrs, as compared to static controls (left frames). The images were quantified using Image J (right frames). The experiments were performed in triplicate, with the image representative of n=3

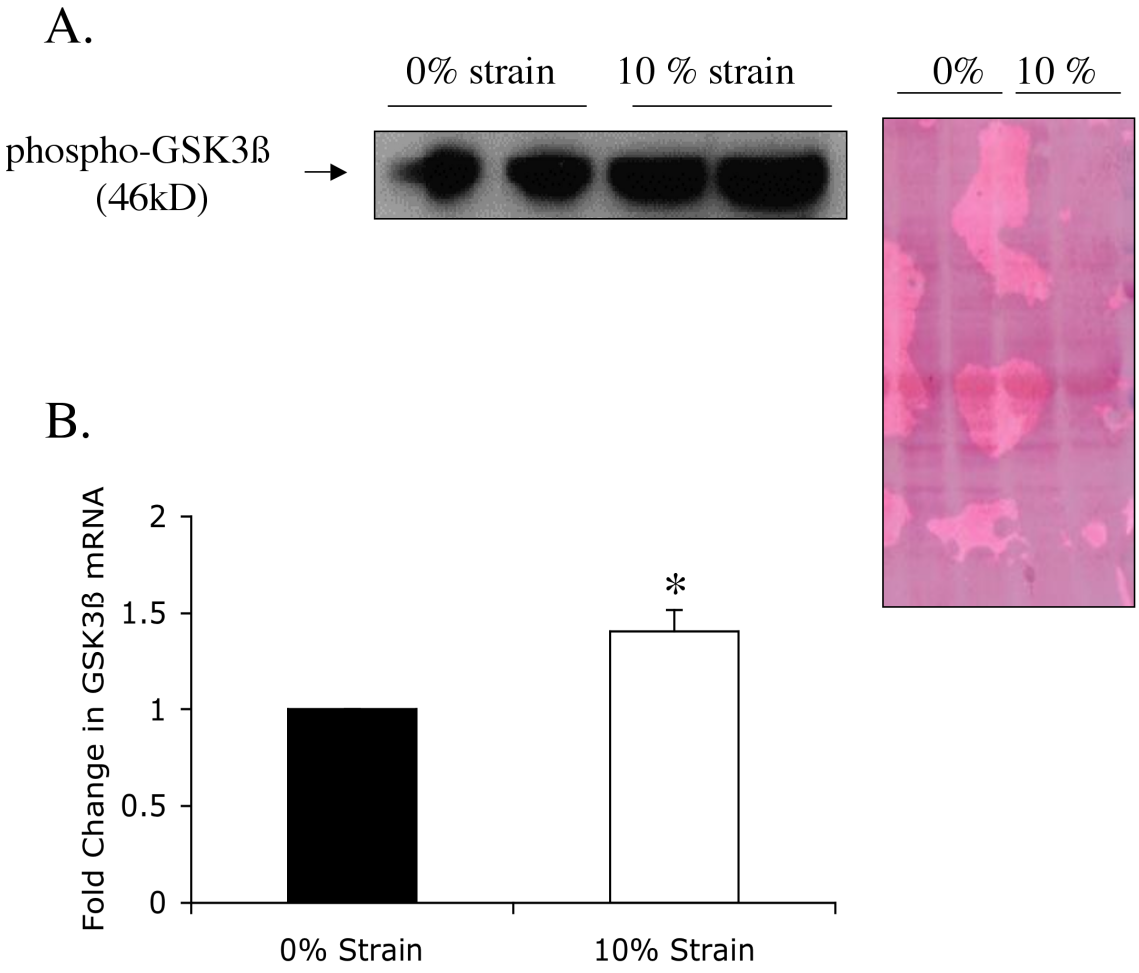


Figure 5.3: Cyclic strain modulates GSK-3β phosphorylation and mRNA Expression. A) Western Blot of phospho-GSK-3β Expression following exposure of RVSMCs to 10% biaxial cyclic strain for 24 hrs, as compared to static controls. Expression was normalized to Ponceau levels (right frame).B) Real-time PCR analysis of GSK-3β mRNA following exposure of RVSMCs to 10% biaxial cyclic strain for 24 hrs, as compared to static controls. Expression was normalized to GAPDH levels, and expressed as fold increase over control. $*p< 0.05$ (student's t-test). The experiments were performed in triplicate, with the image representative of n=3.

5.2.2 Rapid Phosphorylation of GSK-3β, p38 and p44/42 by cyclic strain in VSMCs

Our previous study found that the cyclic-strain dependent change in Notch IC expression is MAPK-dependent (Morrow *et al.*, 2005b). This study, therefore, examined if GSK-3β was involved in this strain-induced effect (Morrow *et al.*, 2005b). Both p38 and p44/42 are MAPKinases, which are activated by phosphorylation, whereas GSK-3β is a serine-threonine kinase which is primarily negatively regulated through phosphorylation on its serine 9 residue, which forms an inactive form of the enzyme (Shaw and Cohen, 1999). Firstly, it was confirmed that p38 and p44/42 are activated by strain by examining their phosphorylation states following 30 min of exposure to 10% cyclic strain (Figure 5.4). Phospho-GSK-3β was also increased following 10% cyclic strain for 24 hrs. GSK-3β mRNA showed only a slight increase following 10% cyclic strain for 24 hrs, indictating that GSK-3β is primarily modified post-translationally by cyclic strain (Figure 5.2).

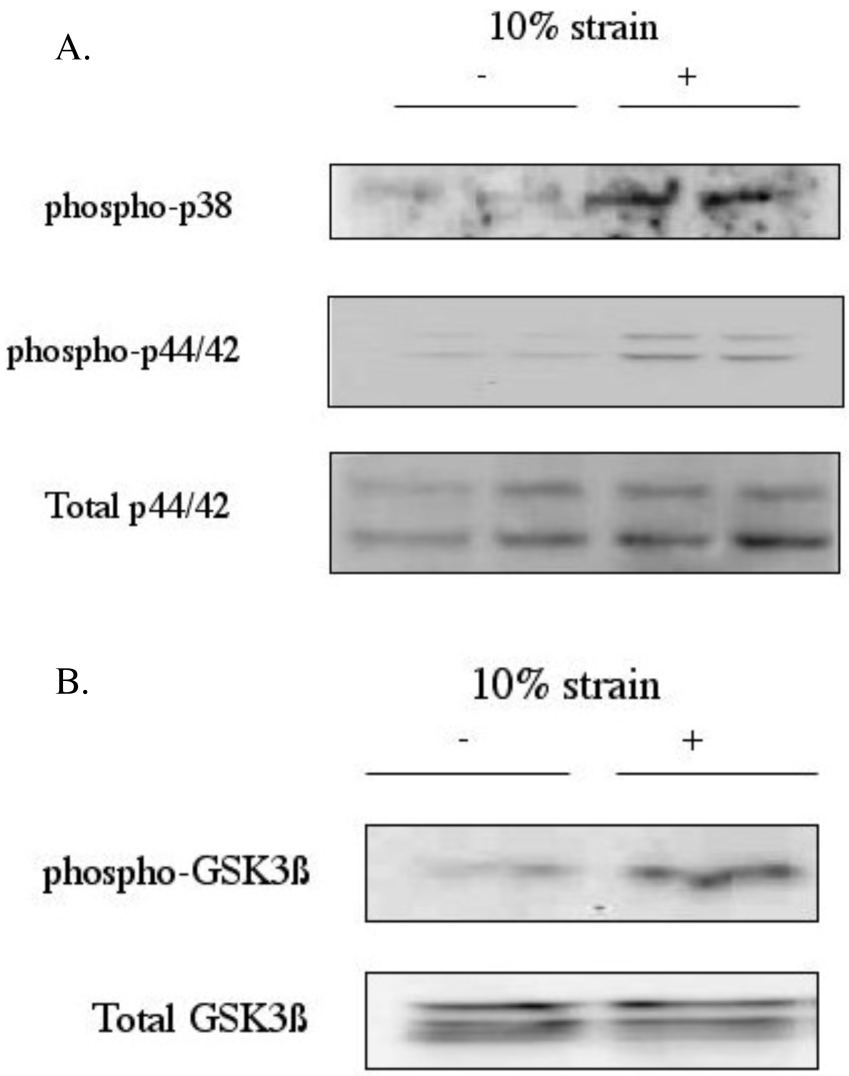


Figure 5.4: Biomechanical Strain modulates MAPK activity. Western blot of phospho-p38, phospho-p44/42 and phospho-GSK-3β and total GSK-3β following exposure of RVSMCs to 10% cyclic strain for 30 mins. The experiments were performed in triplicate, with the images representative of n=3.

5.2.3 Effect of MAPK inhibition on GSK-3β activity in VSMCs

The effect of MAPK inhibition on phosphorylation of the serine 9 residue of GSK-3β was firstly examined following MAPK inhibition using a pharmacological inhibitor of p38, PD169316 (Figure 5.5). No effect on strain-induced phosphorylation of GSK-3β was observed following p38 inhibition in VSMCs. Similarly, when p42/44 was inhibited using the pharmacological inhibitor PD098059, there was no effect on the strain-induced phosphorylation of GSK-3β following p42/44 inhibition in VSMCs (Figure 5.6).

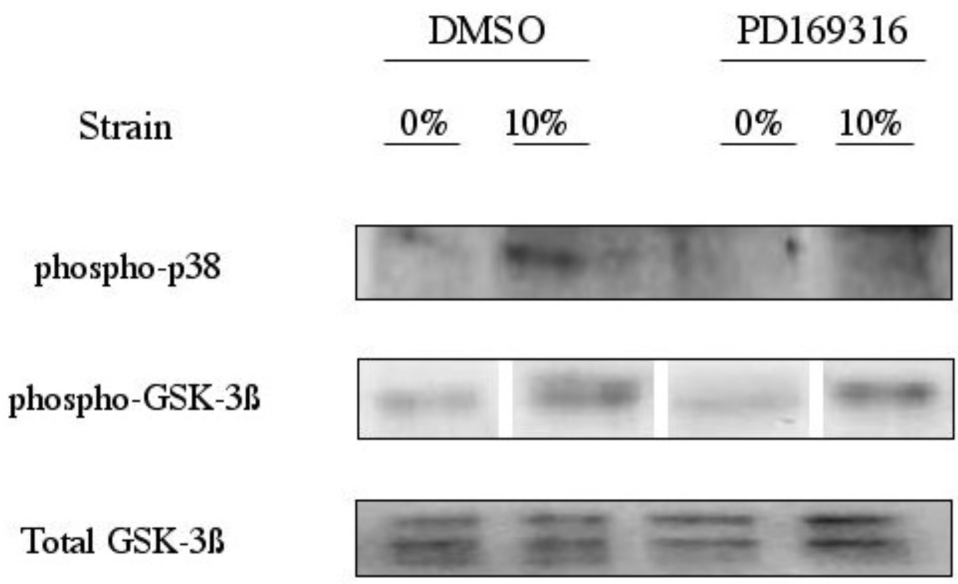


Figure 5.5: p38 inhibition does not modulate GSK-3β phosphorylation. Western blot analysis of phospho-p38, pGSK-3β and total GSK-3β following exposure of quiesced RSMVCs to 10% cyclic strain and treatment of RSVMCs with RPMI containing PD169316 (30μM) for 30 min. The experiments were performed in triplicate, with the images representative of n=3.

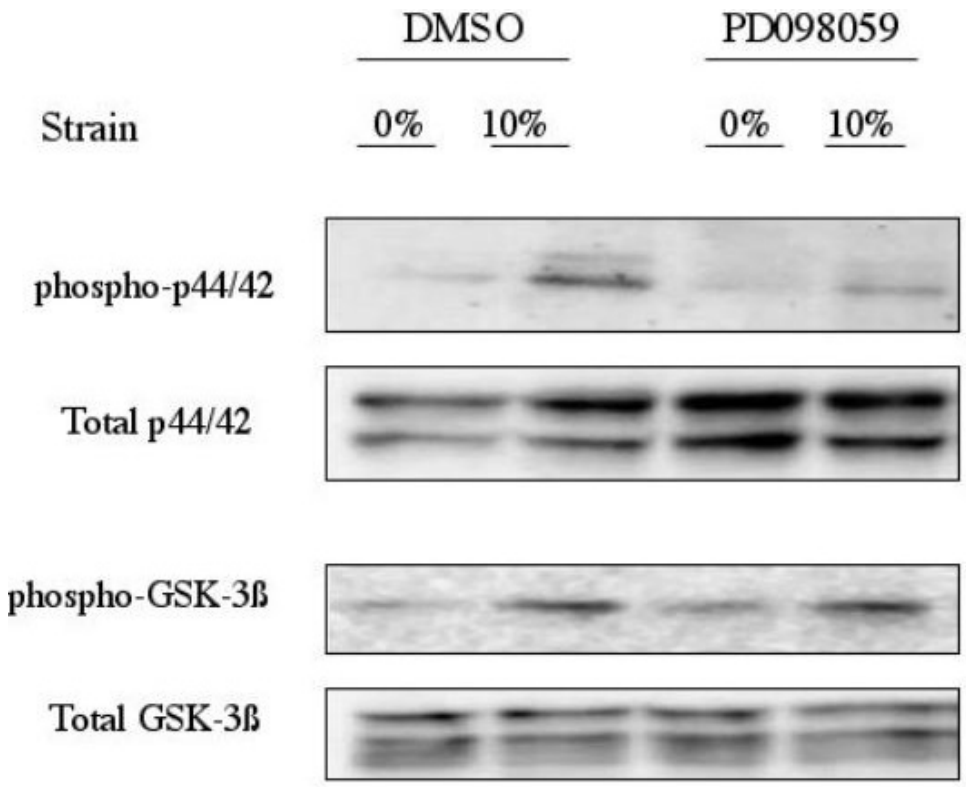


Figure 5.6: p44/42 inhibition does not modulate GSK-3β phosphorylation. Western blot analysis of phospho-p44/42, pGSK-3β and total GSK-3β following exposure of quiesced RSMVCs to 10% cyclic strain and treatment of RSVMCs with RPMI containing PD098059 (30μM) for 30 min. The experiments were performed in triplicate, with the images representative of n=3.

5.2.4 Effect of GSK-3β inhibition on MAPK activity in VSMCs

The effect of GSK-3β inhibition on MAPK activity in VSMCs was examined using the pharmacological inhibitor SB216763. Inhibition of GSK-3β resulted in increased phosphorylation of p38 and p44/42 in static quiesced VSMCs (Figure 5.7). In addition, pharmacological inhibition of GSK-3β with SB216763 attenuated the strain-induced increase in p38 and p44/42 phosphorylation, respectively (Figure 5.8).

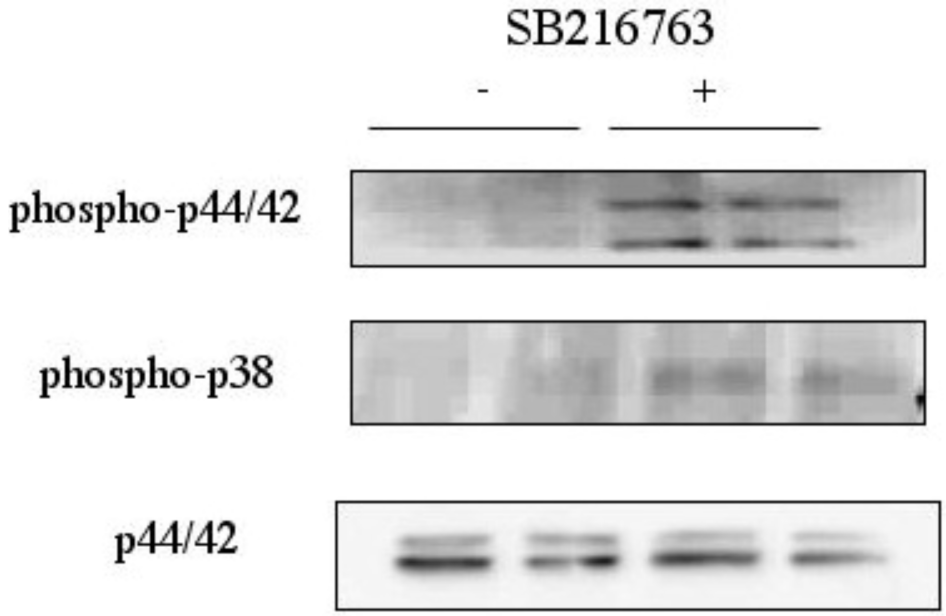


Figure 5.7: pGSK-3β modulates MAPK activity Western blot analysis of phospho-p38, phospho-p44/42 and total p44/42, following treatment of quiesced RSVMCs with RPMI containing SB216763 (25μM) for 30 min. The experiments were performed in triplicate, with the images representative of n=3.

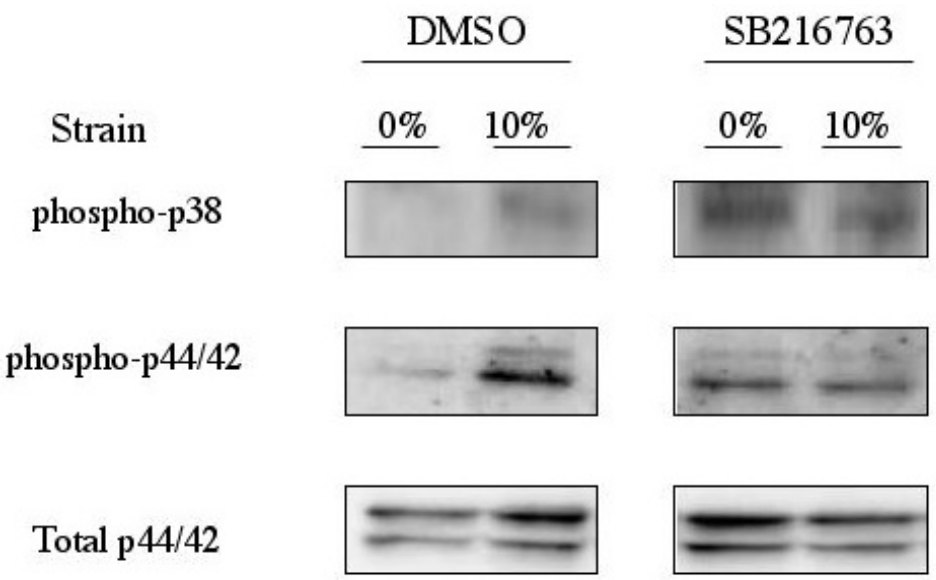
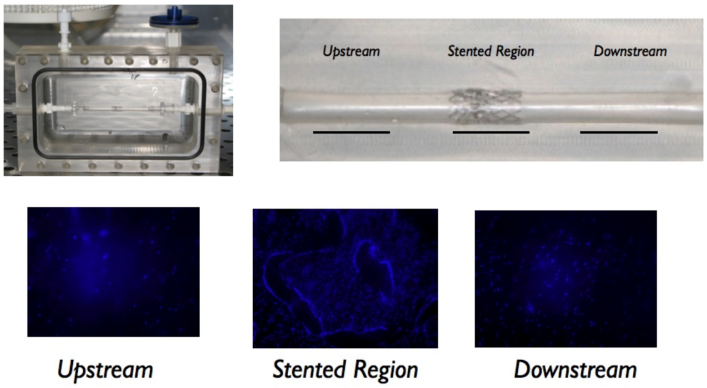


Figure 5.8: Cyclic strain induces phosphorylation of p38 and p44/42 MAPK in a GSK3β dependent manner. Western blot analysis of phospho-p38, phospho-p44/42 and total p44/42, following exposure of quiesced RVSMCs to 10% cyclic strain and treatment with RPMI containing SB216763 (25μM) for 30 min. The experiments were performed in triplicate, with the images representative of n=3.

5.2.5 *In vitro* model of a Stented Artery

Restenosis of an artery is known to occur in many instances following deployment of a stent after angioplasty (Luscher *et al.*, 2007). The mechanism of regrowth is, however, not fully understood. An *in vitro* model of a stented mock coronary artery (MCA) was used to examine the role of the Notch signaling pathway and GSK-3β in in-stent restenosis. The MCA mimics the strain and flow of a carotid artery following expansion of a stent. Due to the rigidity of the stent material, a high mean strain/low strain amplitude exists in the stented region, compared to a low mean strain/high amplitude upstream and downstream of the stent. BVSMC proliferation is increased in the stented region of the MCA, compared to the regions upstream, and downstream of the stent (Figure 5.9 A.)(Images provided courtesy of Alberto Columbo). The regulation of GSK-3β and Notch1 in an in-vitro model of a stented artery was investigated. Phosphorylation of GSK-3β protein was reduced in BVSMCs in the stented region of the Sylgard phantom compared to a region upstream of the stent (Figure 5.9). Notch1 protein was analysed using an antibody targeted against full-length Notch1. The levels of full-length Notch1 is reduced in the stented region of the artery (Figure 5.10). This indicates therefore, that the level of Notch1 IC was increased in the stented region of the tubing compared to a region upstream of the stent (Figure 5.10).

A.



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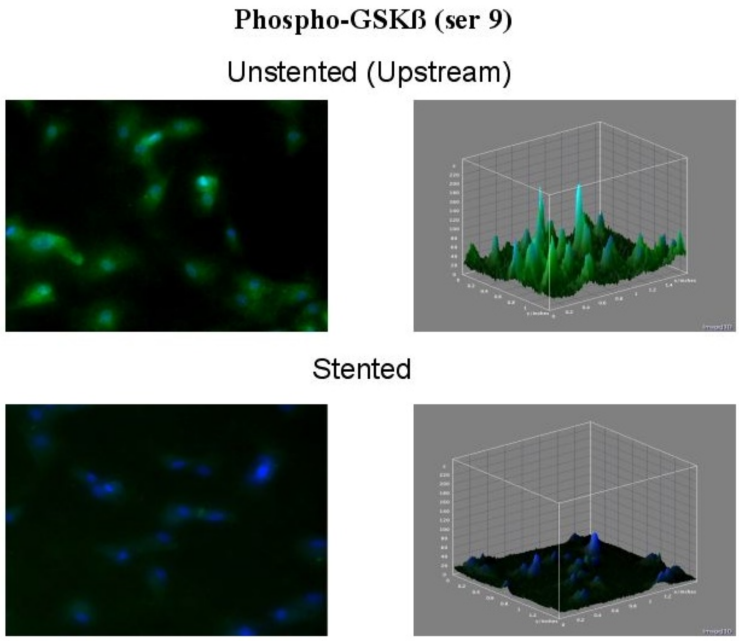

Figure 5.9: GSK-3β modulation in an *in vitro* model of a stented artery. A) DAPI staining of BSMCs in stented, upstream and downstream regions of MCA (Images provided courtesy of Alberto Columbo). B) Immunostaining of phospho-GSK-3β in the stented region and upstream region of MCA (left frame.) The images were quantified using Image J (right frame).

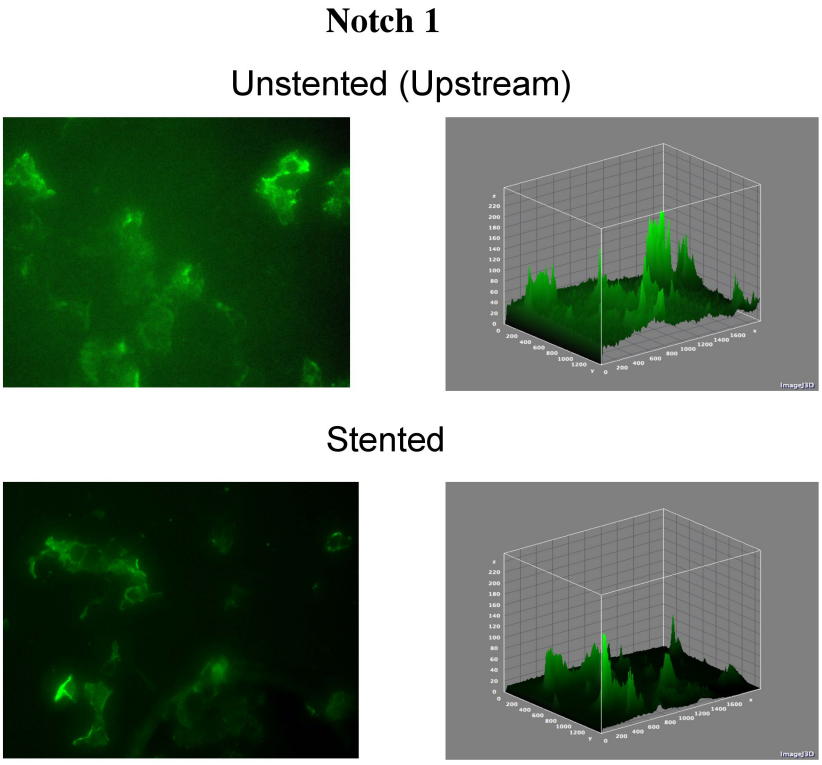

Figure 5.10: Notch1 modulation in an *in vitro* model of a stented artery. Immunostaining of Notch1 in the stented region and upstream region of MCA (left frame.) (20x magnification) The images were quantified using Image J (right frame).

5.2.6 *In vivo* model of biomechanical-induced vascular remodelling

The regulation of GSK-3β and Notch signaling components was investigated in an *in vivo* model of biomechanical-induced vascular remodelling in which the left carotid artery of a rat was completely ligated. Medial thickening and neointima formation is observed in the left carotid artery 14 days after ligation (Morrow *et al.*, 2009). The presence of Notch1 IC, Patched 1 and Gli2 has been previously shown in the neointima and medial artery of the remodelled vessel (Morrow *et al.*, 2009). RNA was isolated 14 days post-ligation and expression levels of GSK-3β, and HRT3 were analysed. GSK-3β mRNA decreased to 0.35 $\pm$ 0.09 fold and HRT3 mRNA levels also decreased to 0.209 $\pm$ 0.06 fold in the ligated vessels compared to the contralateral control(Figure 5.12).

The levels of GSK-3β and HRT3 were investigated in a partially ligated *in vivo* model of biomechanical-induced vascular remodelling and the levels were found to be decreased 3 days post-ligation (to 0.32 $\pm$ 0.06 fold and 0.30 $\pm$ 0.13 fold, respectively, n=3)(Figure 5.11).

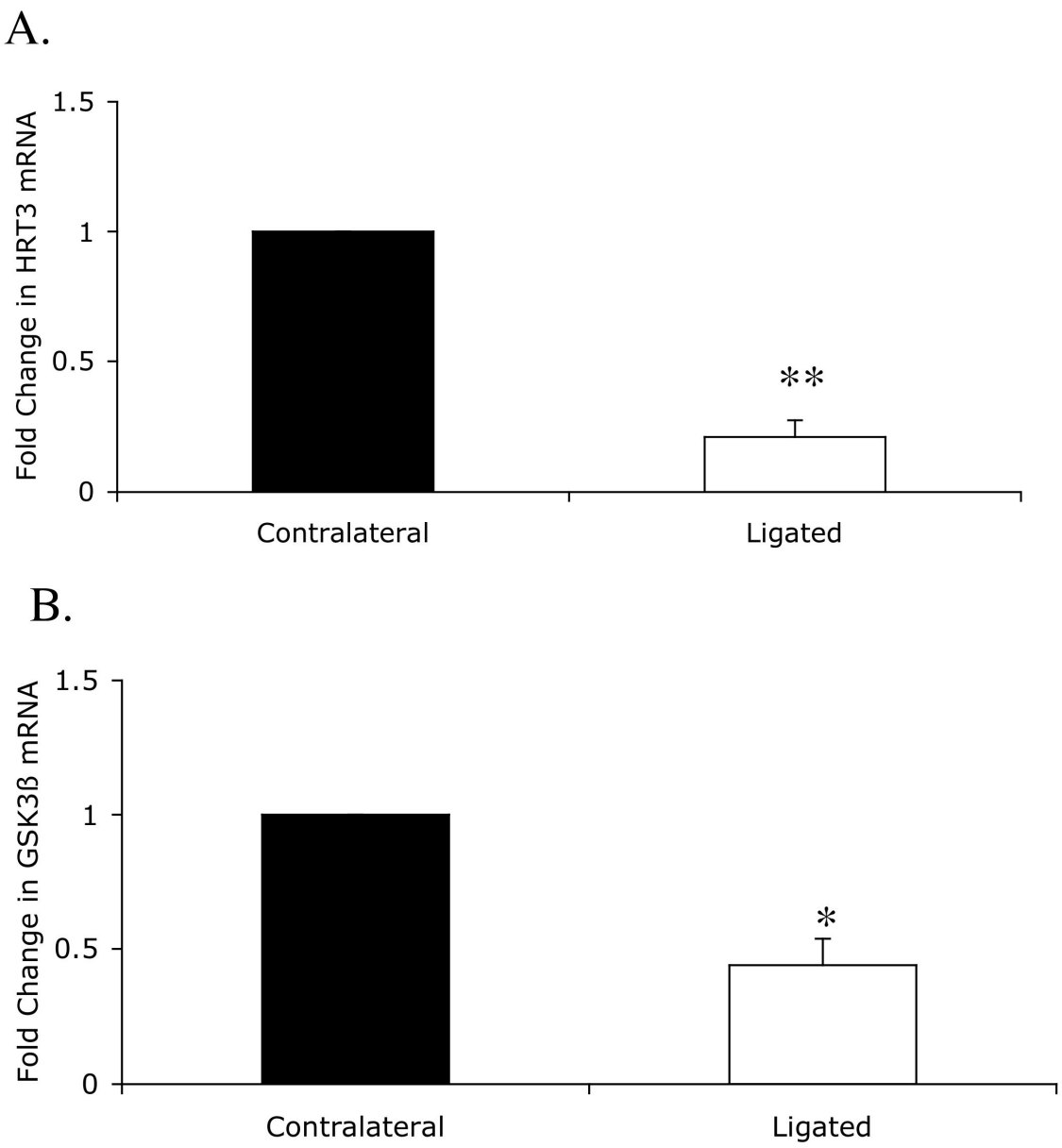

Figure 5.11: GSK-3β and HRT3 are modulated in a totally-ligated mouse carotid artery. Real-time analysis of GSK-3β and HRT3 following total-ligation of the left carotid artery of a C57B16/J mice and isolation of mRNA 14 day post-ligation (4 vessels per preparation, n=2). Expression was normalized to GAPDH levels, and expressed as fold increase over control. $*p< 0.05$, $**$ $p< 0.01$ as compared to contralateral control (students t-test)

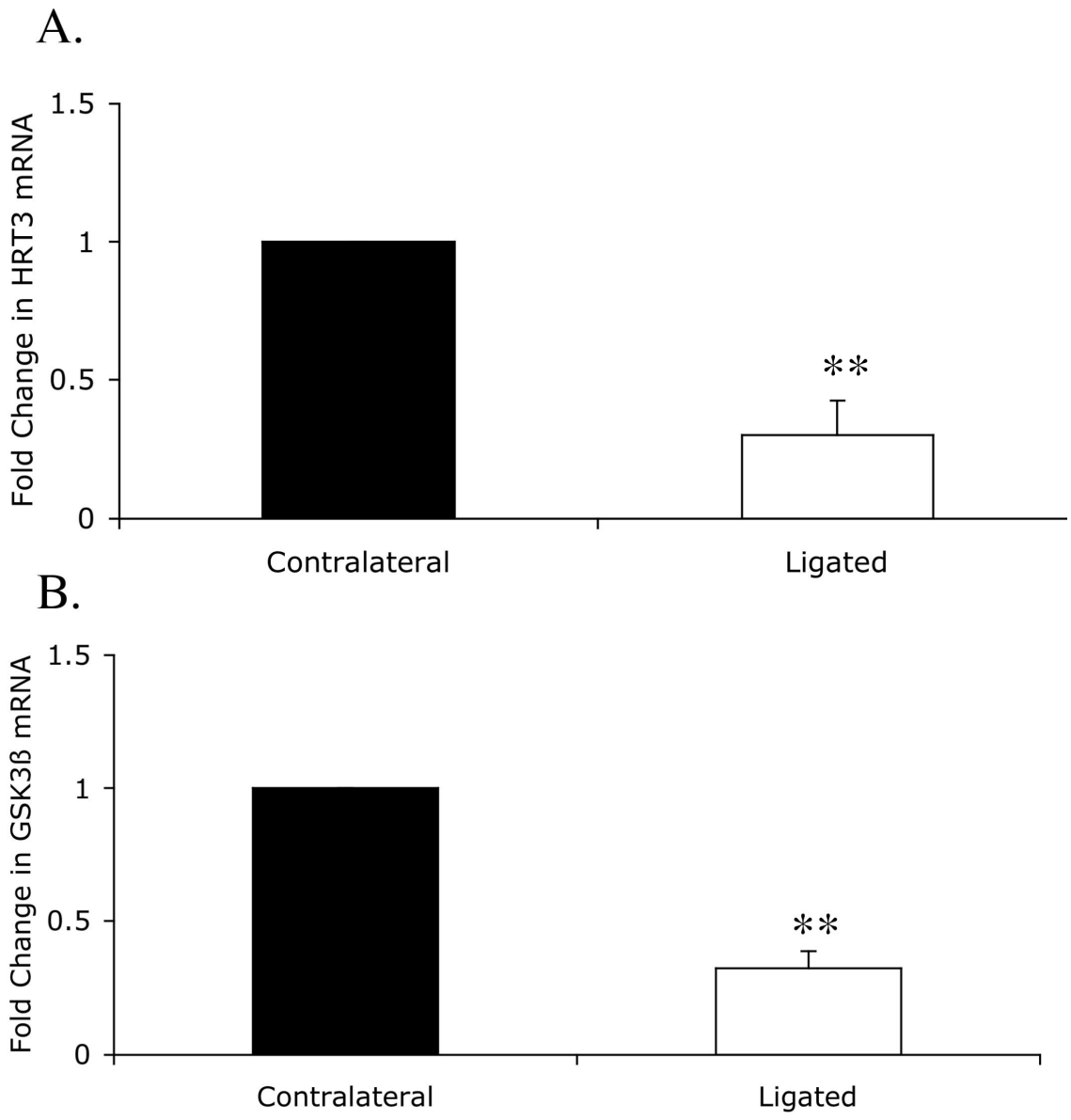

Figure 5.12: GSK-3β and HRT3 are modulated in a partially-ligated mouse carotid artery. Real-time analysis of GSK-3β and HRT3 following total-ligation of the left carotid artery of a C57B16/J mice and isolation of mRNA 3 day post-ligation (4 vessels per preparation, n=3). Expression was normalized to GAPDH levels, and expressed as fold increase over control. $**p< 0.01$ as compared to contralateral control (students t-test)

5.2.7 GSK-3β, HRT3 and Patched1 are modulated in human arteriosclerosis

Human cDNA derived from an arteriosclerotic region of the aorta of a 76 year old, caucasian male was purchased from Biochain. cDNA derived from a non-arteriosclerotic region of the aorta from the same donor was purchased as control. The mRNA expression level of GSK-3β was 0.30 fold lower in the arteriosclerotic tissue compared to the non-arteriosclerotic control. The mRNA expression levels of HRT3 and Patched1 were also lower in the arteriosclerotic tissue compared to the non-arteriosclerotic control (0.79 fold and 0.81 fold respectively) (Figure 5.13).

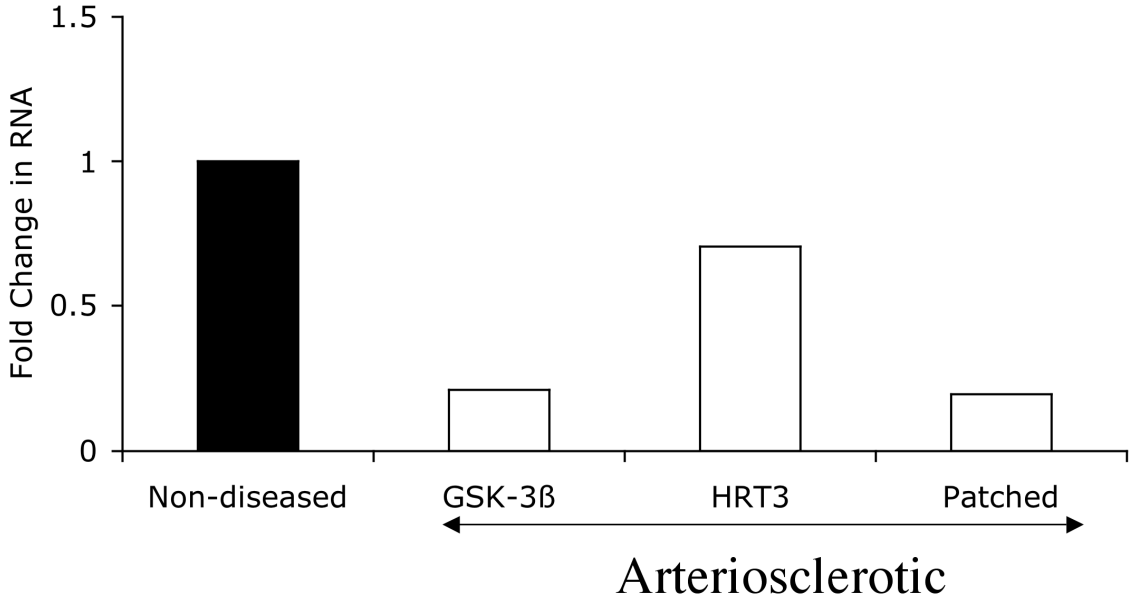

Figure 5.13: GSK-3β, HRT3 and Patched1 are modulated in a human arteriosclerotic aorta. Real-time analysis of GSK-3β, HRT3 and Patched using mRNA derived from an arteriosclerotic region of a human aorta. Expression was normalized to GAPDH levels, and expressed as fold increase over control. Gene expression levels were compared to a non-arteriosclertoic region of the same aorta.

5.3 Discussion

Haemodynamic forces play an important role in vascular pathologies and components of the Notch signalling pathway are regulated in vascular tissue after injury (Wang *et al.*, 2002). Our study confirmed that Notch signalling is decreased following cyclic strain, as previously described (Morrow *et al.*, 2005b). Having established a role for GSK-3β in the regulation of Notch signalling in static cultures, the activity of GSK-3β following exposure to cyclic strain was examined. GSK-3β activity was found to be decreased following cyclic strain through phosphorylation of its serine 9 residue. Interestingly, transcription of GSK-3β was increased following exposure to strain for 24 h. While GSK-3β activity is primarily regulated through phosphorylation, its transcriptional upregulation following strain represents an adaptive mechanism to attenuate the phosphorylation modification exerted by strain.

Cyclic strain has been shown to activate Akt in bovine VSMCs by phosphorylation (Chen *et al.*, 2001) and Akt and GSK-3β are also phosphorylated by strain in endothelial cells (Nishimura *et al.*, 2006). GSK-3β is a direct substrate of Akt through the PI3-kinase pathway (Doble and Woodgett, 2003) and it is possible that GSK-3β is phosphorylated through this PI-3kinase pathway following strain.

MAPKinases, including p44/42 and p38, are serine-threonine kinases that regulate a wide range of cellular activites and are activated by a variety of stimuli. It has been demonstrated in numerous studies that p44/42 and p38 are regulated by strain in VSMCs (Tock *et al.*, 2003), (Li *et al.*, 2000), (Li *et al.*, 1999a). Cyclic strain has a temporal effect on these MAPK in VSMCs with rapid activation of p38 and p44/42 up to 30 mins followed by a decline thereafter that is thought to be

MAPK-phosphatase-1 (MKP-1) dependent (Li *et al.*, 1999a). The rapid induction of p38 and p44/42 activation was demonstrated and it was also demonstrated that GSK-3β is similarly rapidly phosphorylated.

It has been previously demonstrated that the strain-dependent change in Notch signalling in VSMCs is MAPK-dependent (Morrow *et al.*, 2005b), as inhibition of p44/42 activation attenuated the strain-induced decrease in Notch signalling.

As discussed previously, GSK-3β was found to be a regulator of Notch signaling in static VSMCs. It is possible that GSK-3β may regulate Notch signaling in strained cells, through interaction with the intracellular MAPK pathway Therefore, it was investigated if there was a relationship between the strain-induced phosphorylation of GSK-3β and activation of p38 and p44/42 in VSMCs. There was no significant change in strain-induced GSK-3β phosphorylation following inhibition of p44/42 using the MEK inhibition PD098059. Similarly, there was no significant change in strain-induced GSK-3β phosphorylation following inhibition of p38 by the pharmacological inhibitor PD169316. However, as previously discussed GSK-3β is phosphorylated by strain-activated Akt, which may prevent detection of a MAPK- effect. Further investigation using a PI3-kinase inhibitor such as wortmannin, may elucidate further whether MAPK activity contributes to the strain-induced phosphorylation of GSK-3β in VSMCs. Additionally, p44/42 or p38 may regulate GSK-3β activity through a mechanism independent of serine 9 phosphorylation. In mouse embryonic carcinoma F9 cells, p38 mitogen-activated protein kinase regulates canonical Wnt-β-catenin signalling by inactivation of GSK3 β (Bikkavilli *et al.*, 2008) and ERK has a similar effect on the GSK-3beta/beta-catenin pathway in human lung fibroblasts (Caraci *et al.*,

2008). GSK-3β is thought to be regulated in the Wnt pathway independently of serine 9 phosphorylation (Ding *et al.*, 2000). Additionally, an alternative phosphorylation pathway for GSK-3β in the Wnt pathway has been identified, where p38 regulates GSK-3β by direct phosphorylation at its C terminus rather then the serine 9 at the N-terminus (Thornton *et al.*, 2008).

GSK-3 has been shown to be a negative regulator of MAPK in a number of cell types including human colon cancer cells (Wang *et al.*, 2005) , rat skeletal muscle (Macko *et al.*, 2008) and rat primary astrocyes (Kim *et al.*, 2007). Similarly, pharmacological inhibition of GSK-3β in quiesced static VSMCs was found to induce rapid activation of p38 and p44/42. This may occur through inhibition of a kinase upstream of p38 and p44/42, as GSK-3β has previously been shown to reduce MEKK4 stimulation of p38 in COS-7 cells (Abell *et al.*, 2007). It was then determined that under strain conditions, inhibition of GSK-3β through phosphorylation accounts for the rapid activation of p38 and p44/42.

PDGF has the ability to inactivate GSK-3β while activating p44/42 in VSMCs (Huang and Kontos, 2002) and PDGF inhibits Notch signaling in VSMCS through induction of p44/42 (Wang *et al.*, 2002). A novel GSK-3β-MAPK-Notch pathway may exist, therefore in which a stimulus such as PDGF or, in the case of this study, strain can negatively modulate Notch signalling.

The mediator of the MAPK effect on Notch signalling following cyclic strain has yet to be determined. A possible mechanism is through regulation of γ-secretase, as p44/42 but not p38 has been shown to reduce cleavage of Notch1 through modulation of γ-secretase activity in human embryonic kidney cells (Kim *et al.*, 2006). A Hh-VEGF-Notch signaling

pathway in VSMCs, in which Notch is positively regulated was previously identified. A previous study in our lab and further confirmed in the present study has also shown that Hh is regulated by strain (Morrow *et al.*, 2007). It is possible therefore that MAPK exerts its effect on Notch signaling through downregulation of the Hh-VEGF-Notch axis. However, cyclic strain has been shown to induce the expression of VEGF in VSMCs (Smith *et al.*, 2001). Additionally, retinal pericyte VEGF expression is induced by strain independently of p44/42 or Akt (Suzuma *et al.*, 2002). Moreover, it has been previously shown that Notch signaling can rescue the strain-induced decrease in Hh downstream signaling (Morrow *et al.*, 2007). A reduction in this feedback by Notch may, therefore, account for the downregulation of Hh signaling observed in VSMCs following strain.

As discussed previously, Foxc2 is a possible candidate for Notch signalling modulation in VSMCs. Interestingly, ERK activity inhibits Foxc2-dependent upregulation of Dll4 and Hey2 expression in ECs and both Foxc1 and Foxc2 have 10 potential phosphorylation sites for ERK (Hayashi and Kume, 2008). The Foxc transcription factors, therefore, may be involved in the MAPK-dependent strain-induced modulation of Notch signaling in VSMCs, possibly through regulation of Notch ligand expression. An outline of our proposals are summarised in Figure 5.14. Expression of the Notch ligand, Jagged1 was previously shown to be decreased following cyclic strain. p38 MAP kinase negatively regulates Jagged1 expression in FGF-2 stimulated ECs (Matsumoto *et al.*, 2002). Induction of Jagged-1 in bone-marrow derived macrophages (BMM)s by soluble egg antigen (SEA) is , however, increased by ERK signalling (Goh *et al.*, 2008). In any case, the relationship between Jagged1 and MAPK

signaling in VSMCs following exposure to strain warrants further investigation.

In the vasculature, altered smooth muscle cell growth contributes to neointima formation and vascular remodelling during arteriosclerosis, atherosclerosis, and restenosis after angioplasty, including in-stent restenosis (Gosens *et al.*, 2008). Remodeling is a change in the composition of the artery that occurs in response to chronic changes in hemodynamic conditions (Kumar *et al.*, 1997). Restenosis is one of the major limitations of percutaneous transluminal coronary angioplasty (PTCA). It occurs as a combination of neointimal formation and arterial remodeling in response to balloon injury. Although stents are deployed to prevent the closure of the lumen, restenosis may still occur (Post *et al.*, 1997). A direct consequence of stent expansion in a vessel is that the mechanical environment changes from a low mean strain/high amplitude to a condition of high mean strain/low strain amplitude. Recent studies in our lab have identified that a decrease in the amplitude of cyclic strain is more conducive to proliferation of VSCMs than the level of mean cyclic strain (*Alberto Columbo, in discussion*) and may account in part to the restenosis observed in some instances following stent implantation. We investigated the regulation of GSK-3β in VSMCs in an *in vitro* model of a stented mock coronary artery (MCA) and found that GSK-3β activity was increased through reduction of phosphorylation on its serine 9 residue in the stented region compared to a region upstream of the stent. Similarly, Notch1 levels were increased in the stented region compared to a region upstream of the stent. This correlates with our data from the FlexercellTension System, where an increased amplitude of strain (0%-10% compared to 0% static control) decreased the levels of

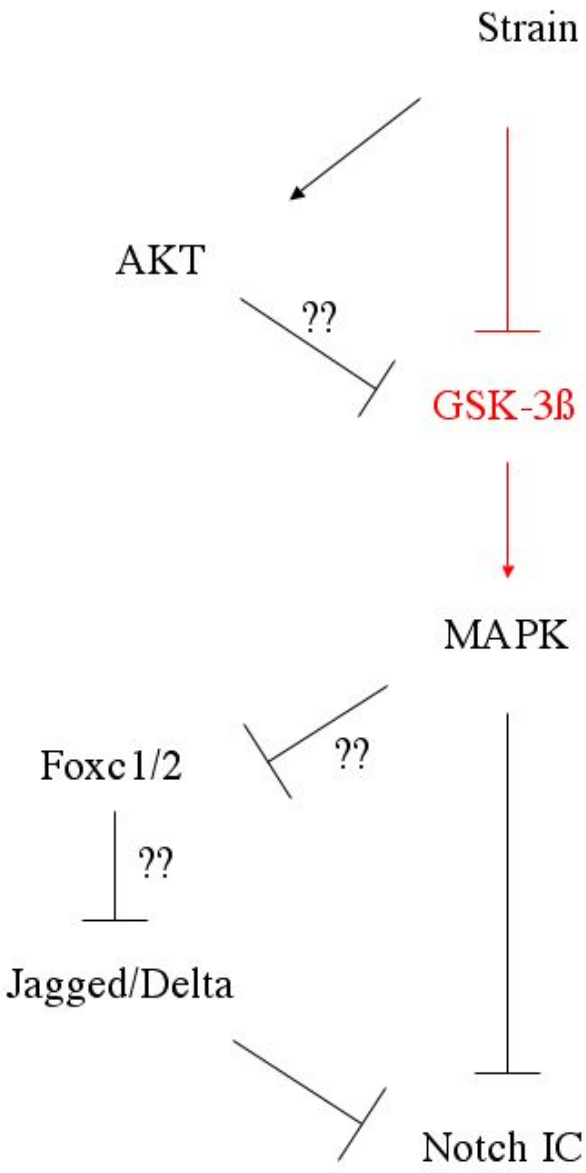

Figure 5.14: Summary of Hypothesised GSK-3β-MAPK-Notch Pathway in VSMCs following Exposure to Strain

Notch 1IC and GSK-3β activity. It suggests that GSK-3β may play a putative role in in-stent restenosis through modulation of Notch signaling. It also highlights the importance of the compliance of a stented vessel in modulating the pro-proliferative Notch signaling pathway and suggests that this is an important factor when considering stent design.

While our *in vitro* models assist in identifying possible key players and the mechanisms involved in vascular diseased states, we can only be confident of their role in pathological conditions through the use of *in vivo* models. In this study, an *in vivo* model was used in which blood flow in the left common carotid artery of the mouse was arrested by ligation, causing blood statis and a low amplitude condition within the ligated artery and a high flow environment in the contralateral non-ligated right carotid artery. In this model, remodeling occurs proximal to the ligation site as a consequence of the altered biomechanical forces. A recent study has shown that Notch1 but not Notch3 mediates this neointimal formation after vascular injury in the same carotid ligation model Li *et al.* (2009). A recent study in our lab has shown that Notch1 and its target genes are increased in the completely ligated models in a time-dependent manner. Notch and downstream target mRNA is initially upregulated in the artery 3 day post-ligation. This is followed by an observed increase in Notch and target gene protein expression 14 days post-ligation, concomitant with a reduction in Notch and target gene mRNA, perhaps as an adaptive mechanism (Figure 5.15 A and B). Patched1, the downstream Hh target gene is also significantly enhanced in the intima and media of remodeled carotid arteries concomitant with a significant increase in the expression of the Hh transcription factor Gli2 (Figure 5.15 A (ii) and B (ii) (Morrow *et al.*, 2009).

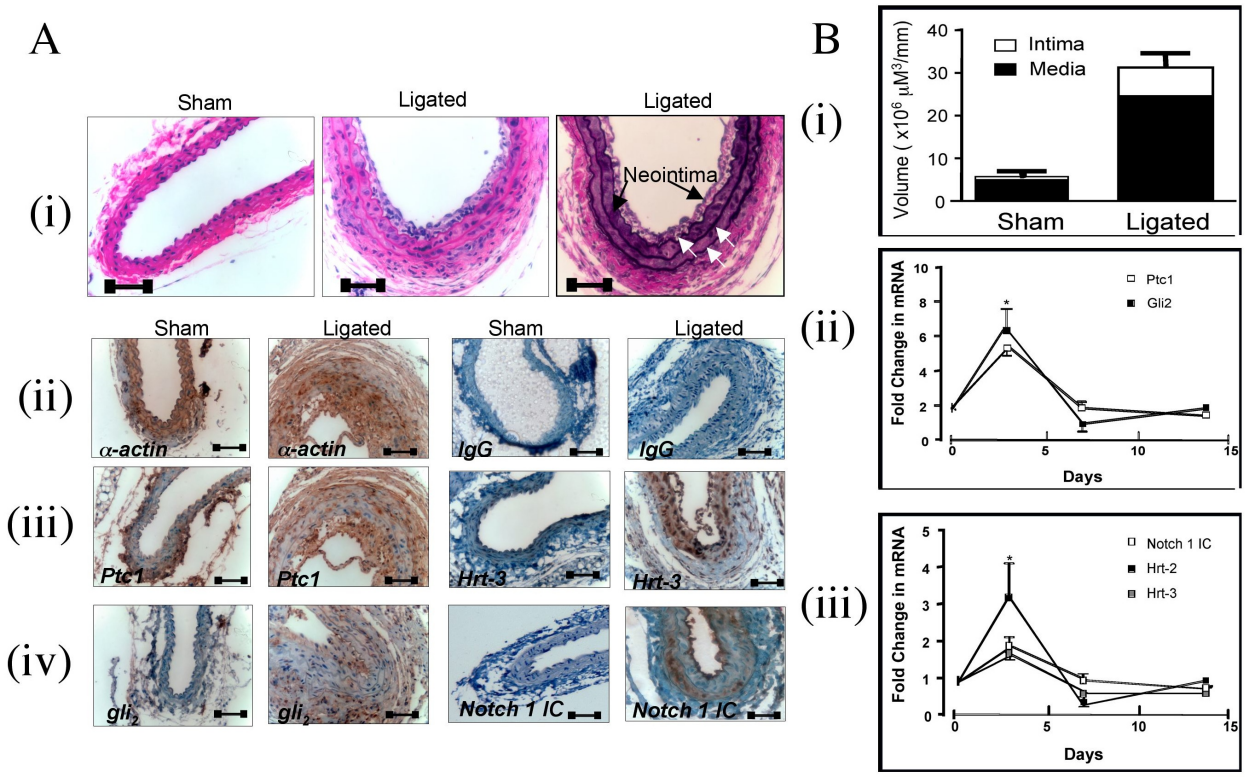

Figure 5.15: Notch and Hh signaling component expression is increased in a biomechanically-induced remodelled carotid artery (Morrow *et al.*, 2009). (a) (i) Haemotoxylin and eosin staining of ligated left carotid artery compared to sham control from C57B16/J mice, 14 days after ligation. (ii) Ptc1 and HRT3 immunohistochemical staining in LCA (iii) Gli2 and N1 immunohistochemical staining in LCA. (B)(i) Quantification of artery volume. (ii) Ptc1 and Gli2 mRNA expression in ligated arteries compared to conlateral control. (iii) Notch1, HRT2 and HRT3 mRNA expression in ligated arteries compared to conlateral control.

This correlates with the hypothesis of a Hh-VEGF-Notch axis in VSMCs that acts to promote VSMC growth. We have already determined that GSK-3β is a regulator of Notch signaling and is modulated by cyclic strain in VSMCs. Here, GSK-3β in two *in vivo* ligated carotid artery (LCA) models was examined. The first involved complete ligation of the carotid artery, causing complete interruption of flow, and the second involved a partial ligation, allowing a low rate of flow within the ligated artery. Notch and its downstream targets were examined in the same models and it was determined that GSK-3β is regulated in the same manner as Notch in these two models of biomechanically-induced vascular remodelling.

GSK-3β has previously been studied in balloon angioplasy models. Balloon injury has been shown to inhibit GSK-3β activity (Park *et al.*, 2003), which is thought to contribute to VSMC survival and subsequent neointimal formation (Hall *et al.*, 2001), (Park *et al.*, 2003). In this model, it is proposed that the growth factors, matrix protein and proteases that are released following mechanical damage by the balloon catheter could inhibit GSK-3β (Gosens *et al.*, 2008). As discussed previously, GSK-3β is known to stimulate VSMC apoptosis following growth factor withdrawal (Beurel and Jope, 2006) and it has also been suggested that inhibition of GSK-3β promotes neointima formation in balloon injury through modification of β-catenin (Park *et al.*, 2003). Interestingly, Notch3 and the downstream HRT effector genes are also down regulated in response to rat carotid artery ballon injury (Wang *et al.*, 2002). Additionally, MAPK are known to be rapidly activated in arteries following balloon angioplasty (Pyles *et al.*, 1997). A dual role for inhibition of GSK-3β following balloon angioplasty may exist, firstly through upregulation of

β-catenin signaling and secondly, through a PDGF-GSK-3β–MAPK-Notch pathway, as previously discussed.

A similar Strain-GSK-3β-Notch pathway may exist in the LCA model. GSK-3β is negatively regulated by strain and here, it was shown that GSK-3β is modulated, similar to Notch in an *in-vivo* model of low flow. It is proposed that GSK-3β is involved in the biomechanical-modulation of Notch signaling to promote vascular remodeling. GSK-3β can promote cell survival of VSMCs through regulation of Notch signalling and GSK-3β may play an important role in controlling changes in VSMC proliferation following biomechanical-induced vascular remodeling.

The final study examined the role of GSK-3β and Notch signaling in human arteriosclerotic disease. The expression of GSK-3β and the downstream targets of the Notch (HRT3) and Hh signaling pathways (Patched1) were reduced in arteriosclerotic tissue from a human aorta compared to non-arteriosclerotic tissue from the aorta of the same donor. Due to restricted availability, it was only possible to perform analysis of tissue from one donor. However, it confirms that GSK-3β and the Notch and Hh pathways are important players in the pathogenesis of vascular disease and that their relationship and regulation warrants further investigation in future in vivo studies.

5.4 Conclusion

Haemodynamic forces play an important role in vascular pathologies and components of the Notch signaling pathway are regulated in vascular tissue after injury (Wang *et al.*, 2002). Our study confirmed that Notch signaling is decreased following cyclic strain, as previously described (Morrow *et al.*, 2005b). Having established a role for GSK-3β in the regulation of Notch signaling in VSMCs, GSK-3β activity was investigated and found to be decreased following exposure of VSMCs to cyclic strain. A strain-dependent change in Notch signaling is MAPK-dependent has previously been demonstrated (Morrow *et al.*, 2005b). Inhibition of GSK-3β was found to increase both p38 and p44/42 activity in RVSMCs and attenuated the strain-induced increase in phosphorylation. The modulation of GSK-3β was investigated in an *in vivo model* of biomechanically-induced remodelling. GSK-3β was found to be regulated in the same manner as Notch and its downstream targets, suggesting that GSK-3β contributes to the altered cell fate that occurs in remodelling due to impaired Notch signalling. GSK-3β and Notch activity was then examined in an *in vitro* model of a stented coronary artery. GSK-3β activity and Notch1 levels were found to be increased in this model, suggesting a role for GSK-3β and Notch1 in in-stent restenosis. GSK-3β, HRT3 and Patched1 expression levels were found to be modulated in human arteriosclerosis. Taken together, these findings implicate GSK-3β in altered vascular cell fate decisions that occur in diseased states due to impaired Notch signaling. Increased understanding of Notch signaling and VSMC fate may contribute to future therapeutic strategies against cardiovascular disease.

Chapter 6

Discussion

Altered vascular cell fate decisions are critical to the pathogenesis of vascular diseases, including hypertension, arteriosclerosis, atherosclerosis and restenosis after angioplasty. Notch signalling has been widely proven to have an important role in controlling cell fate in the development of the vasculature during embryogenesis (Gridley, 2007). A growing body of evidence suggests that Notch signalling is one of a number of signalling pathways recapitulated in adult cells resulting in altered fate decisions leading to vascular disease (Sweeney *et al.*, 2004). Haemodynamic forces play an important role in the physiological control of vascular tone. Altered haemodynamic forces have been implicated in the changes in cell fate associated with vascular disease. Notch signalling is known to be responsive to changes in haemodynamic forces in VSMCs (Morrow *et al.*, 2005b). The canonical Notch pathway involves binding of a ligand to a transmembrane receptor, resulting in the release of an active Notch IC fragment. The Notch IC then translocates to the nucleus where it associates with a CBF-1 DNA-binding protein to assemble a transcription complex that activates downstream target genes. Notch

signalling is a conserved pathway that is highly regulated through a number of mechanisms including cleavage, trafficking and post-translational modification of its ligand and receptors.

Notch IC has been shown to be hyperphosphorylated and phosphorylated forms of Notch have been identified in the nucleus (Foltz and Nye, 2001). GSK-3β is a serine-threonine kinase that was initially identified as a regulator of glucose metabolism (Hall *et al.*, 2001). Its GSK-3α isoform is a structurally similar, but functionally distinct kinase (Lee *et al.*, 2007). Both enzymes are unique kinases in that they are constitutively active in resting cells and are negatively regulated through phosphorylation on a serine 9 residue (β form) or serine 21 residue (α form) (Pearl and Barford, 2002). Additionally, the GSK-3β may require a priming kinase upstream of substrate binding (Liu *et al.*, 2004). Similar to active NotchIC, a predominantly active form of GSK-3β is found in the nucleus and GSK-3β has the ability to phosphorylate Notch1 and Notch2 (Espinosa *et al.*, 2003), (Foltz *et al.*, 2002). The regulation of Notch by GSK-3β in VSMCs was examined and it was determined that GSK-3β positively regulates both Notch1 and Notch3 receptor levels, Notch downstream target gene expression of HRT 1-3 and CBF-1 promoter activity in VSMCs. GSK-3β also regulated transcriptional expression of Notch1 in VSMCs, suggesting that GSK-3β may regulate Notch signaling independently of direct binding or phosphorylation. Alternatively, a positive signalling feedback loop is known to exist in Drosophila and vertebrate Notch signalling and this may account for transcriptional regulation of Notch1 by GSK-3β (De Celis and Bray, 1997), (Del Monte *et al.*, 2007).

The Hh pathway is a developmental pathway involved in

angiogenesis. Shh and VEGF have been shown to act upstream of Notch during arterial endothelial differentiation (Lawson *et al.*, 2002). It was investigated if this crosstalk was conserved in VSMCs and it was determined that Shh upregulates Notch signaling in VSMCs through increased VEGF expression. Hedgehog signalling functions thorough binding of the Hh ligand to its transmembrane receptor, Ptc1 or Ptc2. Binding of Hh ligand to Ptc removes its repressive effect on the second transmembrane Smo. Smo prevents degredation of downstream Gli. Full-length Gli is stabilised and is translocated to the nucleus, where it initates transcription of its downstream targets. The downstream targets of the Gli gene products include both Ptc1-2 and Gli1-2 (Mullor *et al.*, 2002). The role of GSK-3β in Hh signaling in VSMCs was investigated and it was found that GSK-3β negatively modulates Ptc1 expression, Gli2 expression and Gli2 promoter activity. GSK-3β has been shown to phosphorylate Ci, the Drosophila form of Gli, and target it for proteosomal degredation. Therefore, it was proposed that GSK-3β negatively regulates Hh signalling in VSMCs through direct phosphorylation of Gli.

Notch is known to be pro-proliferative and anti-apoptotic in VSMCs (Morrow *et al.*, 2005a) and may be a player in the altered vascular cell phenotype observed in pathophysiological environments, such as vunerable plaques and restenosis after injury (Fung *et al.*, 2007). GSK-3β is a pluripotent kinase involved in many aspects of cell signaling and is known to regulate cell fate in a context and cell-type dependent manner. Specifically, it has been shown to inhibit cell growth in VSMCs in response to hypoxia and GLUT1. The role of GSK-3β in cell growth in serum-stimulated cycling VSMCS was investigated and it was

demonstrated that GSK-3β positively modulates proliferation and prevents apoptosis of VSMCs. Ectopic expression of Notch3 was not shown to attentuate the induction of apoptosis by pharmacological inhibition of GSK-3β. GSK-3β was shown to modulates downstream signalling of Notch and, here, it is suggested that GSK-β modulates apoptosis of VSMCs downstream of Notch signalling. This implicates GSK-3β in the regulation of Notch in the vascular response to injury and the neointimal formation observed in many pathological vascular conditions (Li *et al.*, 2009).

The pulsatile nature of blood flow creates a haemodynamic stimuli of cyclic stretch which is sensed by VSMCs. VSMCs have the ability to convert the biomechanical stimuli of cyclic stretch into a biochemical response, through mechanosensors that include G proteins, integrins, receptor tyrosine kinases and intracellular pathways, including MAPK signalling (Haga *et al.*, 2007). Notch signalling in VSMCs is downregulated by strain (Morrow *et al.*, 2005b). The modulation of GSK-3β by cyclic strain was investigated and it was found that it is also down-regulated in VSMCs through phosphorylation on serine 9. Notch sigalling is modified in VSMCs in a MAPK-dependent manner. A relationship between GSK-3β and the MAPK, p44/42 and p38 in VSMCs following cyclic strain was determined. This suggests an interaction occurs through activation of p44/42 and p38, following inhibition of GSK-3β through phosphorylation on serine 9 following strain.

The focus of this study has been the regulation of Notch signalling by GSK-3β in VSMCs at the level of receptor modulation. The modulation of Notch ligand has yet to be examined and there is a limited number of studies on Notch ligand expression in VSMCs.

The importance of Jag1 in disease pathogenesis is highlighted in Alagille Syndrome. This is an autosomal dominant disorder characterized by development abnormalities of a number of organs including the liver, heart, and eye and most cases are caused by the Notch ligand, Jagged 1, mutations (Kamath *et al.*, 2004). There are five mammalian Notch ligands of which Delta-like 1(Dll1),Delta-like 3 (Dll3) and Delta-like 4 (Dll4) are structurally similar to Drosophila Delta and Jagged1 (Jag1) and Jagged2 (Jag2) are structurally similar to Drosophila Serrate (D'Souza *et al.*, 2008). Notch receptors transduce signals from intimal endothelial cells and adjacent VSMCs to regulate the proliferative, differentiation capacity and survival of VSMCs. Of the five ligands, Dll1, Dll4 and Jag1 are prevalently expressed in EC. Dll1 and Dll4 is critical in Notch signaling between adjacent endothelial cells. Dll-1 is essential for artery-vein distinction, (Limbourg *et al.*, 2007), (Sorensen *et al.*, 2009). Jag1 and Jag2 and, to some degree Dll1, are also found in VSMC (Cristofaro and Emanueli, 2009). A ligand specific role for Jag1 in differentiation of mesenchymal cells to vascular cells was identified when Jag1, but not Dll4 stimulated the SMC marker SMC-mhc, gene expression (Doi *et al.*, 2006). The endothelial expression of Jag1 is crucial for VSMC fate in development. Endothelial-specific deletion of Jag1 resulted in embryonic lethality and cardiovascular defects. The embryos showed striking deficits in vascular smooth muscle, whereas endothelial Notch activation and arterial-venous differentiation appeared normal (High *et al.*, 2008).

Notch ligands are regulated similar to the receptors through mechanisms including glycosylation, ubiquitination, endocytosis, proteolysis and control of ligand expression (D'Souza *et al.*, 2008). There are a number of studies that identify a role of crosstalk of Hh and VEGF

in the regulation of Notch ligand expression levels. Hh is known to stimulate Jagged1 expression in mesenchymal cells (McGlinn *et al.*, 2005). VEGF has been shown to stimulate both Dll1 and Dll4 in endothelial cells during arteriogenesis and angiogenesis (Limbourg *et al.*, 2007), (Seo *et al.*, 2006). As previously discussed, therefore, Jag1 may be a mediator of VEGF upregulation of Notch signaling in VSMCs A role for the forkhead transcription factors, Foxc1 and Foxc2, in the direct activation of Dll4 and HRT2 promoter activity though interaction with VEGF has been established in ECs (Seo *et al.*, 2006). The relationship between VEGF, forkhead transcription factors and Jag1 warrants investigation in VSMCs. The Wnt pathway, which results in GSK-3β inhibition, has additionally been shown to regulate Notch ligand expression. Wnt signaling is known to regulate Dll1 expression in the presomitic mesoderm of mouse embryos (Hofmann *et al.*, 2004). Additionally, the GSK-3β substrate β-catenin targets Jag1 in ectopic hair follicle formation in adult epidermis (Estrach *et al.*, 2006). This mechanism has also yet to be investigated in VSMCs.

In VSMCs, PDGF downregulates Jag1 and downstream Notch target gene expression. (Campos *et al.*, 2002). This downregulation has been shown to be mediated by p44/42 and p38 activity. Campos et al. postulate that this occurs through post-translational modification of Jag1, possibly through glycosylation. We have identified Jag1 as a Notch signalling component that is downregulated following cyclic strain(Morrow *et al.*, 2005b). This suggest that GSK-3β may exert an effect on Notch signalling following cyclic strain through modulation of Jag1 expression through activation of p44/42 and p38 but this has yet to be confirmed.

The pathogenesis of atherosclerosis involves progressive lipid accumulation, inflammation and smooth muscle cell proliferation in a period of many years. Angioplasty and in-stent restenosis differs in that hyperlipidaemia does not play an important role in restenosis and its pathogenesis occurs over the course of a few weeks and months (Kibos *et al.*, 2007). It involves a rapid vasoconstrictive response due to endothelial disruption caused by overstretch of the vessel, known as elastic recoil. This recoil is reduced by stent implantation. Rapid platelet activation, thrombin secretion and an inflammatory response is followed by VSMC proliferation, ECM production, neointimal formation and vascular remodelling (Schillinger and Minar, 2005).

The role of GSK-3β and Notch signaling in in-stent restenosis was investigated using a perfused stented mock coronary artery (MCA). The MCA consists of Sylgard®, which mimics the mechanical properties of a coronary artery. BSMCs were seeded onto the tubing and a bare-metal stent was deployed. The MCA was then exposed to pulsatile flow for 7 days. The environment in a stented artery changes from a low mean strain/high amplitude to a condition of high mean strain/low strain amplitude due to the rigidity of the stent. GSK-3β activity and Notch1 levels were shown to be upregulated in BASMCs in a stented environment compared to the upstream non-stented environment. This suggests that altered cyclic stretch correlates with increased VSMC proliferation observed in in-stent restenosis through impaired Notch signalling mediated by GSK-3β. Additionally, it highlights the importance of the compliance of a stented vessel in modulating the pro-proliferative Notch signaling pathway and suggests that this is an important factor when considering stent design. Moreover, it suggests

that there is an involvement of Notch signaling in vascular conditions in which artery compliance is impaired, such as age-related arteriosclerosis, which warrants further investigation.

A number of factors contribute to impaired vascular cell fate in intimal hyperplasia, vascular remodelling and the arterial response to injury, including the release of growth factors and inflammatory cytokines from ECs, platelets and macrophages and impaired haemodynamic environments. Abbregated Notch signaling is modulated by a number of factors in these disease states. Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction (Lindner *et al.*, 2001). The pro-inflammatory TNF has been shown to induce Jag1 in endothelial cells in a NFκB-dependent manner (Johnston *et al.*, 2009). Notch signaling is also associated with the differentiation of bone-marrow derived cells into SMC or SMC-like cells. These cells attach on injured arteries and are involved in arterial lesion formation (Doi *et al.*, 2009). Dll4 is also associated with Notch signaling and may mediate inflammatory responses in macrophages (Fung *et al.*, 2007). A critical role for Notch signaling in injury-induced vascular remodeling was demonstrated in a study where HRT2 knockout mice showed decreased neointimal formation after vascular injury (Sakata *et al.*, 2004). In the *in vivo* ligated carotid artery(LCA), Notch1, but not Notch3 was identified as a mediator of SMC proliferation and neointimal formation after vascular injury (Li *et al.*, 2009).

The *in vivo* LCA model involves arresting of blood flow in the left common carotid artery of the animal. Remodelling of the artery, leads to stenosis, a narrowing of the lumen and, therefore, a high mean strain

within the artery. However, the arrest of pulsatile flow results in a decrease in the amplitude of the strain. The LCA model, therefore, allows us to examine Notch regulation in an *in vivo* model of low flow/ low amplitude of cyclic strain, similar to the haemodynamic environment of a stented artery. We identified the importance of impaired cyclic stretch on GSK-3β and the Notch signaling modulation of cell fate *in vitro*. Using the *in vivo* model LCA, a biphasic response of Hh, VEGF and Notch signaling components was previously demonstrated in these models and the expression of Notch and components in both the intima and media of carotid arteries (Morrow *et al.*, 2009). In this study, the modulation of GSK-3β in the same manner as Notch signalling was demonstrated in both a partially ligated and totally ligated LCA model. We additionally investigated the role of GSK-3β and Notch and Hh downstream targets in human arteriosclerosis and found that they were similarly modulated, thus backing up the hypothesis that GSK-3β crosstalks with Hh and Notch signaling during neointimal information and the vascular response to injury.

The hypothesis is summarised in Figure 6.1. Under physiological conditions, a high amplitude cyclic strain environment exists that negatively regulates GSK-3β, thus inhibiting Notch signaling and VSMCs proliferation, through activation of p44/42 and p38. In pathological conditions, strain-induced inhibition of GSK-3β is abrogated, leading to a decrease in MAPK-induced Notch signaling. Additionally, Hh signaling is induced which contributes to aberrant Notch signalling through VEGF expression.

Apart from modulation by GSK-3β, new novel methods of regulation of Notch signalling are constantly being researched. These include

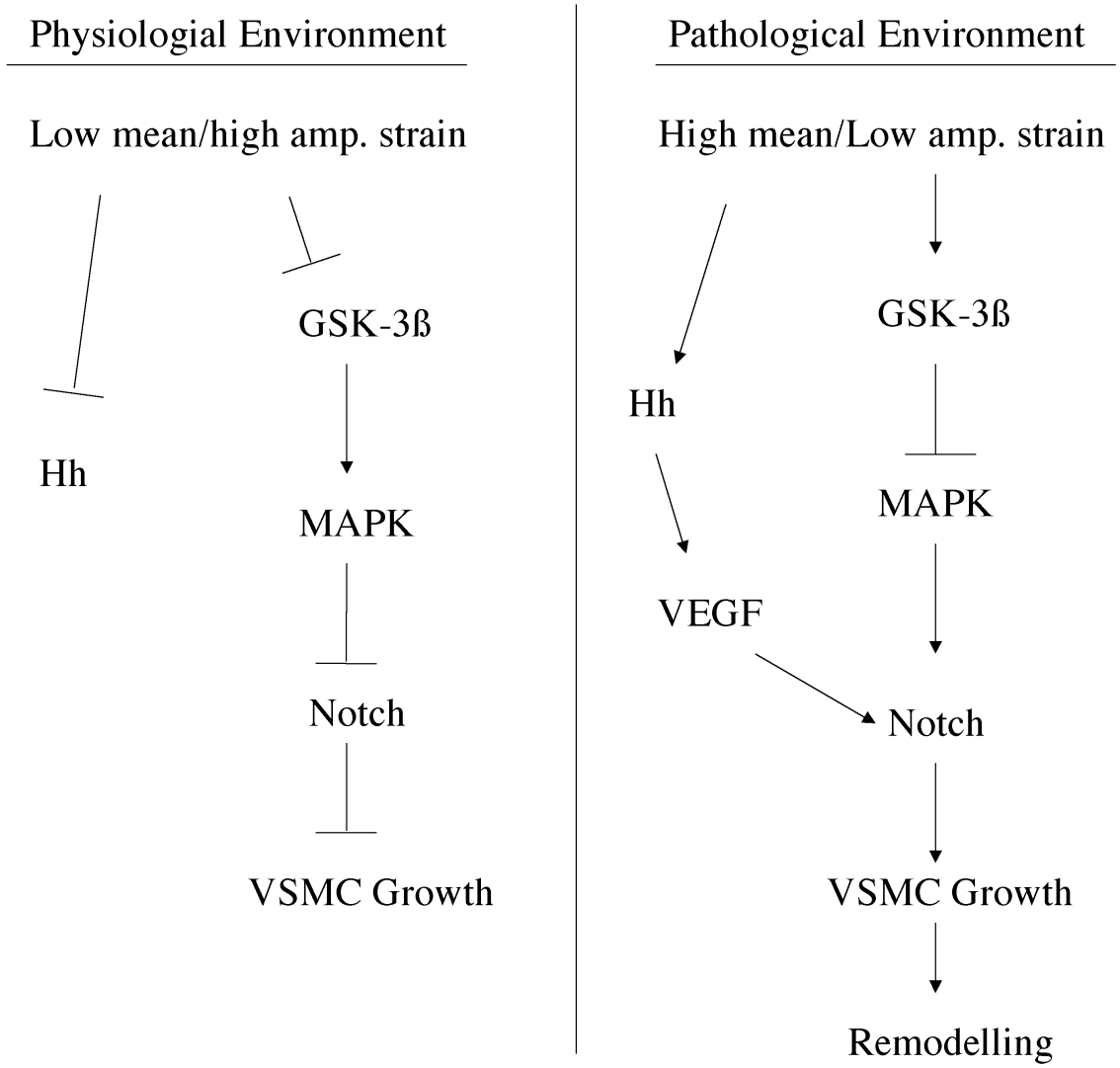

Figure 6.1: Proposed Modulation of Notch Signalling in Altered Haemodynamic Environments.

non-canonical ligand activity by DNER, MAPG1 and MAPG2 (D'Souza *et al.*, 2008) and repression of Notch activation by soluble forms of Dll and Jagged (Urs *et al.*, 2008). While their are many studies on regulation of Notch signaling since it was first identified as a neurogenic gene in Drosophila, a nominal number of studies examine the role of micro-RNAs in its modulation. Micro-RNAs (miRNAs) are approximately 22 nucleotide endogenous non-coding single stranded RNAs which are enocoded within the genome and are predicted to regulate at least one-third of all human genes (Condorelli and Dimmeler, 2008). Following processing by the RNase III enzymes Drosha and Dicer, the mature miRNAs act in gene silencing pathways through the regulation of mRNA stability and protein translation. miRNAs that bind to their mRNA targets with perfect complementarity induce target mRNA cleavage through degredation by endoribonucleases in the RNA Induced-Silencing complex of the RNAi pathway (Nelson and Weiss, 2008). miRNAs that bind with imperfect complementarity within the 3' untranslated region (UTR) region repress translation of the gene, while mRNA levels remains stable (Lim *et al.*, 2005). Expression of miRNAs are tissue-specific. Abundant miRNAs within the rat carotid artery include miR-145, let-7, miR-125b, miR-125a, miR-23 and miR-143 (Zhang, 2008). miRNAs have an essential role in vascular development as Dicer-deficient mice that lose miRNAs suffer seriously impaired vessel development (Zhao *et al.*, 2007). Additionally, Dicer-deficient mice display altered expression of several crucial genes involved in embryonic angiogenesis, including VEGF (Yang *et al.*, 2005). Using microarray analysis it has been established that aberrent miRNA expression is a characteristic of vascular walls after balloon injury, with 113 artery

miRNAs differentially expressed 7 days after injury and 102 miRNAs differentially expressed 28 days after injury Ji *et al.* (2007). Anti-sense mediated knockdown of an aberrantly overexpressed miRNA, miR-21, had a significantly negative effect on neointimal lesion formation in rat artery after angioplasty (Zhang, 2008). These findings indicate that studies of the role of miRNAs in signaling pathways involved in vascular disease could be critical in our search for therapeutic strategies.

A number of studies of miRNA regulation of Notch signaling have revealed the potential of this line of investigation. MicroRNA-199b-5p has been shown to impair cancer stem cells through negative regulation of the Notch downstream target HES1 in Medulloblastoma (Garzia *et al.*, 2009). GY-box-, Brd-box-, and K-box-class microRNAs were determined to regulate Drosophila Notch bHLH target genes (Lai *et al.*, 2005). Additionally, MicroRNA1, in Drosophila, regulates Notch signaling and influences cardiac differentiation(Kwon *et al.*, 2005). miRNAs have also been shown to be haemodynamically responsive and cyclic stretch was shown to be upregulate miR-146a, thereby delaying C2C12 myogenic differentiation through inhibition of the Notch regulator, Numb (Kuang *et al.*, 2009). The Hh pathway has also been shown to be regulated by miRNAs in Drosophila and in mammals (Ferretti *et al.*, 2008), (Friggi-Grelin *et al.*, 2008), (Katoh and Katoh, 2008). Studies in zebrafish have identified miR-214 as a modulator of Hh signalling in specifying muscle cell fate. Su(fu) was identified as a predicted target of miR-214. The effects of knockdown of miR-214 were then found to be mitigated by simulataneous knockdown of Su(fu) (Flynt *et al.*, 2007). A study by Thatcher et al. (2007) has important significance, when the context of our study is taken into account. In this study, knockdown of Hh signaling by

cyclopamine and knockdown of Notch by DAPT in zebrafish identified a number of miRNAs involved in Hh and Notch signalling. Interestingly, simulataneous knockdown of Hh and Notch signaling identified five abbregated miRNAs that had both Hh and Notch predicted targets. These include miR-27b which was positively modulated by both DAPT and cyclopamine treatment and who's predicted targets include ptc1 and the zebrafish homolog of Notch1, notch1b (Thatcher *et al.*, 2007). miRNA regulation of Notch and Hh signalling in VSMCs has yet to be investigated and this study suggests the potential of a common miRNA involved in the crosstalk of Notch and Hh in VSMCs. Microanalysis of miRNAs, therefore, of Notch signaling in VSMCs could provide invaluable information in our investigation of the pathogenesis of vascular diseases.

To date, traditional vascular disease therapies involve preventative hypertension and lipid-lowering medication, and surgical interventions, including angioplasty, stenting and by-pass grafts, which are used in extreme risk cases (Daskalopoulou *et al.*, 2007). Recent-advances in stent design allow the use of slow-release anti-proliferative drugs that include sirolimus and paclitaxel. While these drugs inhibit not only the overeaction of wound healing and restenosis, they also have a pro-thrombogenic potential, as they also inhibit reendothelialization and normal wound healing (Mungamuri *et al.*, 2006). Studies are ongoing to develop potential therapies that will antagonise vascular proliferation, without promoting adverse side effects. Our data, among others identify Notch signalling as a potential key player in vascular disease and therapies that target the Notch signaling pathways may play an important role in the future. Pre-clinical and clinical evidence indicate

that Notch signalling has a major function in many cancers and GSIs (γ-secretase inhibitors) have been investigated as candidate cancer therapeutic agents . While these have oral availability and are low cost, they have considerable off-target effects, An alternate strategy that has been suggested is to identify more context-specific targets within the Notch pathway and design more selective delivery strategies for notch inhibitors (Rizzo *et al.*, 2008). This study highlights GSK-3β as potential modulator of Notch signalling in the control of VSMC fate. Lithium, an antagonist of GSK-3β, is a well-established treatment for bipolar disorders (Martinez, 2008). The maintenance of appropriate physiological levels of GSK-3β activity is crucial, because either too little or too much GSK-3β activity can promote cell death in certain conditions (Doble and Woodgett, 2003). Dose-dependent modulation of GSK-3β inhibition and control of the timing and extent of its inhibition has been suggested as a mechanism to treat cancer, diabetes and mood disorders (Martinez, 2008). A similar strategy may be useful in exploiting the therapeutic capacity of GSK-3β in vascular disease. Alternatively, the emergence of artificial miRNAs which can target several mRNA targets at the same time, may aid in applying intervention strategies in the treatment of vascular disease through the treatment of multiple pathways, including Hhh, VEGF, GSK-3β and Notch.

In conclusion, we have identified GSK-3β as a modulator of Notch signalling in VSMCs. We have, however, yet to address the mechanism by which GSK-3β modulates Notch signalling. As discussed previously, γ-secretase inhibition of Notch receptor activity would confirm modulation of Notch downstream targets by GSK-3β upstream of Notch receptor activity. We demonstrated that GSK-3β is regulated by cyclic

strain in VSMCs, but again the pathway involved has not been defined. Inhibition of PI-3kinase activity would determine if this occurs via the PI3-kinase/Akt pathway. We demonstrated that GSK-3β modulates p44/42 and p38 following strain, but the mechanism in which p44/42, and p38 exert their effect on Notch signalling following strain remains to be determined. We hypothesise that this occurs through modulation of Notch ligand expression. Analysis of Jagged1 expression in VSMCs following strain and MAPK inhibition may confirm this hypothesis. Similarly, the effect of GSK-3β on Jagged1 expression would further enhance our understanding of the role of GSK-3β on Notch signaling *in vitro*. We have demonstrated that GSK-3β mRNA expression is regulated in biomechanically-induced *in vivo* remodelling. While this suggests that GSK-3β activity is altered in remodelled vessels, immunohistochemical staining of remodelled tissue will confirm this finding. Overall, we have identified a novel regulator of Notch signalling in VSMCs that is a potential therapeutic candidate for vascular disease states that display impaired Notch signalling.

Bibliography

Abell, A., Granger, D., Johnson, G. MEKK4 Stimulation of p38 and JNK Activity Is Negatively Regulated by GSK3beta. Journal of Biological Chemistry 2007;**282(42)**:30476.

Abid, M., *et al.* Forkhead transcription factors inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia. Journal of Biological Chemistry 2005;**280(33)**:29864.

Ali, A., Hoeflich, K., Woodgett, J. Glycogen synthase kinase-3: properties, functions, and regulation. Chem. Rev 2001;**101**:2527–2540.

Allard, D., Figg, N., Bennett, M., Littlewood, T. Akt regulates the survival of vascular smooth muscle cells via inhibition of FoxO3a and GSK3. Journal of Biological Chemistry 2008;**283(28)**:19739.

Ambrose, J., Barua, R. The pathophysiology of cigarette smoking and cardiovascular disease-An update. Journal of the American College of Cardiology 2004;**43(10)**:1731–1737.

Andrassy, M., Belov, D., Harja, E., Zou, Y., Leitges, M., Katus, H., Nawroth, P., Yan, S., Schmidt, A., Yan, S. Central role of PKCβ in neointimal expansion triggered by acute arterial injury. Circulation research 2005; **96(4)**:476–483.

Arias, A., Zecchini, V., Brennan, K. CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? Current Opinion in Genetics & Development 2002;**12(5)**:524–533.

Armulik, A., Abramsson, A., Betsholtz, C. Endothelial/pericyte interactions. Circulation research 2005;**97(6)**:512–523.

Aronson, D. Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. Journal of hypertension 2003;**21(1)**:3.

Artavanis-Tsakonas, S., Rand, M., Lake, R. Notch Signaling: Cell Fate Control and Signal Integration in Development. Science 1999; **284(5415)**:770.

Ashley, E., Niebauer, J. *Cardiology explained*. Remedica, London, UK., 1st edn., 2004.

Atkinson, J. Aging of arterial extracellular matrix elastin: etiology and consequences. Pathologie-biologie 1998;**46(7)**:555.

Axelrod, J., Matsuno, K., Artavanis-Tsakonas, S., Perrimon, N. Interaction Between Wingless and Notch Signaling Pathways Mediated by Dishevelled. Science 1996;**271(5257)**:1826.

Baccarelli, A., Zanobetti, A., Martinelli, I., Grillo, P., Hou, L., Lanzani, G., Mannucci, P., Bertazzi, P., Schwartz, J. Air Pollution, Smoking, and Plasma Homocysteine. Environ Health Perspect 2007;**115(2)**:176–181.

Barolo, S. Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. Genes & Development 2002;**16(10)**:1167–1181.

Baron, M., Aslam, H., Flasza, M., Fostier, M., Higgs, J., Mazaleyrat, S., Wilkin, M. Multiple levels of Notch signal regulation (Review). Molecular Membrane Biology 2002;**19(1)**:27–38.

Bassus, S., Herkert, O., Kronemann, N., Gorlach, A., Bremerich, D., Kirchmaier, C., Busse, R., Schini-Kerth, V. Thrombin Causes Vascular Endothelial Growth Factor Expression in Vascular Smooth Muscle Cells Role of Reactive Oxygen Species. Arteriosclerosis, Thrombosis, and Vascular Biology 2001;**21(9)**:1550–1555.

Bax, B., Brown, M., Reith, A. Crystal Structure of Glycogen Synthase Kinase 3beta 2002;WO Patent WO/2002/050,254.

Beatus, P., Lundkvist, J., Öberg, C., Pedersen, K., Lendahl, U. The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity. Mechanisms of Development 2001; **104(1-2)**:3–20.

Bellavia, D., Checquolo, S., Campese, A., Felli, M., Gulino, A., Screpanti, I. Notch3: from subtle structural differences to functional diversity. Oncogene 2008;**27(38)**:5092–5098.

Bennett, M., Evan, G., Schwartz, S. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. Journal of Clinical Investigation 1995;**95(5)**:2266.

Berdnik, D., Török, T., González-Gaitán, M., Knoblich, J. The Endocytic Protein α-Adaptin Is Required for Numb-Mediated Asymmetric Cell Division in Drosophila. Developmental Cell 2002;**3(2)**:221–231.

Beurel, E., Jope, R. The paradoxical pro-and anti-apoptotic actions of GSK3

in the intrinsic and extrinsic apoptosis signaling pathways. Progress in Neurobiology 2006;**79(4)**:173–189.

Bianchi, S., Martinoli, C., service, S. O. *Ultrasound of the musculoskeletal system*. Springer, New York, USA, 1st edn., 2007.

Bijur, G., Jope, R. Proapoptotic Stimuli Induce Nuclear Accumulation of Glycogen Synthase Kinase-3β. Journal of Biological Chemistry 2001; **276(40)**:37436–37442.

Bijur, G., Jope, R. Glycogen synthase kinase-3 beta is highly activated in nuclei and mitochondria. Neuroreport 2003a;**14(18)**:2415–9.

Bijur, G., Jope, R. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. J Neurochem 2003b; **87(6)**:1427–35.

Bikkavilli, R., Feigin, M., Malbon, C. p38 mitogen-activated protein kinase regulates canonical Wnt-{beta}-catenin signaling by inactivation of GSK3 {beta}. Journal of Cell Science 2008;**121(21)**:3598.

Birukov, K., Shirinsky, V., Stepanova, O., Tkachuk, V., Hahn, A., Resink, T., Smirnov, V. Stretch affects phenotype and proliferation of vascular smooth muscle cells. Molecular and cellular biochemistry 1995; **144(2)**:131–139.

Bland, C., Kimberly, P., Rand, M. Notch-induced proteolysis and nuclear localization of the Delta ligand. Journal of Biological Chemistry 2003; **278(16)**:13607–13610.

Blaumueller, C., Qi, H., Zagouras, P., Artavanis-Tsakonas, S. Intracellular

cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell 1997;**90(2)**:281–91.

Bochaton-Piallat, M., Gabbiani, F., Redard, M., Desmouliere, A., Gabbiani, G. Apoptosis participates in cellularity regulation during rat aortic intimal thickening. The American journal of pathology 1995;**146(5)**:1059.

Boo, Y., Jo, H. Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. American Journal of Physiology- Cell Physiology 2003;**285(3)**:499–508.

Boyle, J., Bowyer, D., Proudfoot, D., Weissberg, P., Bennett, M. Human monocyte/macrophages induce human vascular smooth muscle cell apoptosis in culture. Circulation 1998;**98(Suppl I)**:598.

Brou, C. Intracellular trafficking of Notch receptors and ligands. Experimental Cell Research 2008;**315(9)**:1549–55.

Bruckner, K., Perez, L., Clausen, H., Cohen, S. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature 2000; **406(6794)**:411–5.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K., Dickson, B., Basler, K. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell(Cambridge) 1999;**99(7)**:803–816.

Burlacu, A. Regulation of apoptosis by Bcl-2 family proteins. J Cell Mol Med 2003;**7(3)**:249–257.

Bush, G., diSibio, G., Miyamoto, A., Denault, J., Leduc, R., Weinmaster, G.

Ligand-Induced Signaling in the Absence of Furin Processing of Notch1. Developmental Biology 2001;**229(2)**:494–502.

Caballero, A. Endothelial Dysfunction in Obesity and Insulin Resistance: A Road to Diabetes and Heart Disease. Obesity Research 2003;**11**:1278–1289.

Cai, X. Regulation of smooth muscle cells in development and vascular disease: current therapeutic strategies. Expert Review of Cardiovascular Therapy 2006;**4(6)**:789–800.

Campos, A., Wang, W., Pollman, M., Gibbons, G. Determinants of Notch-3 Receptor Expression and Signaling in Vascular Smooth Muscle Cells Implications in Cell-Cycle Regulation. Circulation Research 2002; **91(11)**:999–1006.

Cappadona, C., Redmond, E., Theodorakis, N., McKillop, I., Hendrickson, R., Chhabra, A., Sitzmann, J., Cahill, P. Phenotype dictates the growth response of vascular smooth muscle cells to pulse pressure in vitro. Experimental cell research 1999;**250(1)**:174–186.

Caraci, F., Gili, E., Calafiore, M., Failla, M., La Rosa, C., Crimi, N., Sortino, M., Nicoletti, F., Copani, A., Vancheri, C. TGF-β1 targets the GSK-3β/β-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. Pharmacological Research 2008; **57(4)**:274–82.

Castella, P., Sawai, S., Nakao, K., Wagner, J., Caudy, M. HES-1 Repression of Differentiation and Proliferation in PC12 Cells: Role for the Helix 3-Helix 4 Domain in Transcription Repression. Molecular and Cellular Biology 2000;**20(16)**:6170.

Cattell, M., Anderson, J., Hasleton, P. Age-related changes in amounts and concentrations of collagen and elastin in normotensive human thoracic aorta. Clinica Chimica Acta 1996;**245(1)**:73–84.

Chan, S., Hegyi, L., Scott, S., Cary, N., Weissberg, P., Bennett, M. Sensitivity to Fas-mediated apoptosis is determined below receptor level in human vascular smooth muscle cells. Circulation research 2000;**86(10)**:1038–1046.

Chapman, G., Durante, W., Hellums, J., Schafer, A. Physiological cyclic stretch causes cell cycle arrest in cultured vascular smooth muscle cells. American Journal of Physiology- Heart and Circulatory Physiology 2000;**278(3)**:748–754.

Chastagner, P., Israël, A., Brou, C. AIP4/Itch regulates Notch receptor degradation in the absence of ligand. PLoS ONE 2008;**3(7)**:e2375.

Chen, A., Gortler, D., Kilaru, S., Araim, O., Frangos, S., Sumpio, B. Cyclic strain activates the pro-survival Akt protein kinase in bovine aortic smooth muscle cells. Surgery 2001;**130(2)**:378–81.

Chen, H., Thiagalingam, A., Chopra, H., Borges, M., Feder, J., Nelkin, B., Baylin, S., Ball, D. Conservation of the Drosophila lateral inhibition pathway in human lung cancer: A hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proceedings of the National Academy of Sciences 1997;**94(10)**:5355.

Chen, R., Greene, E., Collinsworth, G., Grewal, J., Houghton, O., Zeng, H., Garnovskaya, M., Paul, R., Raymond, J. Enrichment of transiently transfected mesangial cells by cell sorting after cotransfection with GFP. AJP-Renal Physiology 1999;**276(5)**:777.

Chiba, S. Concise Review: Notch Signaling in Stem Cell Systems. Stem Cells 2006;**24(11)**:2437.

Chien, S. Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell. American Journal of Physiology- Heart and Circulatory Physiology 2007;**292(3)**:H1209.

Chin, M., Maemura, K., Fukumoto, S., Jain, M., Layne, M., Watanabe, M., Hsieh, C., Lee, M. Cardiovascular Basic Helix Loop Helix Factor 1, a Novel Transcriptional Repressor Expressed Preferentially in the Developing and Adult Cardiovascular System. Journal of Biological Chemistry 2000;**275(9)**:6381–6387.

Chomczynski, P., Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 1987;**162(1)**:156–159.

Clifford, R., Deacon, K., Knox, A. Novel Regulation of Vascular Endothelial Growth Factor-A (VEGF-A) by Transforming Growth Factor β 1: Requirement For Smads, β-Catenin, and GSK3 β. Journal of Biological Chemistry 2008;**283(51)**:35337.

Clyman, R., Mauray, F., Kramer, R. Beta 1 and beta 3 integrins have different roles in the adhesion and migration of vascular smooth muscle cells on extracellular matrix. Exp Cell Res 1992;**200(2)**:272–84.

Coghlan, M., Culbert, A., Cross, D., Corcoran, S., Yates, J., Pearce, N., Rausch, O., Murphy, G., Carter, P., Roxbee Cox, L., *et al.* Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chemistry & Biology 2000;**7(10)**:793–803.

Condorelli, G., Dimmeler, S. MicroRNAs: components of an integrated system controlling cardiac development, physiology, and disease pathogenesis. Cardiovascular Research 2008;**79(4)**:551–552.

Consigny, P. Pathogenesis of atherosclerosis. AJR Am J Roentgenol 1995; **164(3)**:553–8.

Costa, R., Assreuy, J. Multiple potassium channels mediate nitric oxide-induced inhibition of rat vascular smooth muscle cell proliferation. Nitric Oxide 2005;**13(2)**:145–151.

Cowin, S., Humphrey, J. *Cardiovascular soft tissue mechanics*. Kluwer Academic Publishers, Norwell, USA, 1st edn., 2001.

Cristofaro, B., Emanueli, C. Possible novel targets for therapeutic angiogenesis. Current Opinion in Pharmacology 2009;**9(2)**:102–8.

Cross, D., Alessi, D., Cohen, P., Andjelkovich, M., Hemmings, B. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;**378(6559)**:785–789.

Cross, D., Culbert, A., Chalmers, K., Facci, L., Skaper, S., Reith, A. Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death. Journal of Neurochemistry 2001;**77(1)**:94–102.

Cross, M., Dixelius, J., Matsumoto, T., Claesson-Welsh, L. VEGF-receptor signal transduction. Trends in Biochemical Sciences 2003;**28(9)**:488–494.

Cunningham, K., Gotlieb, A. The role of shear stress in the pathogenesis of atherosclerosis. Laboratory investigation 2004;**85(1)**:9–23.

Daskalopoulou, S., Daskalopoulos, M., Perrea, D., Nicolaides, A., Liapis, C. Carotid artery atherosclerosis: What is the evidence for drug action? Current pharmaceutical design 2007;**13(11)**:1141–1159.

Davies, M. Stability and instability: two faces of coronary atherosclerosis The Paul Dudley White Lecture 1995. Circulation 1996;**94(8)**:2013–2020.

Davignon, J., Ganz, P. Role of Endothelial Dysfunction in Atherosclerosis. Circulation 2004;**109(90231)**:27–32.

De Celis, J., Bray, S. Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. Development 1997; **124(17)**:3241–3251.

De Nigris, F., Sica, V., Herrmann, J., Condorelli, G., Chade, A., Tajana, G., Lerman, A., Lerman, L., Napoli, C. c-Myc oncoprotein: cell cycle-related events and new therapeutic challenges in cancer and cardiovascular diseases. Cell cycle 2003;**2(4)**:325–328.

Del Monte, G., Grego-Bessa, J., Gonzalez-Rajal, A., Bolos, V., De La Pompa, J. Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. Developmental Dynamics 2007;**236(9)**.

DePaoli-Roach, W. Glycogen synthase kinase-3β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. J Biol. Chem 1994;**269(20)**:14566–74.

Dethlefsen, S., Shepro, D., D'Amore, P. Comparison of the effects of mechanical stimulation on venous and arterial smooth muscle cells in vitro. J Vasc Res 1996;**33**:405–413.

Diederich, R. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development 1994;**120(3)**:473–481.

Diehl, J., Cheng, M., Roussel, M., Sherr, C. Glycogen synthase kinase-3 beta regulates cyclin D1 proteolysis and subcellular localization. Genes & Development 1998;**12**:3499–3511.

Ding, V., Chen, R., McCormick, F. Differential Regulation of Glycogen Synthase Kinase 3β by Insulin and Wnt Signaling. Journal of Biological Chemistry 2000;**275(42)**:32475–32481.

Doble, B., Woodgett, J. GSK-3: tricks of the trade for a multi-tasking kinase. Journal of Cell Science 2003;**116(7)**:1175–1186.

Dohi, Y., Kojima, M., Sato, K., Lüscher, T. Age-related changes in vascular smooth muscle and endothelium. Drugs & aging 1995;**7(4)**:278–91.

Doi, H., Iso, T., Sato, H., Yamazaki, M., Matsui, H., Tanaka, T., Manabe, I., Arai, M., Nagai, R., Kurabayashi, M. Jagged1-selective Notch Signaling Induces Smooth Muscle Differentiation via a RBP-J-dependent Pathway. Journal of Biological Chemistry 2006;**281(39)**:28555–28564.

Doi, H., Iso, T., Shiba, Y., Sato, H., Yamazaki, M., Oyama, Y., Akiyama, H., Tanaka, T., Tomita, T., Arai, M., *et al.* Notch signaling regulates the differentiation of bone marrow-derived cells into smooth muscle-like cells during arterial lesion formation. Biochemical and Biophysical Research Communications 2009;**381(4)**:654–659.

Doi, H., Iso, T., Yamazaki, M., Akiyama, H., Kanai, H., Sato, H., Kawai-Kowase, K., Tanaka, T., Maeno, T., Okamoto, E., *et al.* HERP1 Inhibits

Myocardin-Induced Vascular Smooth Muscle Cell Differentiation by Interfering With SRF Binding to CArG Box. Arteriosclerosis, Thrombosis, and Vascular Biology 2005;**25(11)**:2328–2334.

Donovan, J., Kordylewska, A., Jan, Y., Utset, M. Tetralogy of Fallot and Other Congenital Heart Defects in Hey2 Mutant Mice. Current Biology 2002;**12(18)**:1605–1610.

D'Souza, B., Miyamoto, A., Weinmaster, G. The many facets of Notch ligands. Oncogene 2008;**27(38)**:5148–5167.

Dyczynska, E., Sun, D., Yi, H., Sehara-Fujisawa, A., Blobel, C., Zolkiewska, A. Proteolytic Processing of Delta-like 1 by ADAM Proteases. Journal of Biological Chemistry 2007;**282(1)**:436.

Eichmann, A., Yuan, L., Moyon, D., Lenoble, F., Pardanaud, L., Breant, C. Vascular development: from precursor cells to branched arterial and venous networks. Int J Dev Biol 2005;**49(2-3)**:259–67.

Ellozy, S., Carroccio, A. Drug-eluting stents in peripheral vascular disease: Eliminating restenosis. The Mount Sinai journal of medicine 2003; **70(6)**:417–419.

Elmore, S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;**35(4)**:495–516.

Erl, W. Statin-induced vascular smooth muscle cell apoptosis: a possible role in the prevention of restenosis? Current drug targets. Cardiovascular & haematological disorders 2005;**5(2)**:135–44.

Espinosa, L., Ingles-Esteve, J., Aguilera, C., Bigas, A. Phosphorylation by Glycogen Synthase Kinase-3β Down-regulates Notch Activity, a Link

for Notch and Wnt Pathways. Journal of Biological Chemistry 2003; **278(34)**:32227–32235.

Estrach, S., Ambler, C., Lo Celso, C., Hozumi, K., Watt, F. Jagged 1 is a β-catenin target gene required for ectopic hair follicle formation in adult epidermis. Development 2006;**133(22)**:4427–38.

Ferrara, N., Bunting, S. Vascular endothelial growth factor, a specific regulator of angiogenesis. Current Opinion in Nephrology and Hypertension 1996;**5(1)**:35–44.

Ferretti, E., De Smaele, E., Miele, E., Laneve, P., Po, A., Pelloni, M., Paganelli, A., Di Marcotullio, L., Caffarelli, E., Screpanti, I., *et al.* Concerted microRNA control of Hedgehog signalling in cerebellar neuronal progenitor and tumour cells. The EMBO Journal 2008;**27**:2616–2627.

Fischer, A., Gessler, M. Hey Genes in Cardiovascular Development. Trends in Cardiovascular Medicine 2003;**13(6)**:221–226.

Fischer, A., Gessler, M. Delta Notch and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Research 2007;**35(14)**:4583–96.

Fiúza, U., Arias, A. Cell and molecular biology of Notch. Journal of Endocrinology 2007;**194(3)**:459–474.

Flynt, A., Li, N., Thatcher, E., Solnica-Krezel, L., Patton, J. Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. Current Biology 2007;**39(2)**:259–63.

Foltz, D., Nye, J. Hyperphosphorylation and association with RBP of the intracellular domain of Notch1. Biochem Biophys Res Commun 2001; **286(3)**:484–92.

Foltz, D., Santiago, M., Berechid, B., Nye, J. Glycogen Synthase Kinase-3β Modulates Notch Signaling and Stability. Current Biology 2002; **12(12)**:1006–1011.

Forde, J., Dale, T. Glycogen synthase kinase 3: A key regulator of cellular fate. Cellular and Molecular Life Sciences (CMLS) 2007;**64(15)**:1930–1944.

Frame, S., Cohen, P. GSK3 takes centre stage more than 20 years after its discovery. Biochem J 2001;**359(Pt 1)**:1–16.

Friggi-Grelin, F., Lavenant-Staccini, L., Therond, P. Control of antagonistic components of the hedgehog signaling pathway by microRNAs in Drosophila. Genetics 2008;**179(1)**:429–39.

Frise, E., Knoblich, J., Younger-Shepherd, S., Jan, L., Jan, Y. The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. Proceedings of the National Academy of Sciences 1996;**93(21)**:11925–11932.

Fryer, C., Lamar, E., Turbachova, I., Kintner, C., Jones, K. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes & Development 2002;**16(11)**:1397–411.

Fryer, C., White, J., Jones, K. Mastermind Recruits CycC: CDK8 to Phosphorylate the Notch ICD and Coordinate Activation with Turnover. Molecular Cell 2004;**16(4)**:509–520.

Fung, E., Tang, S., Canner, J., Morishige, K., Arboleda-Velasquez, J., Cardoso, A., Carlesso, N., Aster, J., Aikawa, M. Delta-Like 4 Induces Notch Signaling in Macrophages: Implications for Inflammation. Circulation 2007;**115(23)**:2948–56.

Garg, V. Molecular genetics of aortic valve disease. Current Opinion in Cardiology 2006;**21(3)**:180–4.

Garzia, L., Andolfo, I., Cusanelli, E., Marino, N., Petrosino, G., De Martino, D., Esposito, V., Galeone, A., Navas, L., Esposito, S., *et al.* MicroRNA-199b-5p Impairs Cancer Stem Cells through Negative Regulation of HES1 in Medulloblastoma. PLoS ONE 2009;**4(3)**:e4998.

Gerhard, M., Roddy, M., Creager, S., Creager, M. Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. Hypertension 1996;**27(4)**:849–853.

Gibbons, G., Dzau, V. The emerging concept of vascular remodeling. New England Journal of Medicine 1994;**330(20)**:1431–1438.

Gilbert, J., Weinhold, P., Banes, A., Link, G., Jones, G. Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells in vitro. J Biomech 1994;**27(9)**:1169–77.

Ginger, R., Dalton, E., Ryves, W., Fukuzawa, M., Williams, J., Harwood, A. Glycogen synthase kinase-3 enhances nuclear export of a Dictyostelium STAT protein. The EMBO Journal 2000;**19**:5483–5491.

Giniger, E., Wills, Z., Emerson, M., Rusch, J., Bikoff, J., Baum, B., Perrimon, N., Van Vactor, D. A role for Abl in Notch signaling. Neuron 1998; **20(4)**:667–682.

Goh, F., Irvine, K., Lovelace, E., Donnelly, S., Jones, M., Brion, K., Hume, D., Kotze, A., Dalton, J., Ingham, A., *et al.* Selective induction of the Notch ligand Jagged-1 in macrophages by soluble egg antigen from Schistosoma mansoni involves ERK signalling. Immunology 2008; **127(3)**:326–37.

Goishi, K., Klagsbrun, M. Vascular endothelial growth factor and its receptors in embryonic zebrafish blood vessel development. Current topics in developmental biology 2004;**62**:127–52.

Gordon, W., Arnett, K., Blacklow, S. The molecular logic of Notch signaling-a structural and biochemical perspective. Journal of Cell Science 2008;**121(19)**:3109–19.

Gordon, W., Vardar-Ulu, D., Histen, G., Sanchez-Irizarry, C., Aster, J., Blacklow, S. Structural basis for autoinhibition of Notch. Nature Structural & Molecular Biology 2007;**14**:295–300.

Gosens, R., Meurs, H., Schmidt, M. The GSK-3/β-catenin-signalling axis in smooth muscle and its relationship with remodelling. Naunyn-Schmiedeberg's Archives of Pharmacology 2008;**378(2)**:185–191.

Greenwald, S. Ageing of the conduit arteries. Journal of pathology 2007; **211(2)**:157–172.

Gridley, T. Notch signaling in vascular development and physiology. Development 2007;**134(15)**:2709–18.

Guo, M., Jan, L., Jan, Y. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 1996;**17(1)**:27–41.

Gupta-Rossi, N., Le Bail, O., Gonen, H., Brou, C., Logeat, F., Six, E., Ciechanover, A., Israel, A. Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. Journal of Biological Chemistry 2001;**276(37)**:34371–34378.

Gupta-Rossi, N., Six, E., LeBail, O., Logeat, F., Chastagner, P., Olry, A., Israël, A., Brou, C. Monoubiquitination and endocytosis direct γ-secretase cleavage of activated Notch receptor. The Journal of cell biology 2004;**166(1)**:73–83.

Hadi, H., Carr, C., Al Suwaidi, J. Endothelial Dysfunction: Cardiovascular Risk Factors, Therapy, and Outcome. Vascular Health and Risk Management 2005;**1(3)**:183–98.

Haga, J., Li, Y., Chien, S. Molecular basis of the effects of mechanical stretch on vascular smooth muscle cells. Journal of Biomechanics 2007; **40(5)**:947–960.

Hall, J., Chatham, J., Eldar-Finkelman, H., Gibbons, G. Upregulation of Glucose Metabolism During Intimal Lesion Formation Is Coupled to the Inhibition of Vascular Smooth Muscle Cell Apoptosis: Role of GSK3beta. Diabetes 2001;**50(5)**:1171–9.

Hamet, P., DeBlois, D., Dam, T., Richard, L., Teiger, E., Tea, B., Orlov, S., Tremblay, J. Apoptosis and vascular wall remodeling in hypertension. Canadian journal of physiology and pharmacology 1996;**74(7)**:850–861.

Han, S., Gai, W., Yancovitz, M., Osman, I., Di Como, C., Polsky, D. Nucleofection is a highly effective gene transfer technique for human melanoma cell lines. Experimental Dermatology 2008;**17(5)**:405–11.

Hardt, S., Sadoshima, J. Glycogen Synthase Kinase-3β A Novel Regulator of Cardiac Hypertrophy and Development. Circulation Research 2002; **90(10)**:1055–1063.

Harja, E., Hudson, B., Zou, Y., Lu, Y., Schmidt, A., Yan, S. PKC beta/Egr-1: a central axis in atherosclerosis. FASEB J 2005;**19**:387–20.

Hayashi, H., Kume, T. Foxc transcription factors directly regulate Dll4 and Hey2 expression by interacting with the VEGF-Notch signaling pathways in endothelial cells. PLoS ONE 2008;**3(6)**:e2401.

Hayward, P., Kalmar, T., Martinez Arias, A. Wnt/Notch signalling and information processing during development. Development 2008; **135(3)**:411–24.

High, F., Lu, M., Pear, W., Loomes, K., Kaestner, K., Epstein, J. Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proceedings of the National Academy of Sciences 2008;**105(6)**:1955–9.

Hipper, A., Isenberg, G. Cyclic mechanical strain decreases the DNA synthesis of vascular smooth muscle cells. Pflügers Archiv European Journal of Physiology 2000;**440(1)**:19–27.

Hirata, H., Ohtsuka, T., Bessho, Y., Kageyama, R. Generation of Structurally and Functionally Distinct Factors from the Basic Helix-Loop-Helix Gene Hes3 by Alternative First Exons. Journal of Biological Chemistry 2000;**275(25)**:19083–19089.

Ho, Q., Kuo, C. Vascular endothelial growth factor: Biology and therapeutic applications. International Journal of Biochemistry and Cell Biology 2007;**39(7-8)**:1349–1357.

Hodgkin, P. B cell differentiation and isotype switching is related to division cycle number. Journal of Experimental Medicine 1996; **184(1)**:277–281.

Hoeflich, K., Luo, J., Rubie, E., Tsao, M., Jin, O., Woodgett, J. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature 2000;**406(6791)**:86–90.

Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B., Gossler, A. WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomitic mesoderm of mouse embryos. Genes & development 2004;**18(22)**:2712–2717.

Holmes, K., Roberts, O., Thomas, A., Cross, M. Vascular endothelial growth factor receptor-2: Structure, function, intracellular signalling and therapeutic inhibition. Cellular Signalling 2007;**19(10)**:2003–2012.

Hooper, C., Killick, R., Lovestone, S. The GSK3 hypothesis of Alzheimer's disease. Journal of Neurochemistry 2008;**104(6)**:1433–9.

Hoshi, S., Goto, M., Koyama, N., Nomoto, K., Tanaka, H. Regulation of Vascular Smooth Muscle Cell Proliferation by Nuclear Factor-κB and Its Inhibitor, I-κB. Journal of Biological Chemistry 2000;**275(2)**:883–889.

Hu, Y., Cheng, L., Hochleitner, B., Xu, Q. Activation of mitogen-activated protein kinases (ERK/JNK) and AP-1 transcription factor in rat carotid arteries after balloon injury. Arteriosclerosis, thrombosis, and vascular biology 1997;**17(11)**:2808–2816.

Huang, J., Kontos, C. Inhibition of vascular smooth muscle cell proliferation, migration, and survival by the tumor suppressor protein

PTEN. Arteriosclerosis, thrombosis, and vascular biology 2002; **22(5)**:745–751.

Huangfu, D., Anderson, K. Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. Development 2006;**133(1)**:3–14.

Inoue, M., Itoh, H., Ueda, M., Naruko, T., Kojima, A., Komatsu, R., Doi, K., Ogawa, Y., Tamura, N., Takaya, K., *et al.* Vascular Endothelial Growth Factor (VEGF) Expression in Human Coronary Atherosclerotic Lesions Possible Pathophysiological Significance of VEGF in Progression of Atherosclerosis. Circulation 1998;**98(20)**:2108–2116.

Intengan, H., Schiffrin, E. Vascular Remodeling in Hypertension Roles of Apoptosis, Inflammation, and Fibrosis. Hypertension 2001;**38(3)**:581–587.

Iso, T., Kedes, L., Hamamori, Y. HES and HERP families: Multiple effectors of the notch signaling pathway. Journal of Cellular Physiology 2003;**194(3)**:237–255.

Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H., Chung, G., Kedes, L., Hamamori, Y. HERP, a Novel Heterodimer Partner of HES/E (spl) in Notch Signaling. Molecular and Cellular Biology 2001;**21(17)**:6080–9.

Jani, B., Rajkumar, C. Ageing and vascular ageing. Postgraduate Medical Journal 2006;**82(968)**:357–62.

Jehn, B., Bielke, W., Pear, W., Osborne, B. Cutting Edge: Protective Effects of Notch-1 on TCR-Induced Apoptosis 1. The Journal of Immunology 1999;**162(2)**:635–638.

Jeremy, J., Rowe, D., Emsley, A., Newby, A. Nitric oxide and the proliferation of vascular smooth muscle cells. Cardiovascular research 1999;**43(3)**:580–94.

Ji, R., Cheng, Y., Yue, J., Yang, J., Liu, X., Chen, H., Dean, D., Zhang, C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circulation research 2007;**100(11)**:1579–88.

Jin, Y., Kim, H., Oh, M., Ki, H., Kim, K. Regulation of Notch1/NICD and Hes1 expressions by GSK-3alpha/beta. Molecules and cells 2009; **27(1)**:15–9.

Johnston, D., Dong, B., Hughes, C. TNF induction of jagged-1 in endothelial cells is NFκB-dependent. Gene 2009;**435(1-2)**:36–44.

Jope, R., Johnson, G. The glamour and gloom of glycogen synthase kinase-3. Trends in Biochemical Sciences 2004;**29(2)**:95–102.

Jope, R., Yuskaitis, C., Beurel, E. Glycogen Synthase Kinase-3 (GSK3): Inflammation, Diseases, and Therapeutics. Neurochemical Research 2007;**32(4)**:577–595.

Joutel, A., Andreux, F., Gaulis, S., Domenga, V., Cecillon, M., Battail, N., Piga, N., Chapon, F., Godfrain, C., Tournier-Lasserve, E. The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. Journal of Clinical Investigation 2000;**105(5)**:597–605.

Joutel, A., Vahedi, K., Corpechot, C., Troesch, A., Chabriat, H., Vayssiere, C., Cruaud, C., Maciazek, J., Weissenbach, J., Bousser, M., *et al.* Strong

clustering and stereotyped nature of Notch 3 mutations in CADASIL patients. Lancet(British edition) 1997;**350(9090)**:1511–1515.

Ju, H., Nerurkar, S., Sauermelch, C., Olzinski, A., Mirabile, R., Zimmerman, D., Lee, J., Adams, J., Sisko, J., Berova, M., *et al.* Sustained activation of p38 mitogen-activated protein kinase contributes to the vascular response to injury. Journal of Pharmacology and Experimental Therapeutics 2002;**301(1)**:15–20.

Kaazempur-Mofrad, M., Younis, H., Patel, S., Isasi, A., Chung, C., Chan, R., Hinton, D., Lee, R., Kamm, R. Cyclic strain in human carotid bifurcation and its potential correlation to atherogenesis: Idealized and anatomically-realistic models. Journal of Engineering Mathematics 2003;**47(3)**:299–314.

Kaiser, D., Freyberg, M., Friedl, P. Lack of hemodynamic forces triggers apoptosis in vascular endothelial cells. Biochemical and Biophysical Research Communications 1997;**231(3)**:586–590.

Kalaria, R., Viitanen, M., Kalimo, H., Dichgans, M., Tabira, T. The pathogenesis of CADASIL: an update. Journal of the neurological sciences 2004;**226(1-2)**:35–39.

Kamath, B., Spinner, N., Emerick, K., Chudley, A., Booth, C., Piccoli, D., Krantz, I. Vascular Anomalies in Alagille Syndrome A Significant Cause of Morbidity and Mortality. Circulation 2004;**109(11)**:1354–1358.

Kanwar, R., Fortini, M. Notch Signaling: A Different Sort Makes the Cut. Current Biology 2004;**14(24)**:1043–1045.

Karlstrom, H., Beatus, P., Dannaeus, K., Chapman, G., Lendahl, U., Lundkvist, J. A CADASIL-mutated Notch 3 receptor exhibits impaired

intracellular trafficking and maturation but normal ligand-induced signaling. Proceedings of the National Academy of Sciences 2002; **99(26)**:17119–17124.

Katoh, Y., Katoh, M. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (Review). Int J Mol Med 2008;**22(3)**:271–5.

Katsube, K., Sakamoto, K. Notch in vertebrates–molecular aspects of the signal. Int J Dev Biol 2005;**49(2-3)**:369–74.

Katsumi, A., Orr, A., Tzima, E., Schwartz, M. Integrins in Mechanotransduction. Journal of Biological Chemistry 2004; **279(13)**:12001–4.

Kearney, M., Pieczek, A., Haley, L., Losordo, D., Andres, V., Schainfeld, R., Rosenfield, K., Isner, J. Histopathology of in-stent restenosis in patients with peripheral artery disease. Circulation 1997;**95(8)**:1998–2002.

Kibos, A., Campeanu, A., Tintoiu, I. Pathophysiology of coronary artery in-stent restenosis. Acute Cardiac Care 2007;**9(2)**:111–119.

Kidd, S., Lieber, T., Young, M. Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. Genes & Development 1998;**12(23)**:3728–40.

Kim, S., Park, H., Hong, H., Baik, E., Jung, M., Mook-Jung, I. ERK1/2 is an endogenous negative regulator of the γ-secretase activity. The FASEB Journal 2006;**20(1)**:157–159.

Kim, S., Yang, S., Kim, H., Shin, C., Ko, K. Inhibition of GSK-3β mediates expression of MMP-9 through ERK1/2 activation and translocation of NF-κB in rat primary astrocyte. Brain Research 2007;**1186**:12–20.

Kimble, J., Simpson, P. THE LIN-12/Notch Signaling Pathway and its Regulation. Annual Reviews in Cell and Developmental Biology 1997; **13(1)**:333–361.

Kockeritz, L., Doble, B., Patel, S., Woodgett, J. Glycogen Synthase Kinase-3-An Overview of An Over-Achieving Protein Kinase. Current Drug Targets 2006;**7(11)**:1377–1388.

Kockx, M., Herman, A. Apoptosis in atherosclerosis: beneficial or detrimental? Cardiovascular Research 2000;**45(3)**:736–46.

Koledova, V., Khalil, R. Ca2+, Calmodulin, and Cyclins in Vascular Smooth Muscle Cell Cycle. Circulation Research 2006;**98(10)**:1240–1243.

Kovall, R. Structures of CSL, Notch and Mastermind proteins: piecing together an active transcription complex. Current Opinion in Structural Biology 2007;**17(1)**:117–127.

Kovall, R. More complicated than it looks: assembly of Notch pathway transcription complexes. Oncogene 2008;**27(38)**:5099–5109.

Kovall, R., Hendrickson, W. Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. The EMBO Journal 2004;**23**:3441–3451.

Kraiss, L., Kirkman, T., Kohler, T., Zierler, B., Clowes, A. Shear stress regulates smooth muscle proliferation and neointimal thickening in porous polytetrafluoroethylene grafts. Arteriosclerosis, Thrombosis, and Vascular Biology 1991;**11(6)**:1844–1852.

Krishnan, V., Pereira, F., Qiu, Y., Chen, C., Beachy, P., Tsai, S., Tsai, M.

Mediation of Sonic Hedgehog-Induced Expression of COUP-TFII by a Protein Phosphatase. Science 1997;**278(5345)**:1947–50.

Kuang, W., Tan, J., Duan, Y., Duan, J., Wang, W., Jin, F., Jin, Z., Yuan, X., Liu, Y. Cyclic stretch induced miR-146a upregulation delays C2C12 myogenic differentiation through inhibition of Numb. Biochemical and Biophysical Research Communications 2009;**378(2)**:259–263.

Kumar, A., Hoover, J., Simmons, C., Lindner, V., Shebuski, R. Remodeling and neointimal formation in the carotid artery of normal and P-selectin-deficient mice. Circulation 1997;**96(12)**:4333–4342.

Kwon, C., Han, Z., Olson, E., Srivastava, D. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. Proceedings of the National Academy of Sciences 2005;**102(52)**:18986–18991.

Lacolley, P. Mechanical influence of cyclic stretch on vascular endothelial cells. Cardiovascular research 2004;**63(4)**:577–579.

Laemmli, U., *et al.* Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;**227(5259)**:680–685.

Lai, E., Tam, B., Rubin, G. Pervasive regulation of Drosophila Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. Genes & development 2005;**19(9)**:1067–1080.

Landsberg, J., Yuan, J. Calcium and TRP Channels in Pulmonary Vascular Smooth Muscle Cell Proliferation. Physiology 2004;**19(2)**:44–50.

Lanner, F., Sohl, M., Farnebo, F. Functional Arterial and Venous Fate Is Determined by Graded VEGF Signaling and Notch Status During

Embryonic Stem Cell Differentiation. Arteriosclerosis, Thrombosis, and Vascular Biology 2007;**27(3)**:487–93.

Lardelli, M., Dahlstrand, J., Lendahl, U. The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. Mechanisms of development 1994;**46(2)**:123–36.

Lawson, N., Vogel, A., Weinstein, B. sonic hedgehog and vascular endothelial growth factor Act Upstream of the Notch Pathway during Arterial Endothelial Differentiation. Developmental Cell 2002;**3(1)**:127–136.

Lee, B., Bae, J., Park, R., Kim, J., Park, J., Kim, I. betaig-h3 triggers signaling pathways mediating adhesion and migration of vascular smooth muscle cells through alphavbeta5 integrin. Experimental and molecular medicine 2006;**38(2)**:153–61.

Lee, E., Yu, S., Baker, N. The Scabrous protein can act as an extracellular antagonist of Notch signaling in the Drosophila wing. Current Biology 2000;**10(15)**:931–936.

Lee, H., Tsai, J., Liao, P., Tsai, W., Lin, K., Chuang, C., Sun, C., Chang, W., Tsai, H. Glycogen synthase kinase 3α and 3β have distinct functions during cardiogenesis of zebrafish embryo. BMC Developmental Biology 2007;**7**:93.

Lehoux, S., Castier, Y., Tedgui, A. Molecular mechanisms of the vascular responses to haemodynamic forces. J Intern Med 2006;**259(4)**:381–392.

Li, C., Hu, Y., Mayr, M., Xu, Q. Cyclic Strain Stress-induced Mitogen-activated Protein Kinase (MAPK) Phosphatase 1 Expression in Vascular

Smooth Muscle Cells Is Regulated by Ras/Rac-MAPK Pathways. Journal of Biological Chemistry 1999a;**274(36)**:25273–25280.

Li, C., Hu, Y., Sturm, G., Wick, G., Xu, Q. Ras/Rac-Dependent Activation of p38 Mitogen-Activated Protein Kinases in Smooth Muscle Cells Stimulated by Cyclic Strain Stress. Arteriosclerosis, Thrombosis, and Vascular Biology 2000;**20(3)**:E1–9.

Li, C., Wernig, F., Leitges, M., Hu, Y., Xu, Q. Mechanical stress-activated PKCδ regulates smooth muscle cell migration 1. The FASEB Journal 2003;**17(14)**:2106–2108.

Li, C., Xu, Q. Mechanical stress-initiated signal transductions in vascular smooth muscle cells. Cellular Signalling 2000;**12(7)**:435–445.

Li, F., Chong, Z., Maiese, K. Winding through the WNT pathway during cellular development and demise. Histol Histopathol 2006;**21(1)**:103–24.

Li, Q., Muragaki, Y., Ueno, H., Ooshima, A. Stretch-induced proliferation of cultured vascular smooth muscle cells and a possible involvement of local renin-angiotensin system and platelet-derived growth factor (PDGF). Hypertension research: official journal of the Japanese Society of Hypertension 1997;**20(3)**:217–23.

Li, S., Sims, S., Jiao, Y., Chow, L., Pickering, J. Evidence From a Novel Human Cell Clone That Adult Vascular Smooth Muscle Cells Can Convert Reversibly Between Noncontractile and Contractile Phenotypes. Circulation Research 1999b;**85(4)**:338–348.

Li, Y., Takeshita, K., Liu, P., Satoh, M., Oyama, N., Mukai, Y., Chin, M., Krebs, L., Kotlikoff, M., Radtke, F., *et al.* Smooth Muscle Notch1

Mediates Neointimal Formation After Vascular Injury. Circulation 2009; **119(20)**:2686–92.

Li, Z., Froehlich, J., Galis, Z., Lakatta, E. Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. Hypertension 1999c;**33(1)**:116–123.

Lim, L., Lau, N., Garrett-Engele, P., Grimson, A., Schelter, J., Castle, J., Bartel, D., Linsley, P., Johnson, J. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005;**433**:769–773.

Limbourg, A., Ploom, M., Elligsen, D., Sorensen, I., Ziegelhoeffer, T., Gossler, A., Drexler, H., Limbourg, F. Notch ligand Delta-like 1 is essential for postnatal arteriogenesis. Circulation research 2007; **100(3)**:363–71.

Lindner, V., Booth, C., Prudovsky, I., Small, D., Maciag, T., Liaw, L. Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. American Journal of Pathology 2001;**159(3)**:875–883.

Lipskaia, L., Pourci, M., Delomenie, C., Combettes, L., Goudouneche, D., Paul, J., Capiod, T., Lompre, A. Phosphatidylinositol 3-kinase and calcium-activated transcription pathways are required for VLDL-induced smooth muscle cell proliferation. Circulation research 2003; **92(10)**:1115–1122.

Liu, B., Ryer, E., Kundi, R., Kamiya, K., Itoh, H., Faries, P., Sakakibaria, K., Kent, K. Protein kinase C-delta (PKCδ) regulates migration and proliferation of vascular smooth muscle cells through the mitogen

activated protein kinase ERK1/2. Journal of vascular surgery: official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter 2007;**45(1)**:160–8.

Liu, S., Zhang, J., Li, H., Fang, Z., Wang, Q., Deng, H., Gong, C., Grundke-Iqbal, I., Iqbal, K., Wang, J. Tau Becomes a More Favorable Substrate for GSK-3 When It Is Prephosphorylated by PKA in Rat Brain. Journal of Biological Chemistry 2004;**279(48)**:50078–88.

Liu, Z., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G., Fairman, R., Velazquez, O., Herlyn, M. Regulation of Notch1 and Dll4 by Vascular Endothelial Growth Factor in Arterial Endothelial Cells: Implications for Modulating Arteriogenesis and Angiogenesis. Molecular and Cellular Biology 2003;**23(1)**:14.

Loberg, R., Vesely, E., Brosius, F. Enhanced Glycogen Synthase Kinase-3β Activity Mediates Hypoxia-induced Apoptosis of Vascular Smooth Muscle Cells and Is Prevented by Glucose Transport and Metabolism. Journal of Biological Chemistry 2002;**277(44)**:41667–41673.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N., Israel, A. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci US A 1998;**95(14)**:8108–8112.

Loirand, G., Guerin, P., Pacaud, P. Rho Kinases in Cardiovascular Physiology and Pathophysiology. Circulation Research 2006;**98(3)**:322–334.

Lu, L., Stanley, P. Roles of O-fucose glycans in notch signaling revealed by mutant mice. Methods Enzymol 2006;**417**:127–36.

Luo, J. Glycogen synthase kinase 3β (GSK3β) in tumorigenesis and cancer chemotherapy. Cancer Letters 2009;**273(2)**:194–200.

Luo, J., Chen, J., Deng, Z., Luo, X., Song, W., Sharff, K., Tang, N., Haydon, R., Luu, H., He, T. Wnt signaling and human diseases: what are the therapeutic implications? Laboratory Investigation 2007;**87**:97–103.

Luscher, T., Steffel, J., Eberli, F., Joner, M., Nakazawa, G., Tanner, F., Virmani, R. Drug-Eluting Stent and Coronary Thrombosis: Biological Mechanisms and Clinical Implications. Circulation 2007;**115(8)**:1051–8.

Macko, A., Beneze, A., Teachey, M., Henriksen, E. Roles of insulin signalling and p38 MAPK in the activation by lithium of glucose transport in insulin-resistant rat skeletal muscle. Archives of physiology and biochemistry 2008;**114(5)**:331–339.

Maier, D. Hairless: the ignored antagonist of the Notch signalling pathway. Hereditas 2006;**143(2006)**:212–221.

Maier, M., Gessler, M. Comparative Analysis of the Human and Mouse Hey1 Promoter: Hey Genes Are New Notch Target Genes. Biochemical and Biophysical Research Communications 2000;**275(2)**:652–660.

Majno, G., Joris, I. Apoptosis, oncosis, and necrosis. An overview of cell death. American Journal of Pathology 1995;**146(1)**:3–15.

Martinez, A. Preclinical efficacy on GSK-3 inhibitors: Towards a future generation of powerful drugs. Medicinal Research Reviews 2008; **28(5)**:773–96.

Mason, H., Rakowiecki, S., Raftopoulou, M., Nery, S., Huang, Y., Gridley,

T., Fishell, G. Notch signaling coordinates the patterning of striatal compartments. Development 2005;**132(19)**:4247–4258.

Matsumoto, T., Turesson, I., Book, M., Gerwins, P., Claesson-Welsh, L. p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. Journal of Cell Biology 2002;**156(1)**:149–160.

Matsuno, K. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development 1995;**121(8)**:2633–2644.

McCarthy, N., Bennett, M. The regulation of vascular smooth muscle cell apoptosis. Cardiovascular Research 2000;**45(3)**:747–55.

McCubrey, J., Steelman, L., Chappell, W., Abrams, S., Wong, E., Chang, F., Lehmann, B., Terrian, D., Milella, M., Tafuri, A., *et al.* Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 2007;**1773(8)**:1263–84.

McGlinn, E., van Bueren, K., Fiorenza, S., Mo, R., Poh, A., Forrest, A., Soares, M., Bonaldo, M., Grimmond, S., Hui, C., *et al.* Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. Mechanisms of development 2005;**122(11)**:1218–1233.

Mercer, J., Figg, N., Stoneman, V., Braganza, D., Bennett, M. Endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice. Circulation research 2005; **96(6)**:667–674.

Miele, L. Notch signaling. Clin Cancer Res 2006;**12(4)**:1074–9.

Mignotte, B., Vayssiere, J. Mitochondria and apoptosis. FEBS Journal 1998; **252(1)**:1–15.

Mills, I., Cohen, C., Kamal, K., Li, G., Shin, T., Du, W., Sumpio, B. Strain activation of bovine aortic smooth muscle cell proliferation and alignment: study of strain dependency and the role of protein kinase A and C signaling pathways. Journal of Cellular Physiology 1997; **170(3)**:228–34.

Monaco, C., Paleolog, E. Nuclear factor κB: a potential therapeutic target in atherosclerosis and thrombosis. Cardiovascular research 2004; **61(4)**:671–682.

Moore, A., Mangoni, A., Lyons, D., Jackson, S. The cardiovascular system in the ageing patient. British journal of clinical pharmacology 2003; **56(3)**:254–260.

Morrow, D., Cullen, J., Liu, W., Guha, S., Sweeney, C., Birney, Y., Collins, N., Walls, D., Redmond, E., Cahill, P. Sonic Hedgehog Induces Notch Target Gene Expression in Vascular Smooth Muscle Cells via VEGF-A. Arteriosclerosis, thrombosis, and vascular biology 2009;**29(7)**:1112–8.

Morrow, D., Scheller, A., Birney, Y., Sweeney, C., Guha, S., Cummins, P., Murphy, R., Walls, D., Redmond, E., Cahill, P. Notch-mediated CBF-1/RBP-Jκ-dependent regulation of human vascular smooth muscle cell phenotype in vitro. American Journal of Physiology- Cell Physiology 2005a;**289(5)**:1188–1196.

Morrow, D., Sweeney, C., Birney, Y., Cummins, P., Walls, D., Redmond, E., Cahill, P. Cyclic Strain Inhibits Notch Receptor Signaling in Vascular Smooth Muscle Cells In Vitro. Circulation Research 2005b;**96(5)**:567–575.

Morrow, D., Sweeney, C., Birney, Y., Guha, S., Collins, N., Cummins, P., Murphy, R., Walls, D., Redmond, E., Cahill, P. Biomechanical regulation of hedgehog signaling in vascular smooth muscle cells in vitro and in vivo. AJP-Cell Physiology 2007;**292(1)**:C488–96.

Mullor, J., Sánchez, P., Altaba, A. Pathways and consequences: Hedgehog signaling in human disease. Trends in Cell Biology 2002;**12(12)**:562–569.

Mulvany, M. Vascular remodelling of resistance vessels: can we define this? Cardiovascular Research 1999;**41(1)**:9–13.

Mumm, J., Kopan, R. Notch signaling: from the outside in. Developmental biology 2000;**228(2)**:151–165.

Mungamuri, S., Yang, X., Thor, A., Somasundaram, K. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Research 2006;**66(9)**:4715–4724.

Murphy, E., Steenbergen, C. Inhibition of GSK-3beta as a target for cardioprotection: the importance of timing, location, duration and degree of inhibition. Expert Opin. Ther. Targets 2005;**9(3)**:447–456.

Murre, C., Bain, G., van Dijk, M., Engel, I., Furnari, B., Massari, M., Matthews, J., Quong, M., Rivera, R., Stuiver, M. Structure and function of helix-loop-helix proteins. Biochim Biophys Acta 1994;**1218(2)**:129–35.

Muslin, A. MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. Clinical Science 2008; **115**:203–218.

Nakagawa, O., McFadden, D., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D., Olson, E. Members of the HRT family of basic helix-loop-

helix proteins act as transcriptional repressors downstream of Notch signaling. Proceedings of the National Academy of Sciences 2000; **97(25)**:13655–60.

Nakajima, T., Kitajima, I., Shin, H., Takahashi, I., Shigeta, K., Abeyama, K., Yamashita, Y., Tokioka, T., Soejima, Y., Maruyama, I. Involvement of NF-kappa B activation in thrombin-induced human vascular smooth muscle cell proliferation. Biochemical and biophysical research communications(Print) 1994;**204(2)**:950–955.

Nakamura, A., Isoyama, S., Watanabe, T., Katoh, M., Sawai, T. Heterogeneous smooth muscle cell population derived from small and larger arteries. Microvascular Research 1998;**55(1)**:14–28.

Nelson, K., Weiss, G. MicroRNAs and cancer: past, present, and potential future. Molecular Cancer Therapeutics 2008;**7(12)**:3655–60.

Newby, A. Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. Cardiovascular research 2006;**69(3)**:614–624.

Nichols, W., Denardo, S., Wilkinson, I., McEniery, C., Cockcroft, J., O'Rourke, M. Effects of Arterial Stiffness, Pulse Wave Velocity, and Wave Reflections on the Central Aortic Pressure Waveform. The Journal of Clinical Hypertension 2008;**10(4)**:295–303.

Nicholson, D., Thornberry, N. Apoptosis: Life and Death Decisions. Science 2003;**299(5604)**:214–5.

Nicita-Mauro, V., Maltese, G., Nicita-Mauro, C., Basile, G. Vascular aging and geriatric patient. Minerva cardioangiologica 2007;**55(4)**:497–502.

Nishimura, K., Li, W., Hoshino, Y., Kadohama, T., Asada, H., Ohgi, S., Sumpio, B. Role of AKT in cyclic strain-induced endothelial cell proliferation and survival. American Journal of Physiology- Cell Physiology 2006;**290(3)**:812–821.

Nybakken, K., Perrimon, N. Hedgehog signal transduction: recent findings. Current opinion in genetics & development 2002;**12(5)**:503–511.

Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M., Lendahl, U. The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. Journal of Biological Chemistry 2001; **276(38)**:35847–35853.

Ogden, S., Fei, D., Schilling, N., Ahmed, Y., Hwa, J., Robbins, D. G protein Galphai functions immediately downstream of Smoothened in Hedgehog signalling. Nature 2008;**456(7224)**:967–70.

Ohashi, N., Matsumori, A., Furukawa, Y., Ono, K., Okada, M., Iwasaki, A., Miyamoto, T., Nakano, A., Sasayama, S. Role of p38 mitogen-activated protein kinase in neointimal hyperplasia after vascular injury. Arteriosclerosis, thrombosis, and vascular biology 2000;**20(12)**:2521–2526.

Ordentlich, P., Lin, A., Shen, C., Blaumueller, C., Matsuno, K., Artavanis-Tsakonas, S., Kadesch, T. Notch Inhibition of E47 Supports the Existence of a Novel Signaling Pathway. Molecular and Cellular Biology 1998; **18(4)**:2230–2239.

Orlandi, A., Bochaton-Piallat, M., Gabbiani, G., Spagnoli, L. Aging,

smooth muscle cells and vascular pathobiology: implications for atherosclerosis. Atherosclerosis 2006;**188(2)**:221–230.

Ougolkov, A., Fernandez-Zapico, M., Savoy, D., Urrutia, R., Billadeau, D. Glycogen Synthase Kinase-3β Participates in Nuclear Factor κB-Mediated Gene Transcription and Cell Survival in Pancreatic Cancer Cells. Cancer Research 2005;**65(6)**:2076–2081.

Owens, D., Watt, F. Contribution of stem cells and differentiated cells to epidermal tumours. Nature Reviews Cancer 2003;**3(6)**:444–451.

Owens, G., Kumar, M., Wamhoff, B. Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. Physiological Reviews 2004;**84(3)**:767–801.

Paddison, P., Caudy, A., Bernstein, E., Hannon, G., Conklin, D. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes & development 2002;**16(8)**:948–958.

Pan, Y., Bai, C., Joyner, A., Wang, B. Sonic hedgehog Signaling Regulates Gli2 Transcriptional Activity by Suppressing Its Processing and Degradation. Molecular and Cellular Biology 2006;**26(9)**:3365–3377.

Panin, V., Shao, L., Lei, L., Moloney, D., Irvine, K., Haltiwanger, R. Notch Ligands Are Substrates for Protein O-Fucosyltransferase-1 and Fringe. Journal of Biological Chemistry 2002;**277(33)**:29945–29952.

Park, K., Yang, H., Youn, S., Yang, H., Chae, I., Oh, B., Lee, M., Park, Y., Choi, Y., Kim, H., *et al.* Constitutively Active Glycogen Synthase Kinase-3β Gene Transfer Sustains Apoptosis, Inhibits Proliferation of Vascular Smooth Muscle Cells, and Reduces Neointima Formation After Balloon

Injury in Rats. Arteriosclerosis, Thrombosis, and Vascular Biology 2003; **23(8)**:1364–1369.

Parks, A. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development 2000;**127(7)**:1373–1385.

Paroush, Z., Finley Jr, R., Kidd, T., Wainwright, S., Ingham, P., Brent, R., Ish-Horowicz, D. Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell 1994;**79(5)**:805–15.

Pearl, L., Barford, D. Regulation of protein kinases in insulin, growth factor and Wnt signalling. Current Opinion in Structural Biology 2002; **12(6)**:761–767.

Perlman, H., Maillard, L., Krasinski, K., Walsh, K. Evidence for the rapid onset of apoptosis in medial smooth muscle cells after balloon injury. Circulation 1997;**95(4)**:981–987.

Perlstein, T., Lee, R. Smoking, Metalloproteinases, and Vascular Disease. Arteriosclerosis, Thrombosis, and Vascular Biology 2006;**26(2)**:250–256.

Phiel, C., Wilson, C., Virginia, M., Klein, P. GSK-3α regulates production of Alzheimer's disease amyloid-β peptides. Nature 2003;**423(6938)**:435–439.

Phng, L., Gerhardt, H. Angiogenesis: A Team Effort Coordinated by Notch. Developmental Cell 2009;**16(2)**:196–208.

Plante, G. Vascular response to stress in health and disease. Metabolism 2002;**51(6PB)**:25–30.

Plante, G. Predisease biological markers: early diagnosis and prevention of arterial hypertension. Metabolism 2008;**57**:36–39.

Pola, R., Ling, L., Silver, M., Corbley, M., Kearney, M., Pepinsky, R., Shapiro, R., Taylor, F., Baker, D., Asahara, T., *et al.* The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nature Medicine 2001;**7**:706–711.

Pollman, M., Hall, J., Gibbons, G. Determinants of vascular smooth muscle cell apoptosis after balloon angioplasty injury influence of redox state and cell phenotype. Circulation research 1999;**84(1)**:113–121.

Post, M., de Smet, B., van der Helm, Y., Borst, C., Kuntz, R. Arterial remodeling after balloon angioplasty or stenting in an atherosclerotic experimental model. Circulation 1997;**96(3)**:996–1003.

Potter, H. Transfection by electroporation. Current protocols in molecular biology 2003;**Chapter 9(Unit 9.3)**.

Price, M., Kalderon, D. Proteolysis of the Hedgehog Signaling Effector Cubitus interruptus Requires Phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1. Cell 2002;**108(6)**:823–835.

Proweller, A., Pear, W., Parmacek, M. Notch Signaling Represses Myocardin-induced Smooth Muscle Cell Differentiation. Journal of Biological Chemistry 2005;**280(10)**:8994–9004.

Pyles, J., March, K., Franklin, M., Mehdi, K., Wilensky, R., Adam, L. Activation of MAP kinase in vivo follows balloon overstretch injury of porcine coronary and carotid arteries. Circulation research 1997; **81(6)**:904–910.

Qi, Y., Qu, M., Long, D., Liu, B., Yao, Q., Chien, S., Jiang, Z. Rho-GDP dissociation inhibitor alpha downregulated by low shear stress promotes vascular smooth muscle cell migration and apoptosis: a proteomic analysis. Cardiovascular Research 2008;**80(1)**:114–22.

Rakesh, K., Agrawal, D. Cytokines and growth factors involved in apoptosis and proliferation of vascular smooth muscle cells. international Immunopharmacology 2005;**5(10)**:1487–1506.

Ramain, P., Khechumian, K., Seugnet, L., Arbogast, N., Ackermann, C., Heitzler, P. Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. Current Biology 2001;**11(22)**:1729–1738.

Rampal, R., Arboleda-Velasquez, J., Nita-Lazar, A., Kosik, K., Haltiwanger, R. Highly Conserved O-Fucose Sites Have Distinct Effects on Notch1 Function. Journal of Biological Chemistry 2005; **280(37)**:32133–40.

Rand, M., Grimm, L., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S., Sklar, J., Aster, J. Calcium depletion dissociates and activates heterodimeric notch receptors. Molecular and Cellular Biology 2000; **20(5)**:1825–1835.

Rebay, I., Fleming, R., Fehon, R., Cherbas, L., Cherbas, P., Artavanis-Tsakonas, S. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell 1991;**67(4)**:687–699.

References, S., Cole, A., Frame, S., Cohen, P. Further evidence that the tyrosine phosphorylation of glycogen synthase kinase-3 (GSK3) in

mammalian cells is an autophosphorylation event. Biochem J 2004; **377(Pt 1)**:249–255.

Ricci, R., Sumara, G., Sumara, I., Rozenberg, I., Kurrer, M., Akhmedov, A., Hersberger, M., Eriksson, U., Eberli, F., Becher, B., *et al.* Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. Science 2004;**306(5701)**:1558–1561.

Rizzo, P., Osipo, C., Foreman, K., Golde, T., Osborne, B., Miele, L. Rational targeting of Notch signaling in cancer. Oncogene 2008;**27(38)**:5124–5131.

Robertson, L., Kim, A., Werstuck, G. Mechanisms linking diabetes mellitus to the development of atherosclerosis: a role for endoplasmic reticulum stress and glycogen synthase kinase-3. Can J Physiol Pharmacol 2006; **84(1)**:39–48.

Rogers, S., Wells, R., Rechsteiner, M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science 1986; **234(4774)**:364–368.

Rossi, D., Gaidano, G. Messengers of cell death: apoptotic signaling in health and disease. Haematologica 2003;**88(2)**:212–218.

Rowe, M., Wiest, C., Chuang, D. GSK-3 is a viable potential target for therapeutic intervention in bipolar disorder. Neuroscience and Biobehavioral Reviews 2007;**31(6)**:920–931.

Ruel, L., Bourouis, M., Heitzler, P., Pantesco, V., Simpson, P. Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. Nature 1993;**362(6420)**:557–560.

Sade, H., Krishna, S., Sarin, A. The Anti-apoptotic Effect of Notch-1 Requires p56lck-dependent, Akt/PKB-mediated Signaling in T Cells. Journal of Biological Chemistry 2004;**279(4)**:2937–44.

Sakamoto, K., Ohara, O., Takagi, M., Takeda, S., Katsube, K. Intracellular Cell-Autonomous Association of Notch and Its Ligands: A Novel Mechanism of Notch Signal Modification. Developmental Biology 2002; **241(2)**:313–326.

Sakata, Y., Xiang, F., Chen, Z., Kiriyama, Y., Kamei, C., Simon, D., Chin, M. Transcription factor CHF1/Hey2 regulates neointimal formation in vivo and vascular smooth muscle proliferation and migration in vitro. Arteriosclerosis, thrombosis, and vascular biology 2004;**24(11)**:2069–2074.

Salamanca, D., Khalil, R. Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. Biochemical Pharmacology 2005;**70(11)**:1537–1547.

Scherr, M., Morgan, M., Eder, M. Gene silencing mediated by small interfering RNAs in mammalian cells. Current Medicinal Chemistry 2003;**10**:245–256.

Schiffrin, E. Remodeling of resistance arteries in essential hypertension and effects of antihypertensive treatment. American Journal of Hypertension 2004;**17(12)**:1192–1200.

Schillinger, M., Minar, E. Restenosis after percutaneous angioplasty: the role of vascular inflammation. Vascular Health and Risk Management 2005;**1(1)**:73–8.

Schroeter, E., Kisslinger, J., Kopan, R., *et al.* Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 1998; **393(6683)**:382–385.

Seasholtz, T., Zhang, T., Morissette, M., Howes, A., Yang, A., Brown, J. Increased Expression and Activity of RhoA Are Associated With Increased DNA Synthesis and Reduced p27Kip1 Expression in the Vasculature of Hypertensive Rats. Circulation Research 2001;**89(6)**:488–495.

Sedding, D., Homann, M., Seay, U., Tillmanns, H., Preissner, K., Braun-Dullaeus, R. Calpain counteracts mechanosensitive apoptosis of vascular smooth muscle cells in vitro and in vivo. The FASEB Journal 2008;**22(2)**:579–89.

Seifert, R., Schwartz, S., Bowen-Pope, D. Developmentally regulated production of platelet-derived growth factor-like molecules. Nature 1984;**311(5987)**:669–671.

Seo, S., Fujita, H., Nakano, A., Kang, M., Duarte, A., Kume, T. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. Developmental Biology 2006;**294(2)**:458–470.

Seugnet, L., Simpson, P., Haenlin, M. Transcriptional regulation of Notch and Delta: requirement for neuroblast segregation in Drosophila. Development 1997;**124(10)**:2015–2025.

Shaw, M., Cohen, P. Role of protein kinase B and the MAP kinase cascade in mediating the EGF-dependent inhibition of glycogen synthase kinase 3 in Swiss 3T3 cells. FEBS Letters 1999;**461(1-2)**:120–124.

Shawber, C. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development 1996;**122(12)**:3765–3773.

Sherr, C., Roberts, J. Living with or without cyclins and cyclin-dependent kinases. Genes & Development 2004;**18(22)**:2699–71.

Shin, H., Lee, H., Nishida, M., Lee, M., Tamura, R., Yamashita, S., Matsuzawa, Y., Lee, I., Koh, G. Betacellulin and amphiregulin induce upregulation of cyclin D1 and DNA synthesis activity through differential signaling pathways in vascular smooth muscle cells. Circulation research 2003;**93(4)**:302–310.

Shyu, K., Chang, M., Wang, B., Kuan, P., Chang, H. Cyclical mechanical stretching increases the expression of vascular endothelial growth factor in rat vascular smooth muscle cells. J Formos Med Assoc 2001; **100(11)**:741–7.

Simpson, P. Introduction: Notch signalling and choice of cell fates in development. Seminars in Cell and Developmental Biology 1998; **9(6)**:581–582.

Smelkinson, M., Zhou, Q., Kalderon, D. Regulation of Ci-SCFSlimb binding, Ci proteolysis, and hedgehog pathway activity by Ci phosphorylation. Developmental cell 2007;**13(4)**:481–495.

Smith, J., Davies, N., Willis, A., Sumpio, B., Zilla, P. Cyclic stretch induces the expression of vascular endothelial growth factor in vascular smooth muscle cells. Endothelium 2001;**8(1)**:41–8.

Song, J., Park, S., Kim, M., Shin, I. Down-regulation of Notch-dependent transcription by Akt in vitro. FEBS Letters 2008;**582(12)**:1693–1699.

Sorensen, I., Adams, R., Gossler, A. DLL1 mediated Notch activation regulates endothelial identity in mouse fetal arteries. Blood 2009; **113(22)**:5375–6.

Sotoudeh, M., Li, Y., Yajima, N., Chang, C., Tsou, T., Wang, Y., Usami, S., Ratcliffe, A., Chien, S., Shyy, J. Induction of apoptosis in vascular smooth muscle cells by mechanical stretch. American Journal of Physiology- Heart and Circulatory Physiology 2002;**282(5)**:1709–1716.

Spyridopoulos, I., Andrés, V. Control of vascular smooth muscle and endothelial cell proliferation and its implication in cardiovascular disease. Frontiers in Bioscience 1998;**3**:d269–287.

Stifani, S., Blaumueller, C., Redhead, N., Hill, R., Artavanis-Tsakonas, S. Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. Nature genetics 1992;**2(2)**:119–127.

Stoneman, V., Bennett, M. Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci 2004;**107(4)**:343–54.

Strauss, B., Rabinovitch, M. Adventitial Fibroblasts Defining a Role in Vessel Wall Remodeling. American Journal of Respiratory Cell and Molecular Biology 2000;**22(1)**:1–3.

Strutt, H., Thomas, C., Nakano, Y., Stark, D., Neave, B., Taylor, A., Ingham, P. Mutations in the sterol-sensing domain of Patched suggest a role for vesicular trafficking in Smoothened regulation. Current Biology 2001; **11(8)**:608–613.

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., Eichmann, A. The Notch ligand Delta-like 4 negatively regulates

endothelial tip cell formation and vessel branching. Proceedings of the National Academy of Sciences 2007;**104(9)**:3225–30.

Sugden, P. Ras, Akt, and Mechanotransduction in the Cardiac Myocyte. Circulation Research 2003;**93(12)**:1179–1192.

Sumpio, B., Banes, A. Response of porcine aortic smooth muscle cells to cyclic tensional deformation in culture. The Journal of surgical research 1988;**44(6)**:696–701.

Suzuma, I., Suzuma, K., Ueki, K., Hata, Y., Feener, E., King, G., Aiello, L. Stretch-induced retinal vascular endothelial growth factor expression is mediated by phosphatidylinositol 3-kinase and protein kinase C (PKC)-ζ but not by stretch-induced ERK1/2, Akt, Ras, or classical/novel PKC pathways. Journal of Biological Chemistry 2002;**277(2)**:1047–1057.

Sweeney, C., Morrow, D., Birney, Y., Coyle, S., Hennessy, C., Scheller, A., Cummins, P., Walls, D., Redmond, E., Cahill, P. Notch 1 and 3 receptor signaling modulates vascular smooth muscle cell growth, apoptosis, and migration via a CBF-1/RBP-Jk dependent pathway. FASEB J 2004; **18(12)**:1421–3.

Taelman, V., Van Wayenbergh, R., Sölter, M., Pichon, B., Pieler, T., Christophe, D., Bellefroid, E. Sequences downstream of the bHLH domain of the Xenopus hairy-related transcription factor-1 act as an extended dimerization domain that contributes to the selection of the partners. Developmental Biology 2004;**276(1)**:47–63.

Tagami, S., Okochi, M., Yanagida, K., Ikuta, A., Fukumori, A., Matsumoto, N., Ishizuka-Katsura, Y., Nakayama, T., Itoh, N., Jiang, J. Regulation of

Notch signaling by dynamic changes in the precisio of S3 cleavage of Notch-1. Molecular and Cellular Biology 2008;**28(1)**:165–176.

Takasaki, S., Kawamura, Y., Konagaya, A. Selecting effective siRNA sequences based on the self-organizing map and statistical techniques. Computational Biology and Chemistry 2006;**30(3)**:169–178.

Takeshita, K., Satoh, M., Ii, M., Silver, M., Limbourg, F., Mukai, Y., Rikitake, Y., Radtke, F., Gridley, T., Losordo, D., *et al.* Critical role of endothelial Notch1 signaling in postnatal angiogenesis. Circulation research 2007; **100(1)**:70–8.

Taketo, M. Wnt signaling and gastrointestinal tumorigenesis in mouse models. Oncogene 2006;**25**:7522–7530.

Tang, Y., Liu, Y., Zhou, J., Wang, G., Qiu, Q., Guan, Y. Silence of ClC-3 chloride channel inhibits cell proliferation and the cell cycle via G1/S phase arrest in rat basilar arterial smooth muscle cells. Cell Proliferation 2008a;**41(5)**:775–785.

Tang, Y., Urs, S., Liaw, L. Hairy-Related Transcription Factors Inhibit Notch-Induced Smooth Muscle α-Actin Expression by Interfering With Notch Intracellular Domain/CBF-1 Complex Interaction With the CBF-1-Binding Site. Circulation Research 2008b;**102(6)**:661–8.

Thatcher, E., Flynt, A., Li, N., Patton, J., Patton, J. MiRNA expression analysis during normal zebrafish development and following inhibition of the Hedgehog and Notch signaling pathways. Developmental Dynamics 2007;**236(8)**:2172–80.

Thornton, T., Pedraza-Alva, G., Deng, B., Wood, C., Aronshtam, A., Clements, J., Sabio, G., Davis, R., Matthews, D., Doble, B., *et al.*

Phosphorylation by p38 MAPK as an Alternative Pathway for GSK3 {beta} Inactivation. Science 2008;**320(5876)**:667.

Tilesi, F., Fradiani, P., Socci, V., Willems, D., Ascenzioni, F. Design and validation of siRNAs and shRNAs. Current Opinion in Molecular Therapeutics 2009;**11(2)**:156–164.

Tock, J., Van Putten, V., Stenmark, K., Nemenoff, R. Induction of SM-α-actin expression by mechanical strain in adult vascular smooth muscle cells is mediated through activation of JNK and p38 MAP kinase. Biochemical and Biophysical Research Communications 2003; **301(4)**:1116–1121.

Tortora, G., Grabowski, S. *Principles of anatomy and physiology*. John Wiley & Sons, New York, 8th edn., 1996.

Touyz, R. Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. Experimental Physiology 2005;**90(4)**:449–455.

Tsurumi, Y., Murohara, T., Krasinski, K., Chen, D., Witzenbichler, B., Kearney, M., Couffinhal, T., Isner, J. Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. Nature Medicine 1997; **3(8)**:879–886.

Tung, J., Heydari, K., Tirouvanziam, R., Sahaf, B., Parks, D., Herzenberg, L., Herzenberg, L. Modern Flow Cytometry: A Practical Approach. Clinics in Laboratory Medicine 2007;**27(3)**:453–468.

Ueba, H., Kawakami, M., Yaginuma, T. Shear Stress as an Inhibitor of Vascular Smooth Muscle Cell Proliferation Role of Transforming

Growth Factor-β1 and Tissue-Type Plasminogen Activator. Arteriosclerosis, thrombosis, and vascular biology 1997;**17(8)**:1512–1516.

Uemura, K., Kuzuya, A., Shimozono, Y., Aoyagi, N., Ando, K., Shimohama, S., Kinoshita, A. GSK3beta activity modifies the localization and function of presenilin 1. Journal of Biological Chemistry 2007;**282(21)**:15823–32.

Urs, S., Roudabush, A., O'Neill, C., Pinz, I., Prudovsky, I., Kacer, D., Tang, Y., Liaw, L., Small, D. Soluble Forms of the Notch Ligands Delta1 and Jagged1 Promote in Vivo Tumorigenicity in NIH3T3 Fibroblasts with Distinct Phenotypes. American Journal of Pathology 2008;**173(3)**:865.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D., Kitajewski, J. Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development 1996;**122(7)**:2251–2259.

van den Heuvel, S. Cell-cycle regulation. Wormbook 2005;1–16.

Varadkar, P., Kraman, M., Despres, D., Ma, G., Lozier, J., McCright, B. Notch2 is required for the proliferation of cardiac neural crest-derived smooth muscle cells. Developmental dynamics: an official publication of the American Association of Anatomists 2008;**237(4)**:1144–52.

Virmani, R., Avolio, A., Mergner, W., Robinowitz, M., Herderick, E., Cornhill, J., Guo, S., Liu, T., Ou, D., O'Rourke, M. Effect of aging on aortic morphology in populations with high and low prevalence of hypertension and atherosclerosis. Comparison between occidental and Chinese communities. The American journal of pathology 1991; **139(5)**:1119–29.

Walsh, K., Smith, R., Kim, H. Vascular Cell Apoptosis in Remodeling, Restenosis, and Plaque Rupture. Circulation Research 2000;**87(3)**:184–188.

Wang, Q., Zhou, Y., Wang, X., Evers, B. Glycogen synthase kinase-3 is a negative regulator of extracellular signal-regulated kinase. Oncogene 2005;**25(1)**:43–50.

Wang, T., Baron, M., Trump, D. An overview of Notch3 function in vascular smooth muscle cells. Progress in Biophysics and Molecular Biology 2008;**96(1-3)**:499–509.

Wang, W., Campos, A., Prince, C., Mou, Y., Pollman, M. Coordinate Notch3-Hairy-related Transcription Factor Pathway Regulation in Response to Arterial Injury. Mediator Role of Platelet-Derived Growth Factor and ERK. Journal of Biological Chemistry 2002;**277(26)**:23165–23171.

Wang, W., Prince, C., Hu, X., Pollman, M. HRT1 modulates vascular smooth muscle cell proliferation and apoptosis. Biochemical and Biophysical Research Communications 2003;**308(3)**:596–601.

Wang, W., Struhl, G. Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development 2004;**131(21)**:5367–5380.

Wang, Z., Banerjee, S., Li, Y., Wahidur Rahman, K., Zhang, Y., Sarkar, F. Down-regulation of Notch-1 inhibits invasion by inactivation of Nuclear Factor-κB, Vascular Endothelial Growth Factor, and Matrix Metalloproteinase-9 in pancreatic cancer cells. Cancer Research 2006; **66(5)**:2778–2784.

Ward, M., Pasterkamp, G., Yeung, A., Borst, C. Arterial Remodeling Mechanisms and Clinical Implications. Circulation 2000;**102(10)**:1186–1191.

Watcharasit, P., Bijur, G., Song, L., Zhu, J., Chen, X., Jope, R. Glycogen Synthase Kinase-3β (GSK3β) Binds to and Promotes the Actions of p53*. Journal of Biological Chemistry 2003;**278(49)**:48872–48879.

Weed, M., Mundlos, S., Olsen, B. The role of sonic hedgehog in vertebrate development. Matrix Biology 1997;**16(2)**:53–58.

Weinmaster, G. Notch2: a second mammalian Notch gene. Development 1992;**116(4)**:931–941.

Weinmaster, G. The Ins and Outs of Notch Signaling. Molecular and Cellular Neuroscience 1997;**9(2)**:91–102.

Weis, W., Kobilka, B. Structural insights into G-protein-coupled receptor activation. Current Opinion in Structural Biology 2008;**18(6)**:734–740.

Wernig, F., Xu, Q. Mechanical stress-induced apoptosis in the cardiovascular system. Progress in Biophysics and Molecular Biology 2002;**78(2-3)**:105–137.

Wilson, E. Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. Journal of Cell Biology 1993; **123(3)**:741–747.

Wilson, E., Sudhir, K., Ives, H. Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. Journal of Clinical Investigation 1995;**96(5)**:2364–2372.

Woodgett, J. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J 1990;**9(8)**:2431–8.

Woodgett, J., Cohen, P. Multisite phosphorylation of glycogen synthase: molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). Biochimica et biophysica acta 1984;**788(3)**:339–347.

Woods, Y., Cohen, P., Becker, W., Jakes, R., Goedert, M., Wang, X., Proud, C. The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen synthase kinase 3-priming kinase. Biochem. J 2001;**355(Pt 3)**:609–615.

Wu, G., Lyapina, S., Das, I., Li, J., Gurney, M., Pauley, A., Chui, I., Deshaies, R., Kitajewski, J. SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Molecular and Cellular Biology 2001;**21(21)**:7403–7415.

Wu, J., Bresnick, E. Bare rudiments of notch signaling: how receptor levels are regulated. Trends in Biochemical Sciences 2007;**32(10)**:477–485.

Xu, Q., Liu, Y., Gorospe, M., Udelsman, R., Holbrook, N. Acute hypertension activates mitogen-activated protein kinases in arterial wall. Journal of Clinical Investigation 1996;**97(2)**:508–14.

Yamamoto, N., Yamamoto, S., Inagaki, F., Kawaichi, M., Fukamizu, A., Kishi, N., Matsuno, K., Nakamura, K., Weinmaster, G., Okano, H., *et al.* Role of Deltex-1 as a Transcriptional Regulator Downstream of the Notch Receptor. Journal of Biological Chemistry 2001;**276(48)**:45031–45040.

Yamboliev, I., Ward, S., Mutafova-Yambolieva, V. Canine mesenteric artery and vein convey no difference in the content of major contractile proteins. BMC physiology 2002;**2(1)**:17.

Yan, S., Harja, E., Andrassy, M., Fujita, T., Schmidt, A. Protein Kinase C β/Early Growth Response-1 Pathway A Key Player in Ischemia, Atherosclerosis, and Restenosis. Journal of the American College of Cardiology 2006;**48(9S)**:47–55.

Yang, W., Yang, D., Na, S., Sandusky, G., Zhang, Q., Zhao, G. Dicer is required for embryonic angiogenesis during mouse development. Journal of Biological Chemistry 2005;**280(10)**:9330–5.

Yao, H., Shaw, P., Wong, C., Wan, D. Expression of glycogen synthase kinase-3 isoforms in mouse tissues and their transcription in the brain. Journal of Chemical Neuroanatomy 2002;**23(4)**:291–297.

Yoshida, T., Azuma, H., Aihara, K., Fujimura, M., Akaike, M., Mitsui, T., Matsumoto, T. Vascular smooth muscle cell proliferation is dependent upon upregulation of mitochondrial transcription factor A (mtTFA) expression in injured rat carotid artery. Atherosclerosis 2005;**178(1)**:39–47.

You, L., Lin, F., Lee, C., DeMayo, F., Tsai, M., Tsai, S. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature 2005;**435(7038)**:98–104.

Zachary, I., Gliki, G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovascular research 2001;**49(3)**:568–81.

Zhang, C. MicroRNAs: role in cardiovascular biology and disease. Clinical Science 2008;**114**:699–706.

Zhang, W., Zhao, Y., Tong, C., Wang, G., Wang, B., Jia, J., Jiang, J. Hedgehog-regulated Costal2-kinase complexes control phosphorylation and proteolytic processing of Cubitus interruptus. Developmental cell 2005;**8(2)**:267–278.

Zhao, Y., Ransom, J., Li, A., Vedantham, V., von Drehle, M., Muth, A., Tsuchihashi, T., McManus, M., Schwartz, R., Srivastava, D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 2007;**129(2)**:303–317.

Zhdanov, R., Podobed, O., Vlassov, V. Cationic lipid–DNA complexes—lipoplexes—for gene transfer and therapy. Bioelectrochemistry 2002; **58(1)**:53–64.

Zhou, S., Fujimuro, M., Hsieh, J., Chen, L., Miyamoto, A., Weinmaster, G., Hayward, S. SKIP, a CBF1-Associated Protein, Interacts with the Ankyrin Repeat Domain of NotchIC To Facilitate NotchIC Function. Molecular and Cellular Biology 2000;**20(7)**:2400–10.

Zieman, S., Kass, D. Advanced Glycation Endproduct Crosslinking in the Cardiovascular System: Potential Therapeutic Target for Cardiovascular Disease. Drugs 2004;**64(5)**:459–70.

Zieman Susan, J., Vojtech, M., Kass David, A. Mechanisms, pathophysiology and therapy of arterial stiffness. American Heart Association 2005;**25(5)**:932–943.

Zolkiewska, A. ADAM proteases: ligand processing and modulation of

the Notch pathway. Cellular and Molecular Life Sciences (CMLS) 2008; **65(13)**:2056–2068.

Zou, Y., Hu, Y., Metzler, B., Xu, Q. Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells (review). Int J Mol Med 1998;**1(5)**:827–34.

Appendix 1

Materials

AGB Scientific (Dublin, Ireland)

Whatmann Chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)

Anti-mouse 2^o antibody, HRP conjugated

Anti-rabbit 2^o antibody, HRP conjugated

ECL Hybond nitrocellulose membrane

ECL Hyperfilm

Rainbow molecular weight marker, broad range (6-175kDa)

Applied Biosystems (Warrington, UK)

Silencer Select GSK-3β siRNA

Axxora (Nottingham, UK)

Anti GSK3β monoclonal IgG

BD Biosciences (Oxford, UK)

BD FACSflow^{TM}

FACS Caliber Flowcytometer

BioRAD (Alpha Technologies, Dublin)

iScript^{TM} cDNA Synthesis Kit

Bio-Sciences Ltd (Dun Laoghaire, Ireland)

DEPC-treated water

Trizol® Reagent

Calbiochem (San Diego, CA)

Anti PCNA mouse monoclonal IgG

Anti phospho-p38 rabbit monoclonal IgG

Anti VEGF(A) rabbit monoclonal IgG

DAPT (γ-secretase inhibitor)

Cell Applications (San Diego, CA)

Cryopreserved Rat Aortic Vascular Smooth Muscle Cells (Passage 2)

Cell Signal (Beverley, MA)

Anti-phospho-GSK3β rabbit monoclonal IgG

Anti-p38 rabbit monoclonal IgG

Anti-p44/42 rabbit monoclonal IgG

Anti-phospho-p44/42 rabbit monoclonal IgG

Dako Cytomation (UK)

Dako mounting media

Dunn Labortechnik GmBH (Asbach, Germany)

6-well Bioflex® plates

Flexcell International Corp. (Hillsborough, NC)

Flexercell® Tension Plus™- FX-4000T™ - system

Invitrogen (Groningen, Netherlands)

Anti goat IgG - Alexafluor 488 conjugate

Anti mouse IgG - Alexafluor 488 conjugate

Anti rabbit IgG - Alexafluor 488 conjugate

DH5α chemically competent cells

Vybrant™ Apoptosis Assay Kit # 2

Vybrant™ CFDA SE Cell Tracer Kit

Lab Vision (California, USA)

UltraVision Detection System Anti-Rabbit, HRP/DAB

Lonza, Cologne, Germany

Basic Nucleofector® Kit for Primary Smooth Muscle Cells (SMC)

MWG Biotech (Milton Keynes, UK)

Notch 1 primer set	HRT3 primer set
Ptc 1 primer set	VEGF primer set
HRT 1 primer set	GAPDH primer set
Gli 2 primer set	GSK-3β primer set
HRT 2 primer set	VEGF siRNA

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemilumescent substrate

Promega (Madison, WI)

Luciferase Reporter Assay Kit

R+D Systems (Abingdon, UK)

Recombinant Shh Protein

Recombinant VEGF Protein

Roche (West Sussex, UK)

Anti HA (HRP conjugated) monoclonal IgG

Santa Cruz (Santa Cruz, California)

Anti HRT 1 goat monoclonal IgG

Anti HRT 2 goat monoclonal IgG

Anti HRT 3 goat monoclonal IgG

Anti Patched goat monoclonal IgG

Sarstedt (Drinagh, Wexford, Ireland)

1.5 ml eppendorf tubes

T25 tissue culture flasks

T75 tissue culture flasks

T175 tissue culture flasks

6-well tissue culture plates

5,10 and 25ml serological pipettes

15 and 50ml falcone tubes

cryovials

Scientific Imaging Systems (Eastman Kodak Group, Rochester, NY)

Kodak 1D image analysis software

Sigma Chemical Company (Poole, Dorset, England)

β-glycerophosphate

β-mercaptoethanol

Acetic Acid

Acetone

Agarose

Ammonium Persulphate

Acrylamide/Bis-acrylamide

Anti α-SMC actin

(mouse monoclonal IgG)

Anti-goat 2^{o} antibody,

(HRP conjugated)

Bovine Serum Albumin

Brightline Haemocytometer

Chloroform

DMEM

DMSO

DPX Mounting Media

EDTA

EGTA

Ethidium Bromide

Foetal Calf Serum
Glycerol
Glycine
Hematoxylin
Hydrochloric acid
Isopropanol
Lauryl Sulphate
Lithium Chloride
(GSK3β inhibitor)
Methanol
PBS tablets
PD098059
(p44/42 inhibitor)
PD169316
(p38 inhibitor)
Penicillin-Streptomycin (100x)
Phosphatase Inhibitor Cocktail
Ponceau S
Potassium Chloride
Potassium Iodide
p-Nitroaniline
Protease Inhibitor Cocktail
RPMI-164040
SB216763
(GSK3β inhibitor)
Sodium Acetate
Sodium Chloride
Sodium Doecly Sulphate
Sodium Hydroxide
Sodium Orthovanadate
Sodium Phosphate
SYBR Green Taq Ready Mix ™
TEMED
Tris Base
Tris Chloride
Tween 20
Triton X-100
Trypsin-EDTA solution (10x)

Stratagene (California, USA)

β-galactosidase Assay Kit

Thermofisher Scientific (Langenselbold, Germany)

White 96-well plates

Appendix 2

Transfection of VSMCs with Lipofectamine

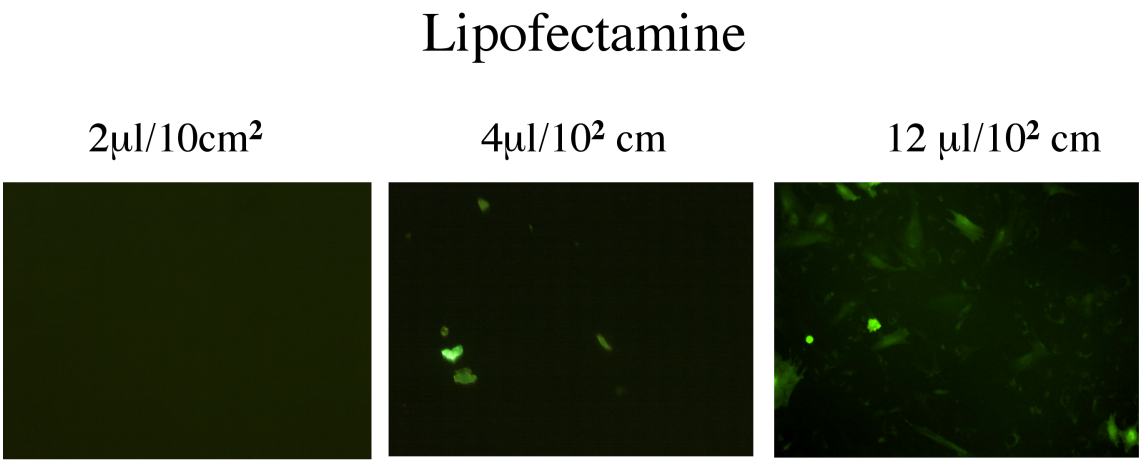

Figure 6.2: Transfection of GFP-Expression Plasmid in VSMCs using Lipofectamine™.

Appendix 3

GSK-3β Knockdown Strategies

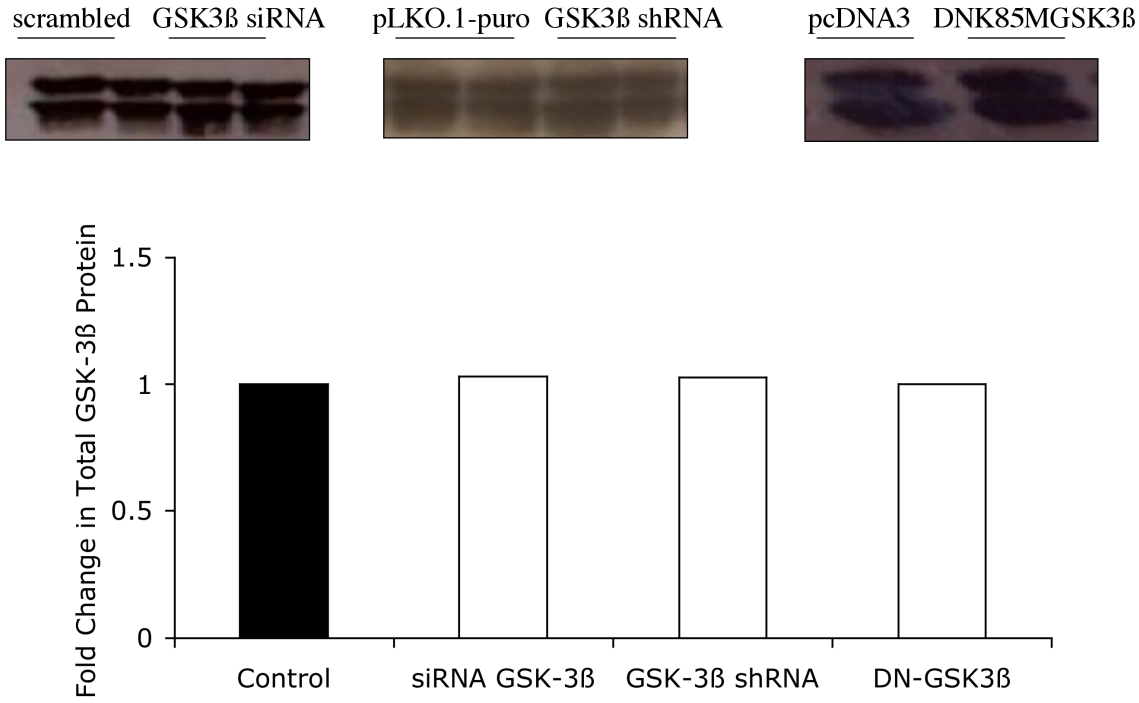

Figure 6.3: GSK-3β Knockdown Stratagies Western blot analysis of GSK-3β in VSMCs following (a) nucleofection of non-commercially designed GSK-3β targeted siRNA siRNA (b) nucleofection of GSK-3β-targed shRNA and (c) nucleofection of DN-GSK-3β plasmid.

Appendix 4

Preparation of Mock Stented Artery in a Novel Vascular Bioreactor

Manufacture of Sylgard® Tube

A Sylgard ® tube, with mechanical properties similar to that of real coronary arteries was manufactured using a custom-made device consisting of a mandrel of 3 mm diameter surrounded co-axially by a plastic tube with internal diameter of 3.3 mm. In brief, Sylgard® was sucked between the co-axial mandrel and tube, which was then placed into an external rig to prevent further deformation. The system was then cured by baking at 120°C. The plastic tube was then peeled off and the cured Sylgard® detached from the mandrel by injection of acetone.

Seeding of BASMCs on MCA

The Sylgard® tubing was initially dipped in a 70% sulphuric acid solution for 60s to create a hyrophilic surface suitable for VSMC adherence. The MCA was cut to a length of 5cm, washed in distilled water and sterilised by autoclaving. The MCA was incubated in fibronectin (8μg/ml) in PBS and rotated for 3h, allowing uniform deposition of the fibronectin. BASMCs were seeded on to the treated MCA and left to attach by rotating for 48hrs in quiescence.

MCA Stenting

A Driver BMS (a cobalt alloy stent with internal diameter of 3mm, and nominal and burst pressure of 9 and 16 atm, respectively) was crimped on an angioplasty balloon catheter of 9mm length. Following

autoclaving, the BMS was deployed inside the MCA under sterile conditions, using a Basix 25 angioplasty inflation syringe (Merit Medical Systems, South Jordan, Utah) to expand the angioplasty balloon.

Preparation and Assembly of the Culture Chamber

The tubing system of the culture chamber was sterilised by ethanol and sterile PBS rinsing for 48 h. All other components of the culture chamber and attachments were sterilised by autoclaving and assembly was carried out in a laminar airflow cabinet. The previously stented and seeded MCA was attached to the culture chamber by luer slips and the extraluminal space of the culture chamber was filled with PBS with 1% primocin. A deformable elastomeric gasket (RTV 102, Radionics) was placed inside a groove around the chamber to ensure sealing. The culture chamber cover was then sealed by a series of screws.

Set-up of CellMax® bioreactor tubing system

The culture chamber was connected to the CellMax® tubing systems (Spectrum Laboratories, Rancho Dominguez, CA) by luer locks (Value Plastic®, Fort Collins, CO) in a laminar airflow cabinet. Media was then perfused through the system by a pulsatile pump. The Cell Max® pump creates a medium flow waveform similar to the stroke of the heart through two pins, which push against the flexible and thin silicon tube. Different pin lengths and pump speed set by an electronic controller allow for different flow rates. For these experiments an inital low flowrate was used for 24 h, to allow the BASMC to adapt to the flow before raising it to a medium flow rate. The system was allowed to run for 7 days before analysis.