The Role of the Notch and Hedgehog Signalling Pathways in Vascular Smooth Muscle Cell Growth

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 programme 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 work.

Signed: David Tomas I.D No.: 51175223

Date: 31/10/06

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Abstract

Vascular Smooth Muscle Cell (SMC) fate decisions are fundamental features in the pathogenesis of vascular disease. We investigated the role of Notch 1 and 3 receptor signalling in controlling adult rat SMC growth *in vitro*. Constitutive expression of active Notch receptors, Notch 1 IC and Notch 3 IC, resulted in significant up-regulation of CBF-1/RBP-Jk-dependent promoter activity and Notch target gene expression concomitant with significant increases in SMC growth. Moreover, inhibition of endogenous Notch mediated CBF-1/RBP-Jk regulated gene expression resulted in a significant decrease in cell growth. Furthermore we examined the specific role of a Hedgehog (Hh)/vascular endothelial growth factor (VEGF) pathway in controlling vascular SMC growth *through* regulation of Notch signalling. We determined that Hh signalling pathway components are constitutively expressed VSMC in culture. Moreover activation of Hh signalling with recombinant Shh resulted in a significant increase in Hh signalling concomitant with an increase in VEGF expression, cell growth and activation of Notch target gene expression in SMC. Inhibition of Hh signalling with cyclopamine resulted in a decrease in Hh signalling concomitant with a decrease in SMC growth while concurrently decreasing Notch target gene expression. Moreover, Shh-mediated stimulation of SMC growth was significantly attenuated following inhibition of Notch target gene expression. In addition, we investigated the role of cyclic strain in modulating Notch/Hh mediated growth of SMC *in vitro*. Rat SMC cultured under the condition of cyclic strain exhibited a temporal and force dependent reduction in Notch and Hh signalling concomitant with a decrease in SMC growth. Furthermore we could reverse this downregulation of Notch/Hh signalling and subsequent decrease in SMC growth by over-expression of Notch 3 IC .To validate these finding we utilized two in *vivo* models of increased biomechanical forces and vascular remodelling where we determined that altered mechanical forces significantly downregulate Notch/Hh signalling concomitant with decreased growth *in vivo*. Collectively, these data suggest that Hh control of SMC fate via VEGF activation of Notch may represent a novel therapeutic target for disease states in which changes in vascular cell fate occur.

Abbreviations

ADAM	A disintegrin and metalloproteinase
AGS	Alagille Syndrome
AIF	Apoptosis-inducing factor
Ang II	Angiotensin II
ANK	Anykrin repeats
AO	Acridine orange
AP	Acid phosphatase
Apaf-1	Apoptosis protease-activating factor-1
ATP	Adenosine tri-phosphate
AV	Annexin V
BART	BamHI-A rightward transcript
BCA	Bicinchoninic acid
bFGF	basic fibroblast growth factor
β-gal	β-galactosidase
bHLH	basic helix-loop-helix
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CADASIL	Cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy
CARD	Caspase recruitment domain
CBF-1	EBV latency C promoter binding factor
cDNA	Complimentary DNA
Ced	Cell death defective
CHF	Cardiovascular helix-loop-helix factor
Ci	Cubitus interruptus
CIR	CBF-interacting repressor
CMV	Cytomegalovirus
c-myc	Cellular-myc
COS2	Costal 2
CR	Cysteine-rich
CRPG	Chlorophenol red-β-D-galactopyranoside

CSL	CBF-1, Suppressor of Hairless, Lag-1
DD	Death domain
DED	Death effector domain
DEPC	Diethylpyrocarbonate
Dhh	Desert hedgehog
DISC	Death-inducing signalling complex
DMEM	Dulbelco's modified Eagle's medium
DMSO	Dimethlysulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside tri-phosphate
ds	Double stranded
DSL	Delta/Serrate/Lag-2
DTT	Dithiothreitol
EBNA-2	EBV nuclear antigen-2
EBV	Epstein Barr Virus
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraaccetic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis-(b-amino-ethylether) N,N,N', N' tetra-acetic acid
ERK	Extracellular-regulated kinase
ET-1	Endothelin-1
EtBr	Ethidium bromide
EU	European Union
ExC	Extracellular
FADD	Fas-associated death domain
FCS	Foetal calf serum
FLIP	Flice inhibitory protein
Fu	Fused
HATS	Histone acetlytransferases
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylase

HERP	Hes-related repressor protein
Hes	Hairy/enhancer of split
HDL	High density lipoproteins
HRT	Hairy related transcription factor
IC	Intracellular
Ihh	Indian hedgehog
IM	Inner membrane
JNK	Jun N-terminal kinase
Kuz	Kuzbanian
LB	Luria-Bertani broth
LDL	Low density lipoproteins
LFA	LipofectAMINE™
LMP-1	Latent membrane protein-1
LNR	LIN12/Notch-related region
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
MEKK	MEK kinase
MEKKK	MEKK kinase
MKP-1	MAPK phosphatase-1
MLV	Murine leukaemia virus
N-CoR	Nuclear receptor co-repressor
OM	Outer membrane
ox-LDL	Oxidized-low density lipoproteins
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF-R	Platelet-derived growth factor-receptor
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C

pNA	pNitroaniline
PS	Presenilin
P/S	Penecillin streptomycin
PTB	Phosphotyrosine-binding
Ptc	Patched
PTX	Pertussis toxin
PVL	Portal vein ligation
RBP-Jκ	Recombination signal binding protein of the Jκ immunoglobulin gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Reverse transcriptase
RVSMC	Rat vascular smooth muscle cells
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
Shh	Sonic hedgehog
SKIP	Ski-interacting protein
Smo	Smoothened
SMRT	Silencing mediator for retinoid and thyroid receptor
SMC	Smooth muscle cells
SSB	Sample solubilization buffer
ss	Single-stranded
ST	Sub-transmembrane domain
Su(Fu)	Suppressor of Fused
SuH	Suppressor of Hairless
TACE	TNF-α converting enzyme
TAD	Transcriptional activator domain
TAE	Tris acetate EDTA
TE	Tris-EDTA
TEMED	N, N, N', N' - Tetramethylethlylenediamine
TF	Transcription factor

TGF-β	Transforming growth factor-β
TLE	Transducin-like enhancer of split
TM	Transmembrane domain
TNF	Tumour necrosis factor
UV	Ultraviolet
VEFG	Vascular endothelial growth factor
VEGF-R	VEGF-receptor
VLDL	Very low density lipoproteins
VSMC	Vascular smooth muscle cells

Units

A/U	Arbitary units
bp	Base pairs
cm	Centimetre
°C	Degrees celsius
kDa	KiloDaltons
µg	Microgram
µl	Microlitre
g	Grams
h	Hours
kg	Kilogram
L	Litre
M	Molar
mA	Milliamperes
mg	Milligrams
mHz	Millihertz
min	Minutes
ml	Millilitres
mM	Millimolar
ng	Nanograms
nm	Nanometres
sec	Seconds
V	Volts
W	Watts
x g	G force

Publications

Papers:

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Morrow, D., Scheller, A., Sweeney, C., Birney, Y., Guha, S., Cummins, P.M., Murphy, R., Walls, Redmond, E.M., & Cahill, P.A. Notch Mediated CBF-1/RBP-Jκ-dependent Regulation of Human Vascular Smooth Muscle Cell Phenotype in Vitro. (2005) *Am J Physiol.* 0: 1982005

Morrow, D., Sweeney, C., Birney, Y., Cummins, P.M., Walls, D., Redmond, E.M., & Cahill, P.A. (2005) Cyclic Strain Inhibits Notch Receptor Signaling in Vascular Smooth Muscle Cells. (2005) *Circ Res.* 96: 567-575.

Sweeney, C., Morrow, D., Birney, Y., Hennessey, C., Coyle, S., Scheller, A., Cummins, P.M., Walls, D., Redmond, E.M., & Cahill, P.A (2004) 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. 18(12):1421-3

Abstracts:

Morrow, D., Sweeney, C., Birney, Y., Guha, S., Cummins, P.M., Walls, D., Redmond, E.M., & Cahill, P.A. Sonic Hedgehog Regulates Vascular Smooth Muscle Cell Fate *In Vitro* Through VEGF Activation of Notch Signaling. *Circulation* 2005

Birney, Y., Morrow, D., Sweeney,C., Guha., S., Cummins, P., Redmond, E., Walls, D., and Cahill, PA.Notch Signalling Regulates Migration in Vascular Smooth Muscle Cells *in vitro. Circulation* 2005

Morrow, D., Sweeney, C., Birney, Y., Guha, S., Cummins, P.M., Walls, D., Redmond, E.M., & Cahill, P.A. Hedgehog regulates Vascular Smooth Muscle Cell Fate Decisions *in vitro* ATVB conference Washington DC, May 2005

Morrow, D , Sweeney, C., Birney, Y , Redmond, E.M and Cahill, PA. The Effect of Hrt1-3 on cell fate decisions American heart Conference New Orleans, Nov 2004

Birney, Y., Sweeney, C., Morrow, D., Scheller, A., Cummins, P., Redmond, E.,Walls, D., and Cahill, PA (2004). [3]The Role of Notch Signalling in Vascular Smooth Muscle Cell Migration[2]. IVBM, Toronto, Canada. Cardiovascular Pathology 13(3) S131:362

Sweeney, C., Morrow, D., Scheller, A., Birney, Y., Redmond, E., Walls, D., and Cahill, PA (2003). [3]Notch Signalling and Cyclic Strain-induced Regulation of Vascular Smooth Muscle Cell Proliferation and Apoptosis[2]. DVB, California, USA. Endothelium 10(6) 347.

Morrow, D., Sweeney, C., Scheller, A., Birney, Y., Cummins, P., Redmond, E., Walls, D., and Cahill, PA (2003). [3]The Role of Notch Signalling in Vascular Smooth Muscle Cell Fate Decisions[2]. DVB, California, USA. Endothelium 10(6) 356.

Sweeney, C., Morrow, D., Scheller, A., Birney, Y., Walls, D., Redmond, E., and Cahill, PA (2003). [3]Notch Signalling and Cyclic Strain-induced Regulation of

Vascular Smooth Muscle Cell Proliferation and Apoptosis[2]. Irish Association of Pharmacology, 13th Annual Meeting, Killiney, Dublin, Nov 2003.

Sweeney, C., Morrow, D., Scheller, A., Birney, Y., Walls, D., Redmond, E., and Cahill, PA (2003). [3]Notch Signalling and Cyclic Strain Induced Changes in Vascular Cell Fate[2]. 4th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology, Washington DC, USA, May 2003. Supplement to Arterioscler Thromb Vasc Biol. 2003; 23(5): P304

Scheller, A., Morrow, D., Sweeney, C., Walls, D., Cummins, P., Redmond, E., and Cahill, PA (2003). [3]Notch Signalling in Vascular Smooth Muscle Cell Differentiation[2]. 4th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology, Washington DC, USA, May 2003. Supplement to Arterioscler Thromb Vasc Biol. 2003; 23(5): P476

Morrow, D., Sweeney, C., Scheller, A., Walls, D., Cummins, P. and Cahill, PA (2003). [3]Notch Receptor Ligand Signalling in Vascular Smooth Muscle Cell Proliferation[2]. Federation of American Societies for Experimental Biology (FASEB), San Diego, USA, Apr 2003. FASEB Journal of Abstracts 7208.

Table of Contents

Chapter 1

Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a term, which describes diseases of the heart and blood vessels. The common types include angina, heart attack (myocardial infarction), diseases of the heart-valves or heart muscle (cardiomyopathy), heart failure, stroke and pain from poor blood flow to the legs (claudication). Overall, CVD is the most common health problem for Irish citizens where vascular diseases currently account for over 40% of all deaths and of this, ischemic heart disease is by far the most, accounting for 25% of all deaths. There have been major improvements in recent years and death rates are about 50-70% lower at any age than 30-35 years ago (World Health Organisation). However, the number of people affected in Ireland has remained the same, although the problem now just occurs at an older age. The single most common cause of these diseases is atherosclerosis, which is the "hardening" of the arteries. Many risk factors can contribute to this condition such as elevated cholesterol levels, particularly low density lipoproteins (LDL), elevated blood triglyceride levels, smoking, high blood pressure, diet, lifestyle, obesity and stress. In addition, other factors in the development of CVD can include medical history, genetic influences, age and ethnicity. At present about 80% of Irish people have at least one of these risk factors.

The pathogenesis of many cardiovascular diseases involves changes in structure, function and integrity of arterial blood vessels. The arterial blood vessel is an active integrate organ composed of endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts, which are divided into 3 structural layers termed the tunicas intima, tunica media and adventitia. The tunica intima comprises of a simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibres. Underlying this is the tunica media, which is comprised primarily of SMC. It is the SMC which play a major role in maintaining vascular tone and function. Endothelial cells function as a semi-permeable barrier and also as a dynamic paracrine and endocrine organ, exerting considerable influence on the underlying vascular smooth muscle cells (VSMC).

Endothelial cells are also involved in maintaining the non-thrombogenic blood –tissue interface by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow.

Figure 1: Structure of an Artery and a Vein

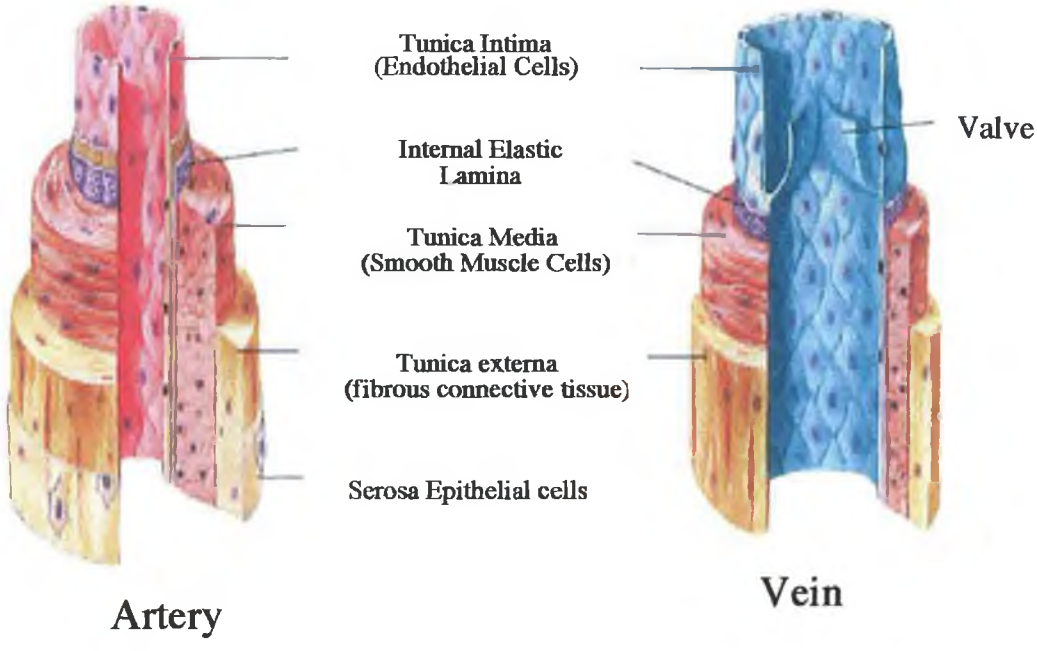


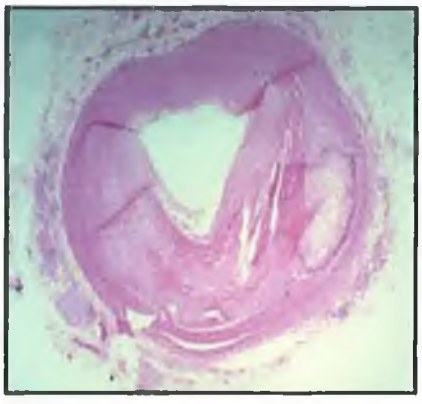
Figure 1: Structure of an Artery and a Vein: Diagrammatic representation of Arterial and Venous blood vessel structure, indicating different structural layers.

1.2 Atherosclerosis

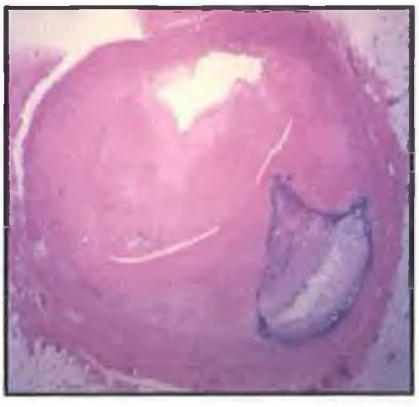
Atherosclerosis is a systemic disease, which involves the intima of large and medium arteries including the aorta, the carotid and the peripheral arteries. A dysfunctional endothelium, chronic inflammation, lipid accumulation and aberrant regulation of VSMC fate decisions (proliferation, apoptosis, differentiation and migration) can clinically manifest as an atherosclerotic plaque. An obstructive coronary plaque can cause a critical reduction in coronary blood flow and subsequently lead to myocardial infarction (Schroeder and Falk *et al.*, 1995).

Figure 2: Atherosclerotic Vessels

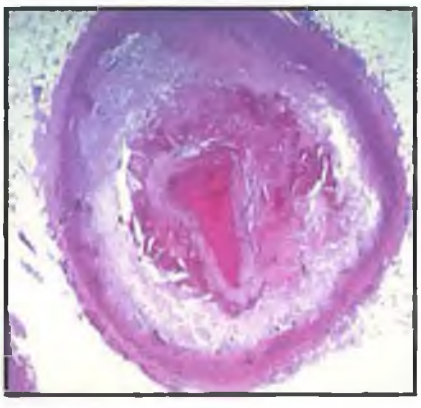
1) This is a normal coronary artery. The lumen is large, without any narrowing by atheromatous plaque. The muscular arterial wall is of normal proportion.



2) The coronary artery shown here has narrowing of the lumen due to build up of atherosclerotic plaque. Severe narrowing can lead to angina, ischemia, and infarction.



3) There is a severe degree of narrowing in this coronary artery. It is "complex" in that there is a large area of calcification on the lower right, which appears bluish in this H&E stain.



4) There is a pink to red recent thrombosis in this narrowed coronary artery. The open, needle-like spaces in the atheromatous plaque are cholesterol clefts. (Internet Pathology library)

Atherosclerosis involves multiple processes including endothelial dysfunction, inflammation, vascular proliferation, apoptosis and matrix alteration. The contribution of vascular proliferation to the pathophysiology of in-stent restenosis, transplant vasculopathy and vein by-pass graft failure is particularly important. Thus, an emerging

strategy for the treatment of these conditions is to inhibit cellular proliferation by targeting cell-cycle regulation. Hence a greater understanding of the signalling pathways and mechanisms that can modulate changes in proliferation, provides new

perspective for preventative and therapeutic strategies. A key process of atherosclerosis involves the proliferation of VSMCs (Ross *et al.,* 1995 and Schwarz *et al.,* 2000). One precursor of lesion development is thought to be focal accumulation of VSMCs within the intima. VSMC may contribute to the development of the atheroma through the production of pro-inflammatory mediators such as monocyte chemoattractant protein 1 and vascular cell adhesion molecule and through the synthesis of matrix molecules required for the retention of lipoproteins. However, VSMC may also be important in maintaining the stability of the plaque through the formation of a fibrous cap. In addition, in lipid–laden lesions in which the fibrous cap is thin and weak, there is evidence of apoptosis, particularly at the "shoulder" region associated with inflammation (Fuster *et al.,* 1994). The local inflammatory milieu can induce expression of proteolytic inhibitors, thus rendering the fibrous cap weak and susceptible to rupture (Schwarz 2000). However, in advanced lesions, fibroblasts and VSMCs with extracellular calcification form a fibrocalcific plaque.

Apoptosis has been observed at many stages of the development of an advanced plaque (Kockx *et al.,* 1998). Apoptosis is important in the progression of atherosclerotic lesion resulting in the formation of a mature lesion containing a dense extracellular matrix (ECM) and a relatively sparse population. Apoptosis is concentrated in the lipid-rich core of the plaque and occurs in both macrophage/foam cells and VSMCs. However, this high percentage of apoptosis in the lesion does not translate to a decrease in tissue volume, therefore it is postulated that the system of phagocytosis of apoptotic cells operates poorly in an atherosclerotic plaque. A possible reason for this is that the intracellular accumulation of lipids may decrease the ability of macrophages and SMC to phagocytose apoptotic cells. Moreover, the increase in apoptosis of macrophages in the lesion may decrease the population of apoptotic scavenging cells.

The function of the intimal SMC in the natural history of the atherosclerotic lesion seems to be to act as a nidus for development of the lesions, perhaps by accelerating lipid accumulation or macrophage chemotaxis. VSMC proliferation is more than likely an early event followed by a chronic process that provides an essential fibrous

cap that prevents plaque rupture. Most thrombus formations are due to a fracture in the protective fibrous cap of the atherosclerotic plaque, which usually occurs at the shoulder region. Therefore it is the integrity of the fibrous cap that fundamentally determines the stability of a plaque and its clinical implications. It is now well established that the fibrous cap can undergo continuous remodelling, which is largely influenced by VSMC (Libby *et al.,* 1995). Therefore apoptosis of VSMC and subsequent reduction in cell number can seriously compromise the integrity of the fibrous cap. With the realization that this probability of atherosclerotic plaque rupture, rather than the severity of plaque stenosis is what determines its clinical complications, this has led to the classification of plaques as either stable or vulnerable. Furthermore, with most myocardial infarctions occurring in lesions with less than 70% stenosis, it is plaque rupture which is believed to be the underlying pathological event (Falk *et al.,* 1995). Moreover, with the recognition of the essential involvement of VSMC proliferation and apoptosis in the conditions mentioned here, an improved understanding of the molecular mechanisms of VSMC proliferation and apoptosis has become a major focus of research and development.

1.3 Proliferation

The proliferation of VSMCs plays a crucial role in the formation of vascular lesions, such as fibrous plaques in atherosclerosis as described in the previous section and intimal thickening after balloon angioplasty (Wang *et al.,* 2002). Accelerated VSMC proliferation is also a characteristic feature in arteries of hypertensive patients and animals. Therefore, the inhibition of VSMC proliferation represents a potentially important therapeutic strategy for the treatment of diseases such as atherosclerosis and restenosis. All mitogenic growth factors share a final common signalling pathway; the cell cycle. The eukaryotic cell cycle is regulated by cyclins, cyclin-dependent kinases (Cdks) and their inhibitors.

Figure 3: Eukaryotic Cell Cycle

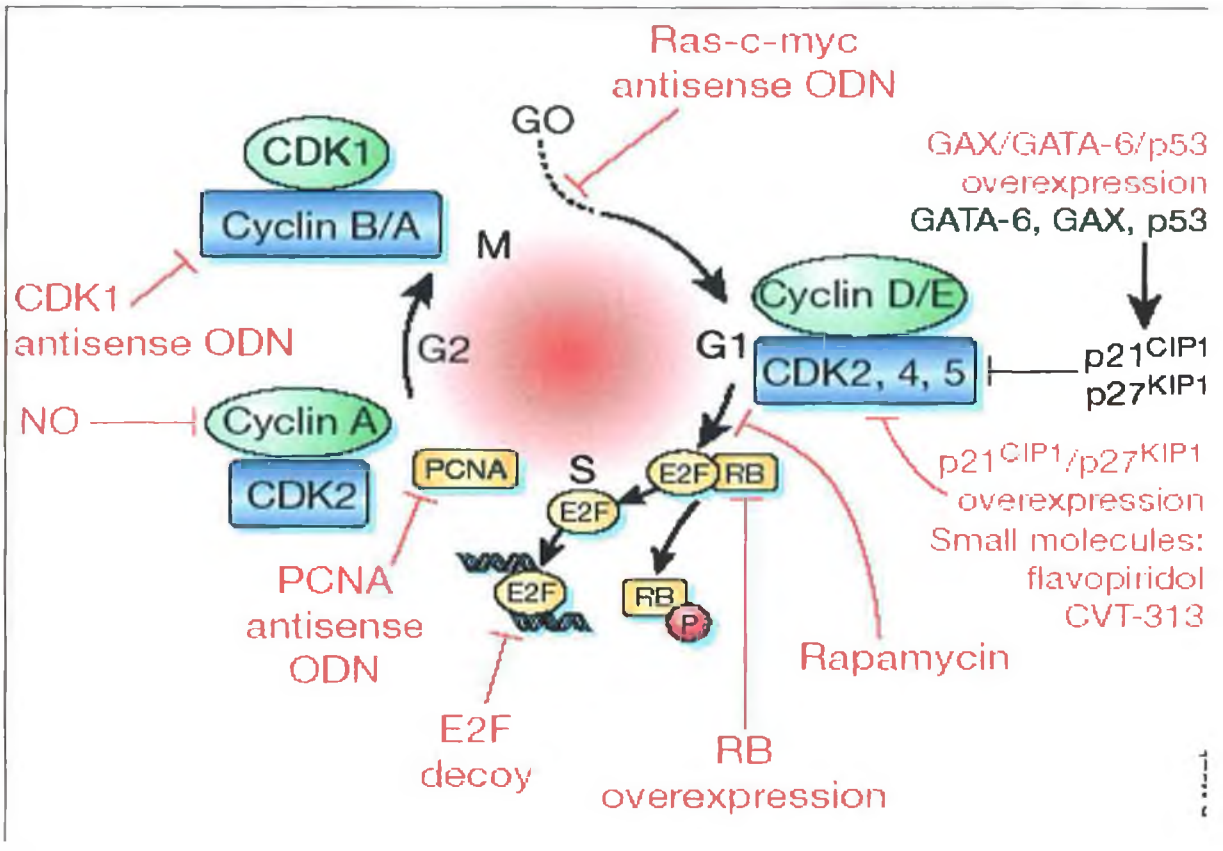


Figure 3: The Cell Cycle: Diagrammatic representation of the Cell Cycle

Quiescent (G_o) cells enter a gap period (G1), during which the factors necessary to DNA replication for the subsequent (S) phase are assembled. After DNA replication is completed, the cells enter another gap phase (G2) in preparation for mitosis (M). Restriction points at the G1-S and G2-M interphases ensure orderly cell cycle progression (Elledge *et al.*, 1996). Cell cycle phases are co-ordinated by CDKs that form holoenzymes with their regulatory subunits, the cyclins. Cyclin-CDK complex activity is dependent on the phosphorylation status of the CDKs and the expression levels of the cyclins. Phase- specific cyclin-CDK complexes confer specificity and orderly progression through the cell cycle. Initial increased accumulation of cyclin D-CDK4 and cyclin E-CDK2 complexes, in co-operation with proliferating cell nuclear antigen (pCNA), co-ordinates DNA replication by regulating the transition through the G1 and S phases (Sherr *et al.*, 1995). The subsequent G2-M transition is regulated by cyclin A-CDK-2 and cyclin B-CDK1 complexes. In addition, cell cycle progression is regulated by CDK inhibitors such as $p27^{KIP1}$ and $p21^{CIP1}$, which can bind

to CDKs and prevent their activation. Antimitogenic signals can also activate transcription factors such as p53, which induce expression of CDK inhibitors and consequently inhibit result in G1-phase arrest. Conversely the E2F family of transcription factors controls expression of genes in the S-phase. In quiescent conditions, E2F members exist in inactive complexes with retinoblastoma protein (RB). However after mitogenic stimulation, the cyclin D-CDK4 and cyclin E-CDK2 complexes hyper-phosphorylate RB, This leads to the dissociation of E2F, which in turn activates the expression of genes which encode cyclins E and A and CDK1. GAX and GATA-6 are also integral cell-cycle associated transcription factors in VSMCs as GAX, which regulates differentiation, migration and proliferation, is expressed in quiesced VSMCs (Smith *et al.,* 1997). Both GAX and GATA-6 stimulate $p21^{CIP1}$ expression and induce cell cycle arrest. Nitric oxide also has the ability to inhibit proliferation by upregulation of $p21^{CIP1}$ (Ishida *et al.,* 1997). With the knowledge that GAX, GATA-6 and nitric oxide all have antiproliferative effects, it is not surprising therefore that they may prove attractive therapeutic targets for vascular diseases caused by aggressive VSMC proliferation.

1.4 Apoptosis

1.4.1 Introduction

Apoptosis, or programmed cell death, is recognized as an important physiological process, both during development and in the maintenance of homeostasis in the adult. This mode of cell death allows for the removal of damaged, injured, infected and incompetent cells from the body in both a quick and efficient manner. There are two forms of cell death, which are characterized depending on the context and cause of death. These two forms of cell death which are termed apoptosis and necrosis are defined and contrasted on their individual mechanisms, biochemistry, and altered cellular morphology (Hetts 1998). Firstly, apoptosis is an active, contained process, which results from stimuli from either an internal or external source (Kuan and Passaro, 1998). The apoptotic process is characterized by cell shrinkage and subsequent membrane blebbing, chromatin condensation around the nuclear membrane, and cleavage of the DNA into regular repeating 180-200 base pair

units (Yeh, 1997). This then results in the formation of "apoptotic bodies" due to the cleavage of the membrane. These apoptotic bodies are then phagocytosed and digested by macrophages or neighbouring cells. Since there is no release of cytosolic components into the extracellular space, no inflammatory response is initiated (Hetts, 1998). Apoptosis-inducing stimuli can be either extrinsic or intrinsic and can result in apoptosis through the activation of a number of pathways. However in most cases, these pathways converge on the caspase system of enzymes to execute their final function. Extrinsic triggers of apoptosis include the activation of receptor-mediated death signalling pathways. This can include for example the activation of the Fas ligand, exposure to DNA damaging substances such as chemotherapeutic agents and ionizing radiation (Rich *et al.*, 2000). In addition apoptosis can be induced due to the removal of death-inhibiting ligands. VSMCs for example can undergo apoptosis due to the removal of growth factors such as insulin-like growth factor and PDGF (Best *et al.*, 1999). Intrinsic signals on the other hand include increased oxidative stress, which lead to the initiation of apoptosis within the cell (Designer and Martinou, 2000).

The second well-characterized form of cell death is necrosis. In contrast to apoptosis, necrosis is a form of passive cell death. Necrosis results from external noxious stimuli and is pathologic in that localized injury and inflammation is produced. Necrosis is characterized by severe cell swelling, breakdown of the membrane barrier and a resulting release of the cellular components into the extracellular space with random degradation of nuclear DNAs. The release of extracellular kinins results in a localized inflammatory response, edema, capillary dilation and macrophage aggregation (Kuan and Passaro, 1998). An example of necrosis can include ischemic necrosis of the cardiomyocyte during acute myocardial infarction (Yeh, 1997), however although necrosis may be important in acute injury and in certain acute inflammatory responses, it is not the normal mechanism for cell death.

1.4.2 Receptor-Mediated Death Signaling Pathways

Death receptors belong to the tumour necrosis factor (TNF) superfamily and are responsible for playing a central role in instructive apoptosis (Ashkenazi and Dixit, 1998). Members of the death receptor family contain one to five cysteine–rich repeats in their extracellular domain, and a death domain (DD) in their cytoplasmic tail. This DD is essential for the initiation of the apoptotic signal by these receptors. Two such receptors that initiate apoptosis include TNF receptor 1 and Fas. These receptors initiate apoptosis once activated by their respective ligands, TNF-α and Fas–L, or by agonist-like antibodies (Maclellan and Schneider, 1997; Gupta, 2003). Following receptor-ligand interaction, the receptor oligomerizes, recruits adaptor molecules, which forms a death inducing signalling complex (DISC). This in turn recruits and activates the caspase cascade and can culminate in apoptosis of the cell (Yeh, 1997; Gupta, 2003).

1.4.3 Apoptosis due to DNA Damage

Apoptosis, growth arrest and repair are all legitimate cellular responses to DNA damage. The choice of cell fate in each instance depends on cell type, location, environment, and extent of the damage. p53 is a transcription factor that has been implicated in cell cycle arrest and in some forms of apoptosis (Maclellan and Schneider, 1997). Under normal conditions, levels of p53 remain at a low level, which is due to its interaction with the Mdm-2 protein, which marks it for ubiquitin-mediated destruction (Mayo *et al.,* 1997). Phosphorylation of either p53 or Mdm-2 by DNA damage prevents the interaction of these two proteins. This leads to the stabilization and subsequent activation of p53 (Evan and Littlewood, 1998). As a result of DNA damage, p53 levels increase within minutes and subsequent growth arrest or apoptosis of the cell can occur (Lundberg and Weinberg, 1999). Several cell-cycle regulators can be induced by p53. These include the cyclin-dependent kinase inhibitor p21, GADD 45 and members of the 14-3-3 family all of which result in growth arrest followed by either DNA repair or cell death (Rich *et al.,* 2000). However, p53 alone will not induce apoptosis, but acts as a transcription factor activating the expression of numerous apoptosis-mediating genes. These can include

Fas molecules as well as members of the Bcl-2 family of apoptosis regulators. Increased levels of p53 result in the up-regulation of Fas expressed on the cell surface, thus rendering the cell susceptible to increased levels of Fas-mediated apoptosis. In addition, p53 induces apoptosis by increasing the expression of a pro-apoptotic member of the Bcl-2 family, Bax, whilst limiting the expression of anti-apoptotic Bcl-2 protein within the cell. The regulation of p53 expression has also been linked to cellular myc (c-myc) and E1A proteins, which have been implicated in the regulation of apoptosis and proliferation (Evan and Littlewood, 1998).

Figure 4: Central Role of p53 in Controlling Cell Growth and Apoptosis

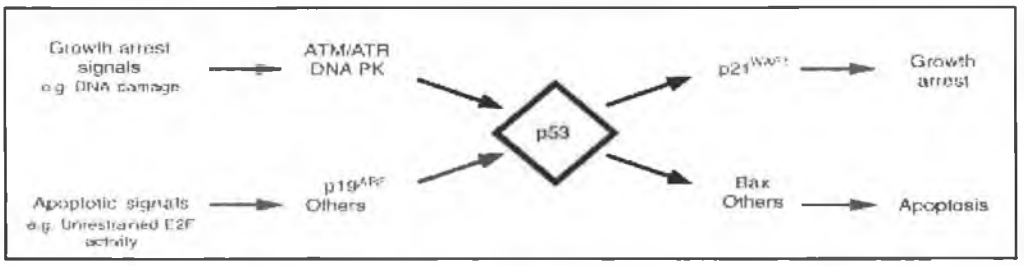


Figure 4: Schematic representation of the central role of p53 in the regulation of cellular growth and apoptosis (Lundberg and Weinberg 1999).

1.5 Effectors of Apoptosis

1.5.1 Caspase cascade

Caspases, which are viewed as the "central executioners" in apoptosis, are an evolutionary conserved family of cysteine proteases. Caspases are synthesized as enzymatically inert zymogens, which require proteolytic cleavage at an internal aspartate residue to induce their activation (Gibbons and Pollman, 2000). These zymogens are composed of three domains, an N-terminal pro-domain, and two domains termed p10 and p20. The activation of caspases results in a serial sequence of caspase activation, referred to as the caspase cascade, which commonly ends in apoptosis. Caspase activation generally occurs by three mechanisms. These include proximity-induced activation, processing by an upstream caspase, and association

with a regulatory subunit (Hengartner, 2000). Proximity-induced activation occurs in the case of caspase 2 and 8. The aggregation of a number of caspase proteins via adaptor proteins renders the caspases capable of auto–proteolytic cleavage and subsequent activation (Hengartner, 2000). In addition, activation of the initiator caspases 8 and 9,results in subsequent cleavage and activation of downstream effector caspases such as caspase 3, caspase 6 and caspase 7. It is these effector caspases, which are responsible for the induction of the biomechanical and morphological changes that are associated with apoptosis.

Caspase 9 is activated through the association with a regulatory subunit, known as an apoptosome. The apoptosome consists of cytochrome c, an adapter molecule Apaf-1 (apoptosis protease-activating factor), and pro-caspase 9 (Gupta, 2003).

Cytochrome c release is an essential component for the formation of the apoptosome, and the subsequent activation of caspases 9 and 3 (Liu *et al.*, 1996). Apaf-1, another essential component of the apoptosome, appears to be activated by p53 and E1A (Fearnhead *et al.*, 1998; Moroni *et al.*, 2001). Apaf-1 has an N-terminal caspase recruitment domain (CARD), adjacent Walker's A- and B-box sequences, and c-terminal to this, twelve WD-40 repeats (Cai *et al.*, 1998). Following binding of cytochrome c to these WD-40 repeats, a conformational change of Apaf-1 occurs, thus exposing the CARD domain. Pro-caspase 9 subsequently binds to the CARD domain, resulting in its activation. Caspase 9 subsequently cleaves and activates caspase 3, caspase 6 and a number of other substrates resulting in the biochemical and morphological changes associated with apoptosis. These substrates include laminins, cytoskeletal proteins and caspase-activated DNase (CAD) among others. It is this active nuclease, which is subsequently responsible for the characteristic "DNA laddering" of apoptosis. Cleavage of cytoskeletal proteins such as fodrin and gelsolin result in overall loss of cell shape. Nuclear laminin cleavage is responsible for the characteristic nuclear shrinkage and budding seen in apoptosis

A number of caspase-independent inducers of apoptosis have also been identified as central to this process. For example reactive oxygen species (ROS) are involved in mitochondrial permeability and release of molecules other than cytochrome c which are integral in the execution of apoptosis (Suzuki *et al.*, 1997) AIF, apoptosis inducing factor is one such molecule that is released from the mitochondria that can induce caspase-independent apoptosis. Once transported to the

nucleus, AIF can cause ATP- independent large DNA fragmentation and chromatin condensation (Gupta 2003).

1.5.2 The Bcl-2 Family

The Bcl-2 family are considered the primary regulators of mitochondria-induced apoptosis, controlling mitochondrial membrane permeabilization and cytochrome c release (Thompson 1995; Desagher and Martinou 2000; Marsden *et al.*, 2002). There has been at least 15 member of the Bcl-2 family identified, and these are divided into two functional groups, pro-apoptotic and anti-apoptotic Bcl-2 family members. Examples of pro-apoptotic family members include Bax, Bad, Bik and Bid, whilst examples of anti-apoptotic family members include Bcl-x_L, Bcl-2 and Bfl-1. Structural analysis of the Bcl-2 family of proteins has identified four conserved regions within the family, known as Bcl-2 homology domains (BH1-BH4) (Muchmore *et al.,* 1996). All Bcl-2 family members contain at least one of these domains, which are formed by α-helices and thus enable different members of the family to form either homo- or heterodimers and regulate each other (Oltvai *et al.,* 1993). In addition the majority of Bcl-2 family members share sequence homology at the c-terminal region, with a -20 residue hydrophobic domain, which targets the Bcl-2 family of proteins to intracellular membranes, most principally the mitochondrial membrane. It is the variable sequence homology, which exists between the BH1 to BH4 domains, which determine whether a family member acts to promote or prevent cell death (Kirshenbaum 2000). The BH4 domain is restricted to anti-apoptotic family members thus making this particular domain fundamental in preventing apoptosis. Hunter *et al.,* 1996 carried out a number of studies whereby deletion of the BH4 domain resulted in anti-apoptotic Bcl-2 protein being defective in suppressing apoptosis.

Table 1: Inhibition and Promotion of Apoptosis by Bcl-2 Family Proteins

Protein	Effect on Apoptosis	Protein-Protein Interactions
Bcl-2	↓	Bax, Bak
Bcl-x_L*	↓	Bax, Bak
Bcl-W	↓	
Bax	↑	Bcl-2, Bcl-x_L
Bad	↑	Bcl-2, Bcl-x_L
Bak	↑	Bcl-2, Bcl-x
Bcl-x_S*	↑	Bax, Bak

*Alternatively spliced. ↑ increases, ↓ decreases.
Table 1: Pro- and anti-apoptotic members of the Bcl-2 family of proteins
Table adapted from Mc Lellan and Schneider, 1997.

Cell fate is determined by the ratio of pro-and anti-apoptotic members of the Bcl-2 family within any given cell (Reed, 1997). Furthermore the Bcl-2 family can delay or prevent apoptosis by a diverse number of death signals, again highlighting their potential influence over whether a cell lives or dies. It is however through the regulation of mitochondrial potential and corresponding cyctochrome c release where the Bcl-2 family primarily exert their pro- or anti-apoptotic influence. Once apoptosis has been stimulated, many pro-apoptotic Bcl-2 family members translocate from the cytoplasm to the mitochondria. These proteins can then disrupt membrane integrity by inserting into the mitochondrial membrane and causing a conformational change. This disruption in mitochondrial membrane integrity and subsequent increase in membrane potential, results in the release of several mitochondrial proteins fundamental to caspase activation and other apoptotic events (Zamzami and Kroemer, 2001). In contrast many of the anti-apoptotic Bcl-2 family members are associated with the mitochondrial membrane, where they act to inhibit increases in mitochondrial membrane potential, and hence prevent apoptosis by maintaining membrane integrity.

Both pro- and anti-apoptotic Bcl-2 family members can also, in part regulate each other. Bcl-2 can form a heterodimer with Bax, thus inhibiting the ability of Bax to increase mitochondrial membrane potential. Similarly, pro-apoptotic members can exert their effect by binding to their anti-apoptotic counterparts. Bad has the ability to bind to Bcl-x_L for example and hence inhibit its anti-apoptotic function (Ferri and Kroemer, 2001). The importance of both pro-apoptotic Bax and anti-apoptotic Bcl-x_L will form an integral part of this study as we investigate the direct effect of both the Notch and Hedgehog signalling pathways on their regulation.

Figure 5: The Major Apoptotic Pathways in Mammalian Cells

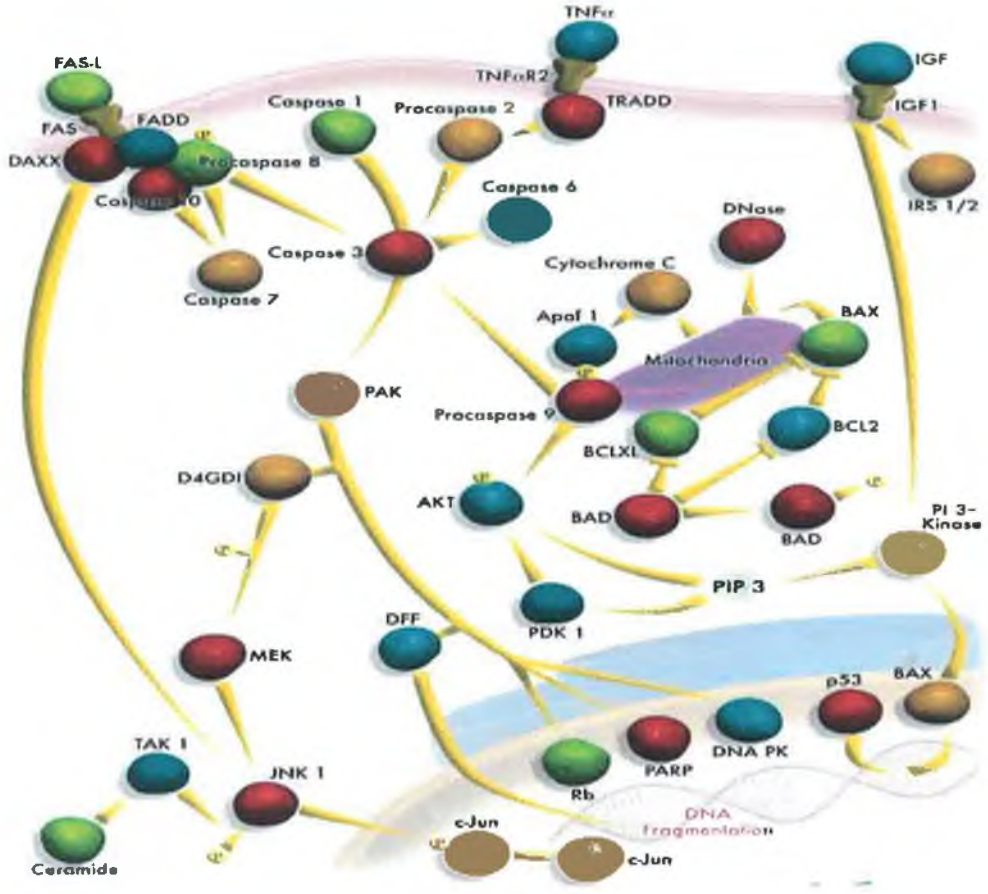


Figure 5: Diagrammatic representation of the major Apoptotic pathways in mammalian cells. (Upstate)

1.6 Mechanical Forces

1.6.1 Introduction

Haemodynamic forces play an important role in maintaining cardiovascular homeostasis. These forces are known to exert both physiological and non-physiological effects on the vascular wall and play a critical role on the regulation of vascular tone, remodelling and influencing vascular cell homeostasis (Li and Xu, 2000). However, haemodynamic forces can also be pathophysiological factors in conditions such as atherosclerosis. The involvement of these haemadynamic forces in atherogenesis is manifested by the focal distribution of atherosclerotic lesions in the bifurcations and curved regions of the arterial tree where blood flow is disturbed with flow separation (Li *et al.,* 1997). In contrast, venous vessels do not develop atherosclerosis when maintained in their normal low-pressure environment, however atherosclerosis can be observed following arterial vein grafts due to increased biomechanical force on the venous vessel (Xu, 2000). Haemadynamic forces associated with blood flow have also been implicated in the regulation of VSMC fate. It is alterations in VSMC fate decisions, which have been associated with pathogenesis of numerous vascular disease states. For example increased proliferation of VSMC is central to the pathogenesis of hypertension, intimal hyperplasia, atherosclerosis and the arterial response to injury (Vinters and Berliner, 1987; Thubrikar and Robicsek, 1995; Traub and Berk, 1998). Furthermore increased apoptosis has been proven to have a direct relationship with increased levels of haemadynamic forces. Analysis of atherosclerotic lesions in both human and animal models have revealed high levels of VSMC apoptosis (Kockx, 1998; Mayr and Xu, 2001). Haemodynamic forces have been implicated in alterations in VSMC phenotype, resulting in an altered response of the cells to mechanical forces (Cappadona *et al.,* 1999) and subsequent vascular remodelling. Therefore, since altered haemadynamic forces play such a critical role in the pathogenesis of vascular disease, a greater understanding of the effect of these forces on VSMC fate and the subsequent pathways that govern these changes in cell fate, proves a major target in the fight against cardiovascular disease.

1.6.2 Haemodynamic Forces

There are two types of haemodynamic forces, which a normal vessel is exposed to. Firstly, a circumferential stretch acting tangentially on the vascular wall and directly related to pressure and dimensions of the vessel and secondly, a shear stress, acting longitudinally at the blood/endothelium interface which is related to the velocity of flow. Both of these factors are essential for the maintenance of a healthy vessel. Blood pressure is described as the force that the circulating blood exerts on the walls of the arteries. It is the major determinant of vessel stretch, which involves the rhythmic distension of the vessel wall. Blood pressure creates strain on the vessel wall in a direction perpendicular to the endoluminal surface. These forces are counterbalanced by intraparietal tangential forces in longitudinal and circumferential directions exerted by different elements of the vessel wall, opposing the distending effects of blood pressure. All elements of the arterial wall are exposed to circumferential tension, each layer bearing differing degrees of this tension. In addition, blood flow exerts a frictional force on the luminal surface of the endothelium. This frictional drag is referred to as shear stress and is defined in terms of blood viscosity and velocity.

Figure 6: Haemodynamic Forces Experienced by Arterial Blood Vessels

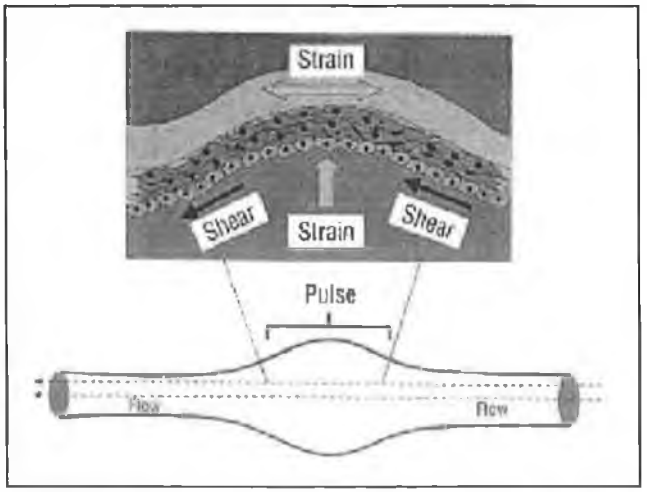


Figure 6: Haemodynamic Forces Experienced by Arterial Blood Vessels. Diagrammatic representation of the force exerted by shear stress and cyclic strain on arterial blood vessels (Davis *et al.*, 1995).

The relationship between circumferential stress and the structure of the vessel wall has been well established. Increases in arterial pressure are associated with SMC hypertrophy and increases in ECM production. Conversely decreases in arterial pressure result in vessel atrophy (Bomberger et al., 1980). In addition, continual mechanical stimulation appears to be essential to maintaining a contractile phenotype in SMC. Whilst a certain level of stretching may also be essential for SMC maintenance, for over stretching may initiate adaptive processes (Lehoux et al., 1998). The fact that endothelial cells are the principal recipients of shear stress does not imply that mechanical stretch has no influence on the endothelium. Cyclic stretch increases EC sensitivity to shear stress resulting in a lowered threshold level required to provoke structural changes and ultimately, both cyclic stretch and shear stress are required to produce maximal responses in the vessel (Zhao et al., 1995). Cyclic strain is a powerful stimulus and can regulate cell fate decisions. Exposure of vascular smooth muscle to cyclic strain leads to apoptosis via a p53 dependent pathway, conversely cyclic strain can suppress EC apoptosis via Aktphosphorylation (Mayr et al., 2002; Persoon-Rothert et al., 2002; Haga et al, 2003). Similarly cyclic strain has been linked to inhibition of proliferation in addition to increases in angiogenesis associated with TGF-β, MMP-2 and VEGF (Rivilis et al., 2002; Zheng et al., 1999; Vailhe et al., 1996; Banai et al., 1994). These studies clearly demonstrate the importance of cyclic strain in coordinating and regulating cell function by mediating changes in gene transcription, signalling molecule activation and release of vasoactive compounds.

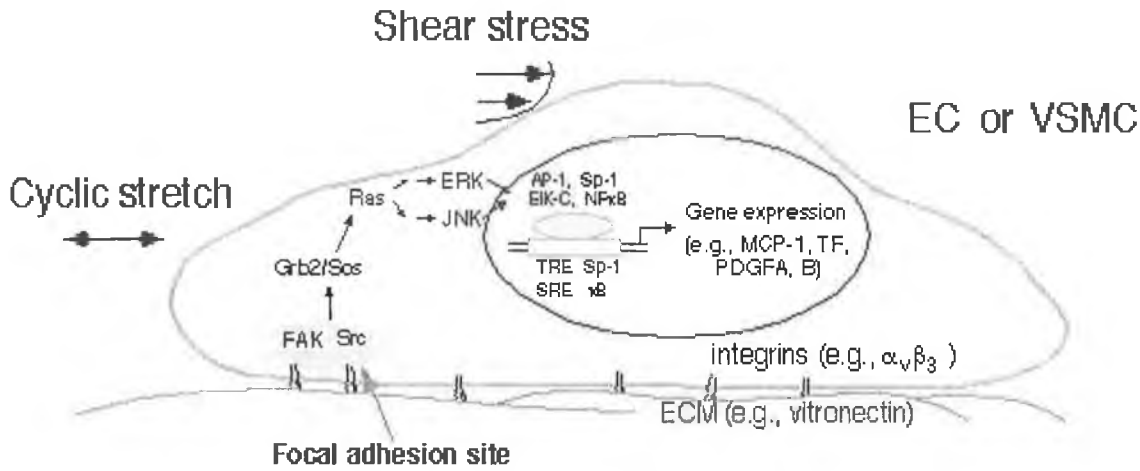
Shear stress is described as the dragging frictional force resulting from blood flow. Under normal physiological conditions, EC are primarily subjected to this haemadynamic shear stress. However, under conditions of endothelial dysfunction or denudation, shear stress can also exert its affect on the underlying VSMC. Changes in shear stress can result from changes in pulse pressure, which is defined as the difference between peak systolic and diastolic pressure. EC, which contain shear stress response elements, respond to physiological or pathological alterations in shear stress by releasing vasoactive agents and pro- or anti-atherogenic substances (Traub and Berk 1998). To attempt to elucidate the role of shear stress in atherogenesis, studies by (Davies *et al.,* 1995), used flow channels as *in vitro* systems to study functional changes of ECs in response to shear stress. These studies indicated that

shear stress induces a rapid induction of immediate early genes. These include c-Src and RAS, which are important mediators of shear stress activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinases (ERKs) and c-jun N-terminal kinases (JNKs), also known as stress-activated protein kinases, to activate these early genes (Jalali *et al.*, 1997). Other mechanisms have also been proposed to explain how EC respond to shear stress, including those via G-proteins, integrins and membrane-associated K^+ channels, which will be explained in further detail in the next section. The ability of vessels to detect and respond to changes in their heamodynamic environment involves a process, which is referred to as mechanotransduction.

1.7.1 Mechanotransduction in VSMC

In order for VSMC to respond to alterations in their haemodynamic environment, they must first have the ability to detect these changes. Mechanotransduction is a process whereby mechanically sensitive receptors present in vascular cells can elect a signalling pathway, which then culminates in the recruitment of an effector molecule(s), subsequently mediating a cellular response. These mechanically sensitive receptors in VSMC include integrins, G-proteins, protein tyrosine kinases and ion channels.

Figure.6: An overview of Mechanotransduction



1.7.2 Integrins

The Integrin family comprise of heterodimeric cell surface receptors most widely known for their role as receptors for extracellular matrix proteins. The heterodimer comprises of one of eighteen α and one of eight β subunits not including splice variants. There are different binding specificity and signalling properties for each possible combination of subunits (Giancotti and Ruoslahti, 1999). These subunits can form twenty-four different integrins. Sixteen of the known integrins are reportedly involved in the vasculature, with seven expressed in EC (Rupp and Little, 2001). The cytoplasmic tail of integrins is generally devoid of enzymatic activity. As a result of this, integrins transduce signals via adaptor proteins which connect the integrin to the cytoskeleton, cytoplasmic kinases and transmembrane growth factors (Giancotti and Ruoslahti, 1999).

1.7.3 G-Proteins

G-proteins signal via a highly sophisticated molecular system with the ability to receive, integrate, and process information from extracellular stimuli. The G-protein signalling machinery include a G-protein coupled receptor (GPCR), a heterotrimeric G-protein complex itself, and effector proteins, in addition to the more recently identified regulators of G-protein signalling (RGS-proteins) and activators of G-protein signalling (AGS-proteins) (Offermanns, 2003). G-protein signalling is known to play a pivotal role in cardiovascular signalling. All of these receptors have seven membrane spanning elements that use intracellular loops and their C-terminal tails for interaction with heterotrimeric G-proteins, which consist of α, β and γ subunits. The α and β subunit forms an undissociable complex, which represents a functional subunit. Ligand activated receptors catalyse the GDP/GTP exchange at the α subunit of a coupled G-protein and promote dissociation of the α and βγ components (Wieland and Mittmann, 2003). The duration of a G-protein activation is controlled by the intrinsic GTPase activity of Gα. Following GTP hydrolysis the Gα subunit returns to the GDP-bound conformation and reassociates with the Gβγ subunit.

1.7.4 Ion-channels

Ion-channels, though regulated by G-proteins, can also function as receptor molecules themselves. Ther are two different types of mechano-sensitive channels which have been identified in vascular cells: shear stress activated potassium channels and stretch activated cationic channels. Inhibition of ion channel activation can attenuate strain induced SMC proliferation. Stretch activated phospholipase C activity was found to involve the influx of calcium via gadolinium sensitive channels. Similarly, Ang II activation of mitogen activated protein kinasesis calcium dependent in VSMC (Lehoux and Tedgui, 1998). The exact mechanisms by which mechanical forces regulate ion channel conformation remains vague, though deformation of the cytoskeleton is thought to be an important contributor in this regulation.

1.7.5 Protein Tyrosine Kinases (PTKs)

Signal transduction events once initiated by "shear-stress-sensitive receptors" leads to a cascade of downstream signalling events many of which are mediated by protein tyrosine kinases. PTKs are crucial in the shear stress regulation of cell shape and stress fibers. This can be demonstrated by inhibition of shear stress induced ERK and JNK activation by genistein, a PTK inhibitor. Once the kinases are activated they relay signals downstream by phosphorylating other protein kinases and transcription factors. These kinases are turned off by activation of specific phosphatases. Mitogen activated protein kinases (MAPKs) are the most well studied kinases in response to haemodynamic forces, and they were first identified as microtubule associated kinases, due to their involvement with the cytoskeleton (Berk et al., 1995).

The Notch Signalling Pathway

1.8.1 Introduction

Notch receptor signalling is a conserved fundamental mechanism controlling cell fate during the development of many tissues, through interaction with ligands of the delta/serrate family (Weinmaster 2000). Although there have been extensive genetic studies performed in *Drosophilia* and *Caenorhabditis elegans*, the mammalian paralogs have also been characterized to display similar complex functions. The main components of the Notch signalling pathway include the Notch receptors (Notch 1-4 in vertebrates) and ligands (Delta 1-4, Jagged 1 and 2 in vertebrates) among others. The Notch receptor and its ligands were first identified in *Drosophila* and the Notch gene was first cloned by Wharton *et al.,* in 1985). The components of the Notch signalling pathway are expressed in a variety of cells and tissues (Saxena *et al.,* 2001), including cells of the immune system, the nervous system, and the vasculature. Vascular expression of Notch receptors and ligands has, to date, been described as being restricted to arterial vessels (Villa *et al.,* 2001). Villa *et al* report that Notch 1, Notch 3, Notch 4, Delta 4, Jagged 1 and Jagged 2 are all expressed in arteries and not in veins. In addition, vascular expression of Notch 3 has been localized specifically to SMC (Joutel *et al.,* 2000), while Notch 4 (Uyttendaele *et al.,* 1996) and Delta 4 (Shutter *et al.,* 2000) have been described as EC specific. Components of the Notch signalling pathway are upregulated in injured arteries. This was first described in 2001 by Lindner *et al.,* who observed increased expression of Notch signalling pathway genes, in both SMC and EC, following balloon catheter denudation of rat carotid arteries. Analysis of Notch receptor expression within the cell reveals that although Notch is a cell surface protein, the majority of Notch within the cell is found intracellularly (Fehon *et al.,* 1991; Aster *et al.,* 1994), and that a significant portion of Notch is retained in the endoplasmic reticulum (Aster *et al.,* 1994; Weinmaster, 1997).

There are two types of signalling primarily employed by the Notch pathway, termed lateral signalling and inductive signalling (Simpson, 1998). Lateral signalling causes two initially equivalent cells to adopt different fates or differentiate to different tissues. This occurs when one cell expressing a Notch receptor is stimulated by

another cell expressing a Notch ligand. Activation of the receptor in the first cell causes it to adopt a specific developmental fate and, through a negative feedback loop, suppresses its own Notch ligand expression. The adjacent cell is then exposed to less Notch ligand and adopts an alternative differentiation program. This allows a single cell or group of cells to be singled out from the surrounding cells, and it is this mode of signalling that is involved in preventing neuronal precursors from differentiating towards the neuronal lineage (Artavanis-Tsakonas *et al.,* 1995; Simpson 1998; Kojika and Griffin 2001). Inductive signalling occurs between non-equivalent cells and can lead to a specific response at the interface between the two, leading to the formation of sharply defined boundaries of gene expression. In this case, activation of the Notch receptor promotes production of Notch ligand, thus causing increased Notch activation in the adjacent cell (Artavanis-Tsakonas *et al.,* 1995; Lewis 1998; Simpson 1998). This occurs, for example, along the wing margin in *Drosophila*, where cells on one side of the margin signal via Notch to cells on the other side. Notch is only activated in each group by the signal coming from cells on the other side. This is possible as the two populations produce a different ligand, and the cells are rendered insensitive to the ligand produced by equivalent cells (Simpson 1998).

1.7.4 Notch Signalling in the Vasculature

There have been at least three identified disorders that are caused by altered function of components of the Notch signalling pathway. One of these leads to cell transformation and cancer and the two involve changes including defects in the cardiovasculature system. Chromosomal breakpoints in the Notch 1 gene have been shown to give rise to the over expression of a truncated protein containing the intracellular portion of Notch 1, leading to T-cell acute lymphoblastic leukemias/lymphomas in patients (Pear *et al.,* 1996). Mutations in the human Jagged 1 gene cause the Alagille syndrome, a genetic disease characterized by liver failure, cardiac abnormalities and vertebral arch defects (Li *et al.,* 1997). The incidence of this disorder is 1:70,000 live births (Joutel and Tournier-Lasserve 1998), however it is likely to be a cause of death *in utero*, as is evident with homozygous mouse models of AGS (Xue *et al.,* 1999). Congenital heart defects, the majority of which affect the

pulmonary circulation, significantly contribute to mortality in AGS patients. Most patients (97%) have a heart murmur and 67% of these have peripheral pulmonary stenosis (Loomes *et al.,* 1999). Lastly, the importance of Notch 3 in VSMC was highlighted in 1996 when Joutel *et al.*, (1996), through positional cloning, found the genetic cause of CADASIL to be point mutations in the human Notch 3 gene. CADASIL is a cerebral autosomal-dominant adult onset arteriopathy, with the mean onset age being approximately 45 years (Gridley 1997; Joutel *et al.,* 2000). Affected individuals exhibit a variety of symptoms including recurrent subcortical ischemic strokes, usually in the absence of any vascular risk factors, leading to progressive cognitive decline, dementia and premature death (Joutel and Tournier-Lasserve 1998; Gridley 2003). Other symptoms include migraine with aura (approx. 30% of patients), mood disorders and psychiatric disturbances (approx. 20% of patients) (Brulin *et al.,* 2002). The vascular lesions underlying CADASIL are non-atherosclerotic, non-amyloid angiopathies preferentially affecting the small arteries and arterioles of the brain (Rubio *et al.,* 1997). However, vascular pathological changes in CADASIL patients are not only confined to the brain, but are also observed in systemic arteries and some veins, as well as in muscle, nerve vessels and skin (Brulin *et al.,* 2002). CADASIL is therefore a systemic vasculopathy. The identification of the genetic alterations involved in these human diseases indicates that perturbation of Jagged/Notch signalling leads to dysfunctional cell and tissue behaviour *in vivo*.

The importance of the Notch signalling pathway in the vasculature is highlighted due to the fact that it is involved in multiple aspects of vascular development. Recent studies have implicated the Notch signalling pathway in the regulation of vasculogenesis and angiogenesis (Tallquist *et al.,* 1999; Gridley 2001; Luttun *et al.,* 2002). It is postulated that the Notch pathway contributes to the establishment of two distinct sub-populations at different stages of vasculogenesis and angiogenesis. These could include EC versus SMC, artery versus vein, pulmonary versus systemic vessels, and large vessels versus capillaries (Iso *et al.,* 2003a). In addition, mutations of Notch receptors and ligands lead to abnormalities in many tissues, including in the vascular system. Similar vascular phenotypes are observed in mice with both increased and decreased Notch signalling, suggesting that the level of

signalling is critical for proper blood vessel development. Alterations in Notch signalling produces abnormalities in vessel structure, branching and patterning of the vasculature, suggesting that Notch signalling does not function early in vasculogenesis, but regulates subsequent events that pattern the vascular network (Villa *et al.,* 2001). Mutations in components of the Notch signalling pathway are often embryonically lethal. Murine genetic studies generally null mutations of the jagged/notch genes have indicated that the vascular system seems to be developmentally reliant on intact Notch signalling pathways. This is the case, for example, with Jagged 1 null mutant mice displaying profound defects in the vasculature (Xue *et al.,* 1999). Furthermore, a Notch1 null or processing-deficient allele (Huppert 2000), in addition to Notch 1 and 4 double mutants exhibit defects in vascular remodelling and angiogenesis (Krebs 2000). Taken together, these observations in combination with the vascular defects seen in the human conditions in which Notch signalling is impaired suggest that responses to cardiovascular injury may also be regulated by Notch gene family members.

1.8.3 Structure of Notch Signalling Pathway Receptors and Ligands

The Notch receptor in *Drosophila* is a 300Kda cell surface protein which is predicted to transverse the membrane once, which consists of an extracellular, ligand-binding domain and a cytoplasmic domain which is required for signal transduction (Figure 1.7). Notch receptors are proteolytically processed into heterodimeric (180Kda and 120Kda) forms which are presented on the cell surface (Weinmaster *et al.*, 1997). In addition to *Drosophilia* Notch, the Notch receptor family includes LIN-12 and GLP-1 in *C-elegans*, mNotch 1 and mNotch 2 in mouse, as well as Notch 1-4 in humans. All of these Notch receptors exhibit the same overall structure, however, although their general architecture is conserved across species, variances in specific domain size can be observed within and among species (Greewald and Rubin, 1992).

Figure 7: Schematic Representation of the Notch Family of Receptors

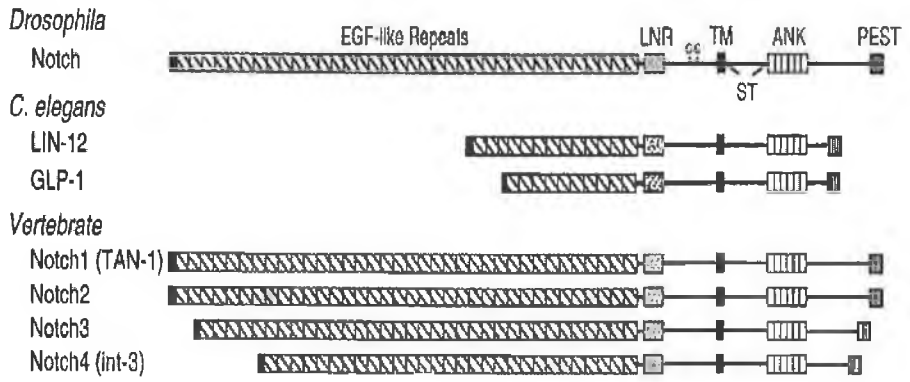


Figure 7: Schematic representation of the Notch family of receptors. The family members have the same overall arrangement of structural motifs. The extra-cellular domains contain multiple epidermal growth factor (EGF)-like repeats, in addition to LIN12/Notch-related region (LNR) and two conserved cysteines. The sub-transmembrane domain (ST) lies between the transmembrane domain (TM) and the 6 ankyrin repeats (ANK). A region rich in proline, glutamine, serine and threonine (PEST) lies at the C terminal (Weinmaster 1997).

Notch receptors are characterized by their extracellular domains, which contain multiple repeats which are related to epidermal growth factors (EGFR) and it is these domains, which are thought to be involved in ligand binding. In addition to these tandemly arranged EGF-like repeats, Notch ExC domain contain a cysteine-rich region termed the LNR (LIN-12/Notch-related region) and a pair of conserved cysteines located between the LNR and TM regions (Weinmaster *et al.*, 1997; Fleming *et al.*, 1998; Baron *et al.*, 2002). Similarly to *Drosophilia* Notch, the Notch 1 and Notch2 proteins contain 36 tandemly arranged EGFRs, while Notch 3 and Notch 4 contain 34 and 29 tandemly arranged EGFRs respectively. It is interesting to note that the order of the EGFRs has been conserved among Notch proteins which suggests that the spatial arrangement of these repeats is important for receptor function (Weinmaster *et al.*, 1997). The cysteine rich LNR region is located immediately downstream of the EGFR domain. The LNR region appears to negatively regulate receptor activation. Studies by Greenwald and Seydoux, 1990 and Lyman and Young,

1993, support this idea whereby deletion of the LNR region or missense mutations in this region produces constitutively active receptor proteins. Located between the LNR and the TM domain there is a pair of conserved cysteines. Through genetic studies in *C elegans* by Greenwald and Seydoux, 1998, they have been proposed to function in receptor dimerization. Moreover, through sequencing analysis, a conserved valine residue identified within the TM domain has been established as a cleavage site of Notch during receptor activation.

There have been four important functional regions identified with the Notch IC domain. These include, the RAM domain which is located in the subtransmembrane region, the ANK repeat domain, a transcriptional activator domain (TAD) and a PEST (Proline, Glutamate, Serine, Threonine-rich sequence). Two nuclear localization sequences are also present in the Notch IC domain and these are located on either side of the ANK repeat domain (Stifani *et al.*, 1992; Fortini *et al.*, 1993). The RAM domain is the main site of CSL interaction (Tamura *et al.*, 1995). The ANK repeats are both necessary and sufficient for Notch activity as they provide a lower affinity binding site to the CSL proteins. This facilitates the RAM/CSL interaction necessary for Notch IC function (Tamura *et al.*, 1995; Roehl *et al.*, 1996). In addition the ANK repeat domain provides a binding site for a number of positive regulators of the Notch signaling pathway including the Deltex protein (Matsuno *et al.*, 1995). The PEST sequence of the Notch receptor has been reported to be involved in Notch protein turnover (Greenwald et al., 1994). Deletion of the PEST domain results in the inactivation of Notch, however, this deletion does not produce a dominant negative form of the receptor as is what is found with all deletions of the ANK repeats or sequences which encode the entire cytoplasmic domain (Rebay *et al.*, 1993; Lieber *et al.*, 1993). There are different numbers of amino acids between the ANK repeats and the PEST sequences among different Notch proteins. These variances in amino acid numbers account for the different sizes in cytoplasmic domains. These differences may reflect why different Notch receptors can be regulated by interaction with different cellular proteins.

Notch receptors are activated by the DSL family of ligands. This family of ligands are defined by the invertebrate ligands including Delta and Serrate in

Drosophilia, Lag-2 in *C-elegans* and Delta 1-3 and Jagged 1-2 in vertebrates (Dunwoodie *et al.*, 1997; Weinmaster *et al.*, 1997; Haddon *et al.*, 1998). The Notch family of ligands are similar in structure to Notch receptors as they are also single-pass transmembrane proteins, which possess multiple EGFRs in their extracellular domains. However, in contrast to the Notch receptors, Notch ligands posses a characteristic degenerate EGFR-N-terminal to the EGFRs, which is known as the DSL domain (Figure 1.8).

Figure 8: Schematic representation of the DSL family of Notch ligands

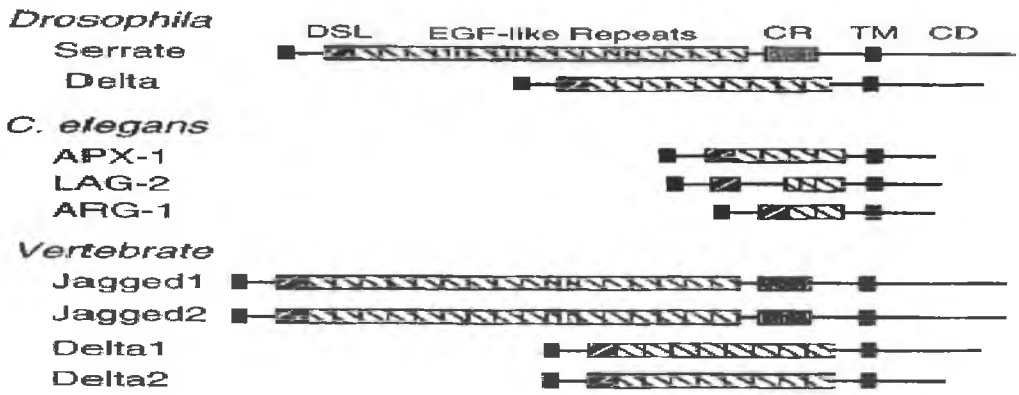


Figure 8: Schematic representation of the DSL family of Notch ligands.
The family members have the same overall arrangement of conserved structural motifs. Like the Notch/LIN-12/GLP-1 receptors, the extracellular domains of the ligands contain multiple (2 to 16), tandemly arrayed EGF-like repeats; however, the Serrate-like proteins (Serrate, Jagged1, and Jagged2) have inserts in some of their repeats and LAG-2 has two half EGF-like repeats. The Serrate-like ligands also have an additional cysteine-rich region (CR) between the EGF-like repeats and the single transmembrane domain (TM). The cytoplasmic domains do not share significant amino acid identities (Weinmaster et al., 1997).

The DSL domain is thought to be required for function in invertebrates (Henderson *et al.*, 1994). This DSL domain is a modified EGF-like repeat domain, which is believed to be the part of the ligand responsible for receptor activation. Point mutations that affect conserved cysteines in this domain have resulted in strong loss-

of function phenotypes (Henderson *et al.*, 1994). Delta and Serrate have different expression patterns and appear to regulate different developmental decisions following activation of a Notch receptor (Couso *et al.*, 1995). However, though Delta and Serrate are structurally related, Serrate contains additional EGFRs and a cysteine region (CR) which may be modulating the binding of the ligand to Notch as cell aggregates formed between Delta and Notch expressing cells, are more stable than cell aggregates formed between Serrate and Notch expressing cells (Rebay *et al.*, 1991).

Overall arrangement of structural motifs is conserved across ligand family members. As with the Notch receptors, the extracellular domains of the ligands contain multiple (2-16) tandemly arranged EGF-like repeats, however, Serrate-like proteins (Serrate, Jagged 1and Jagged 2) have inserts in some of their repeats and LAG-2 has two half EGF-like repeats. In addition, these Serrate-like ligands posses an additional CR region between the EGF-like repeats and the single transmembrane domain. The IC domain of the Notch ligands do not however share significant amino acid identities. The IC-domain is relatively short comprising of approximately 70-215 amino acids in length where no significant homology is evident within or among species (Flemming *et al.*, 1998). Mutant forms of Delta and Serrate in which the IC domain has been deleted, produced dominant negative phenotypes, which indicate that these sequences are required for normal ligand function (Chitnis *et al.*, 1995). In addition to the normal transmembrane ligands, proteolytically cleaved, secreted forms of the proteins have been identified (Klueg *et al.*, 1998) which served as dominant negative molecules. These dominant negative molecules competed with membrane bound ligands and hence blocked Notch activation (Sun and Artavanis-Tsakonas, 1996). However, studies by Wang *et al*, 1998 have described soluble ligands as agonists of the Notch signaling pathway. The question of whether the soluble form of the Notch ligand serves as an agonist or antagonist of Notch signaling remains to be answered. However the phenotypes produced by the secretion and diffusion of these soluble forms not seen with membrane bound ligands, is presumably due to the physical restrictions which are imposed by their cell surface localization.

1.8.4 Processing of the Notch Receptor

The activation of the Notch receptor requires at least three proteolytic cleavage events. These three proteolytic cleavage events termed S1, S2 and S3 (Baron *et al.*, 2002) result in the cleavage of full length Notch protein (300Kda) in to two fragments, an N-terminal fragment, Notch ExC that contains most of the extracellular domain and a C-terminal fragment, which consists of the TM and IC regions (Blaumueller *et al.*, 1997). S1 cleavage occurs within the secretory pathway and is carried out by Furin, which is a calcium -dependent serine protease. This cleavage event results in a processed form of the Notch receptor being transported to the cell surface (Bush *et al.*, 2001) and the two resulting subunits of the heterodimer are held together at the plasma membrane by non-covalent interactions, which require calcium (Rand *et al.*, 2000). Furin inhibition has been shown to interfere with heterodimer formation resulting in diminished ability of Notch to activate CSL transcription factors (Jarriault *et al.*, 1998). The binding of a ligand to the Notch receptor induces a conformational change in the receptor, which is necessary to facilitate S2 cleavage (Weinmaster *et al.*, 1998). The S2 cleavage has been confirmed as a conserved valine which is located twelve amino acids N-terminal of the TM region (Brou *et al.*, 2000; Mumm *et al.*, 2000).There are two proteins which have been identified as S2 cleavage enzymes. TACE (TNF-α-converting enzyme) and Kuzbanian (Kuz) are both members of the ADAM (a disintigrin and metalloproteinase) family are both thought among other possibilities to be involved in this cleavage event. TACE has been identified as being involved in S2 cleavage in both *Drosophilia* and in vertebrates (Brou *et al.*, 2000). This study suggests that Kuz may act at a site distinct to that of TACE, however Kuz has been found to be an important element of Notch signaling as transgenic mice deficient in either Kuz or Notch 1 have shown to display the same phenotype (Mumm *et al.*, 2000). Moreover Kuz RNA knockdown in *Drosophilia* resulted in inhibited S2-like cleavage in vitro proving the importance of this enzyme in Notch receptor cleavage (Lieber *et al.*, 2002). However, studies by Qi *et al*, (1999) showed that expression of a dominant negative form of Kuz had no effect on Notch processing while additional studies by Mumm et al, (2000) proved proteolytic processing was evident in Kuz deficient cells. The membrane tethered Notch product produced by S2 cleavage and the resulting strength of the Notch signal may depend

whether S2 cleavage was carried out by TACE or Kuz (Lieber *et al.*, 2002). The cleavage event at S3 which results in an active form of the Notch receptor, Notch IC, is performed by Presenilins, γ-secretases (Struhl and Greenwald, 1999). There have been two forms of presenilins identified, PS 1 and PS 2 (Kojika and Griffin, 2001) and several studies have implicated PS 1 to be a requirement for S3 cleavage of Notch (De Strooper *et al.*, 1998). Studies by De strooper have shown that dominant-negative mutagenesis of PS 1 reduces the extent of S3 cleavage while Ray and co-workers (1999) have shown that Notch 1 and PS 1 physically interact in both mammalian and *Drosophilia* cells, however pesenilin-independent signalling has also been documented (Berechid *et al.*, 2002). It is important to note that Kadesch et al., 2000 reported that cleavage of the Notch receptor is a mutually dependent event whereby S1 cleavage is required prior to S2 cleavage and S2 cleavage is required prior to S3 cleavage.

1.8.5 Modulators of the Notch Signalling Pathway

The activation of Notch is regulated both by the temporal and spatial distribution of the ligands and by the expression of proteins such as Fringe, Scabrous, Numb and Wingless among others that can exert either a positive or negative effect on the Notch signaling pathway. This was first evident in the developing wing where Notch activity results in the expression of genes such as wingless at the dorsoventral boundry (Blair et al., 1995). The Wingless-signalling pathway is known to act as a negative regulator of the Notch signaling pathway through activation of a cytoplasmic protein, Dishevelled. Dishevelled exerts its effect by binding to the carboxy terminus of the Notch receptor. Fringe exerts its effectiveness of the interactions between Notch and it's ligands by preventing ser-mediated activation and potentiating Notch activation by Delta (Panin *et al.*, 1997). Fringe also impedes the activation of Jagged-Notch activation by binding to and modifying the EGF-like repeats of the Notch receptor in a cell autonomous manner (Moloney *et al.*, 2000). In addition, the effect of Fringe on Delta has the consequence that ventral Delta-expressing cells signal primarily to dorsal cells (Doherty *et al.*, 1996). Other modulators of Notch signaling

include Numb, which has been shown to block CSL protein translocation to the nucleus in response to Notch activation. However, it does not interfere with CSL cellular localization in the absence of Notch-receptor ligand interactions (Frise *et al.*, 1996). Scabrous also acts as a negative regulator of Notch signaling by binding to the EGF-like repeats and modulating receptor-ligand binding (Powell *et al.*, 2001). Although only a few possible modulators of the Notch signaling pathway have been mentioned here, the list continues to grow and this study will hopefully help provide further insight into how this signaling pathway is regulated.

Figure 9: Modulators of the Notch Signalling Pathway

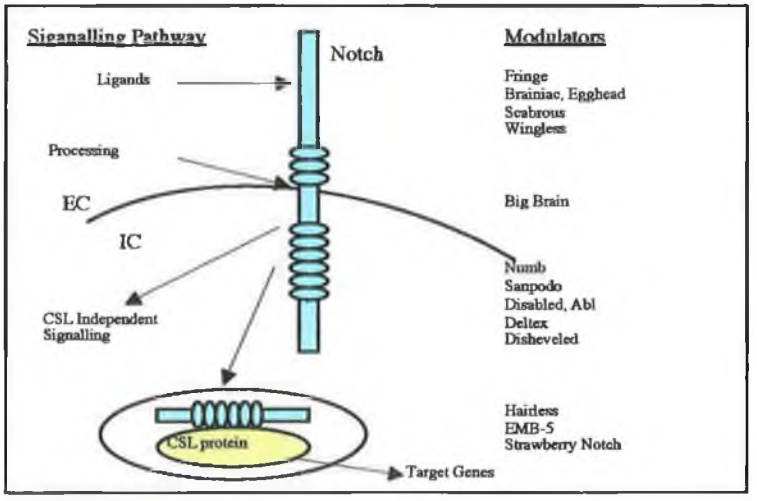


Figure 9: Modulators of the Notch signalling pathway. Schematic representation of sites of action of some modulators of the Notch signalling pathway (Panin and Irvine 1998).

1.8.6 Notch Signalling Transduction

Activation of Notch receptors by their ligands is accompanied by proteolytic processing that releases an intracellular fragment, Notch IC, from the membrane. Notch IC can then enter the nucleus and interact directly with CBF-1. The presence of Notch IC inside a cell stimulates transcription from enhancers containing CBF-1 binding sites. Initial studies of CBF-1 indicated this mammalian homologue of Su(H)

as a repressor of transcription (Hsieh *et al.*, 1995). However addition of Notch IC in cell culture transcription assays converted CBF-1 into a transcriptional activator, thus presenting the model that activation of Notch switches CBF-1 from a transcriptional repressor to activator. Upon nuclear localization, Notch IC cannot bind directly to DNA but forms a tertiary complex with a CBF-1 complex (CSL proteins) and DNA (Goodbourn, 1995). A CBF-1 interaction site has been mapped to the RAM23 region of Notch IC (Tamura *et al.*, 1995) with a lower-affinity binding site in the ankyrin repeat region (Aster *et al.*, 1994). Hence, CBF-1 serves to act as a docking protein that directs Notch IC to promoter targets and consequently the transcription of downstream genes (Struhl and Adachi, 1998).

CBF-1-mediated transcriptional repression involves the destabilization of the transcription factor IID (TFIID)-transcription factor IIA (TFIIA) interactions (Brulin *et al.*, 2002), which result in TFIID being unable to interact with TFIIA and form a functional complex (Kadesch, 2000). CBF-1 repression also requires the recruitment of a histone deactylase (HDAC) co-repressor complex to the promoter (Zhou *et al.*, 2000). HDACs cause the reduction of histone acetylation which is linked to repressed, transcriptionally inactive chromatin. This has the effect of rendering the chromatin less accessible to the transcription machinery (Kadesch, 2000). The HDAC co-repressor complex is made up of several co-repressors, which have the ability to bind to and partially mediate the transcriptional repressor activity of CBF-1. These include SKIP (Ski-interacting protein (Kuroda *et al.*, 1999; Kadesch 2000)), SMRT (silencing mediator for retinoid and thyroid receptor, 78,54), N-CoR (nuclear receptor co-repressor (Lindner *et al.*, 2001)) and CIR (CBF-interacting repressor (Hsieh *et al.*, 1999)). SKIP acts as both a tethering point for the CBF-1 co-repressor complex in order to mediate repression and also as a tethering point for the ankyrin repeat domain of Notch IC activation (Zhou *et al.*, 2000). SMRT and N-CoR have both been shown to act as co-repressors for a variety of transcription factors and both have been shown to bind directly to CBF-1 and antagonize the ability of Notch IC to stimulate CBF-1 dependent gene expression. Moreover, SMRT and N-CoR can bind directly to SKIP and additional co-repressors such as Sin 3A, HDAC and SAP 30 (Lai, 2002).

Figure 10: CBF-1 Co-Repressor Complex

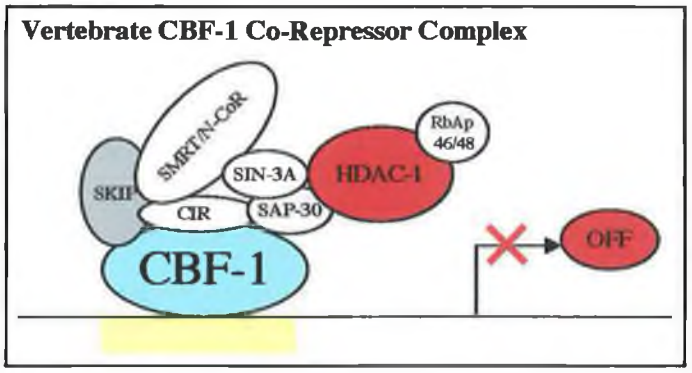


Figure 17: Schematic diagram of the vertebrate CBF-1 co-repressor complex. CBF-1 binds to promoter sequence within the nucleus, and also binds a HDAC-containing co-repressor complex resulting in a repression of transcription (Lai 2001)

Figure 10: CBF-1 Co-Repressor Complex. Schematic representation of the CBF-1 Co-Repressor Complex

Notch IC activation of CBF-1 involves firstly, the loss of CBF-1 mediated transcriptional repression and secondly, the activation of gene expression through the Notch IC activation domain (Apelqvist *et al.*, 1999; Zhou *et al.*, 2000). In order for CBF-1 to be converted from transcriptional repression to activation, SMRT and Notch IC must compete for binding to the CBF-1 complex. Hence, both CBF-1 and SKIP must exchange the SMRT containing co-repressor complex for the Notch IC activation complex (*Zhou et al.*, 2000). As previously mentioned, Notch IC binds to CBF-1 via its RAM23 region and bind to SKIP via its Ankyrin repeat domain. Zhou et al, (2000) showed that a mutation in the fourth ankyrin repeat of Notch IC served to abolish Notch IC-SKIP interaction but not CBF-1 proving that this binding site is necessary for Notch IC biological activity.

Figure 11: Model for Notch Activation of CBF-1-repressed Promoters

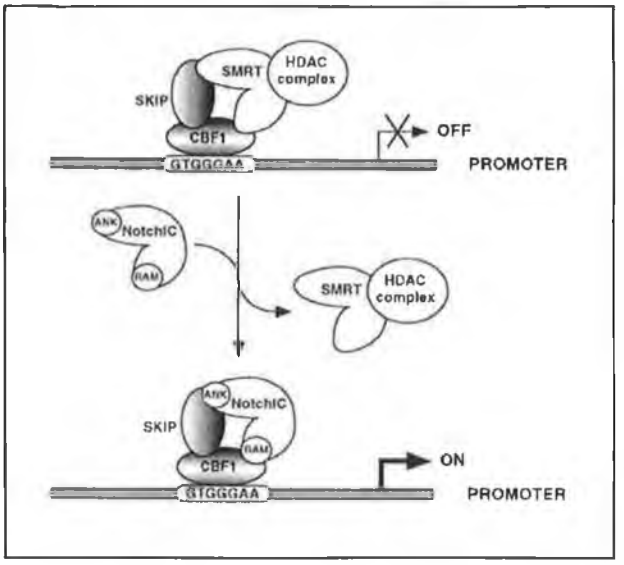


Figure 11: Model for activation of CBF-1 repressed promoters. CBF-1 binds promoter sequence within the nucleus, SKIP interacts with CBF-1. The SMRT/HDAC co-repressor complex binds to both SKIP and CBF-1, mediating transcriptional repression. Notch IC competes with SMRT for contacts on both SKIP and CBF-1. Displacement of the co-repressor complex relieves repression, and Notch IC further activates promoter through its transactivational domain (Zhou *et al.,* 2000).

Moreover, Notch's ability to inhibit muscle cell differentiation was also blocked with the co-expression of SKIP antisense in these cells. Once displacement of the co-repressor complex relieves repression, Notch IC subsequently further activates CBF-1 promoter activity through the presence of its endogenous activation domain (Apelqvist *et al.*, 1999; Zhou *et al.*, 2000). Notch IC recruits a co-activator complex which includes the nuclear protein Mastermind and histone acetyltransferases (HATS) (Baron, 2003). Mastermind is an integral component of Notch signaling as it forms a complex with Notch IC and CBF-1, which results in stabilizing the complex (Nakagawa et al., 2000). Mastermind recruits HATS to the co-activator complex, which is believed to act catalytically to produce an open chromatin conformation, thus

promoting transcription. (Baron, 2003). It is interesting to note that loss-of- function mutations in *Drosophilia* Mastermind generates a similar phenotype to that of loss-of-function of Notch (Iso *et al.*, 2003), further highlighting the importance of this nuclear protein in Notch signalling.

Figure 12: CBF-1 Co-Activator Complex

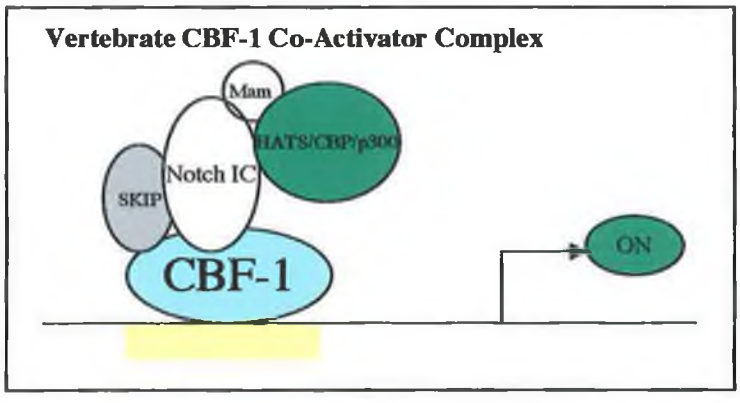


Figure 12: CBF-1 Co-Activator Complex. Notch IC recruits a co-activator complex to facilitate transactivation at the CBF-1 promoter (Lai 2002).

In addition to a signalling pathway which involves members of the CSL family of transcription factors as an intermediary, Notch signalling activates an additional pathway, one of which does not involve CBF-1.i.e. a CBF-1-independent pathway. It has been suggested that the full length (300Kda), uncleaved form of the Notch receptor is the mediator of this pathway (Bush *et al.*, 2001). CBF-1-independent signalling can include Notch IC interaction with alternative transcription factors to CBF-1. One such mediator of CBF-1-independent signalling is thought to be Deltex. Deltex encodes a cytoplasmic protein containing three domains, domain I, a SH3 binding domain (domain II) and a zinc-finger domain (domain III) (Matsuno *et al.*, 1998). Studies by Matsuno *et al.*, (1995) has shown that over-expression of deltex produces a similar dominant phenotype to that produced with over-expression of Notch IC. This study also revealed that deltex binds to the conserved ankyrin repeats within Notch via domain I. In addition, deltex exerted a two-fold stimulation of Notch target gene expression in cultured cell assays. Notch IC has been shown to antagonize

signalling through a JNK MAP kinase pathway and that this effect is mediated through deltex (Ordentlich *et al.*, 1998). However, though these studies do prove interesting, a greater deal of research is required to further elucidate the signalling relationship between Notch and Deltex.

1.8.7: Notch Target Genes

The activation of CBF-1 by Notch IC leads to the expression of primary Notch target genes. These primary target genes of Notch signalling include *Hairy/Enhancer of Split (Hes)* and *Hairy Related Transcription Factor (HRT) genes*. These genes are members of a family of basic helix-loop-helix (bHLH) type of transcriptional repressors that act as Notch effectors by negatively regulating expression of downstream target genes. The members of the Hes and Hrt families share many similarities yet they are still distinct from each other. This is evident, both in their similar but distinct structures, and their mechanism of transcriptional repression. Both the Hes and HRT proteins contain three functional domains, a basic domain containing a conserved proline in the case of the Hes family and a conserved glycine at a corresponding position in the case of the HRT family (Iso *et al.*, 2003). Both families contain another domain, the orange domain, located carboxy to the bHLH region. Furthermore, Hes family members contain a highly conserved tetrapeptide (WRPW) motif at or adjacent to the carboxy terminus, whereas this motif is replaced in HRT proteins by either a YRPW or YQPW motif (Iso *et al.*, 2001).

To date, there are seven described members in the mammalian Hes family, however, only two of these, Hes-1 and Hes-5, are known to be involved in Notch signalling (Gridley 1997; Kojika and Griffin 2001; Iso *et al.*, 2003b).In addition, a number of studies have described the HRT family as downstream targets of the Notch signalling pathway (Maier and Gessler 2000; Iso *et al.*, 2001b). Furthermore, many studies have proven that Notch IC activation of both the Hes and HRT promoters occurs in a CBF-1 dependent manner. Studies by Iso et al., (2001) showed, for example, that the transactivation of Hes-1 and HRT-2 promoters by Notch IC was reduced in the presence of the CBF-1 mutants, R218H-RPB-Jκ and RY227GS in cultured mammalian cells. Moreover, this study also demonstrated that over-expression of Notch IC failed to induce HRT-2 and Hes-1 mRNA expression in CBF-

1 deficient cells. Therefore this study proves that CBF-1 is essential for both Hes and HRT expression in response to Notch signalling. This study will conclusively show that Hrt-1, 2 and 3 are constitutively expressed in adult VSMC and that their expression can be enhanced following over-expression of Notch IC

Studies by Leimeister *et al.,* (2000) have indicated that a single Notch ligand or receptor can participate in the up-regulation of multiple members of the Hes and HRT families, for example, Notch 1 deficient mice have been shown to exhibit a decreased expression of Hes-5 and HRT-1-3, but not Hes-1. Furthermore, activation of different Notch receptors can result in different levels of transactivation of the target gene. Recent studies have shown that Notch 3 IC, in contrast to Notch 1 IC, to be a poor transactivator of the Hes-1 and –5 promoters, and that Notch 3 IC can act as a repressor of Notch 1 IC mediated Hes activation (Beatus *et al.,* (1999). The Hes and HRT families act as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors such as Cbfa-1, a *Drosophila* Runt related protein involved in the regulation of a variety of cell differentiation events (McLarren *et al.,* 2000), hence showing a role of the Notch signaling pathway in determining cell fate decisions . Furthermore, the Hes and HRT families have been shown to repress the expression of bHLH transcriptional activators that drive the expression of Notch ligands (Kimble *et al.,* 1998; Weinmaster 1998; Martinez Arias *et al.,* 2002). Notch signalling can therefore play a role in decreasing the expression of its own ligand.

With the abundance of recent studies, the importance of Hes and HRT proteins as Notch effectors in the vasculature is increasingly being recognized. The HRT family, for example, is expressed in specific regions of the developing heart, vasculature, pharyngeal arches and somites, and the periodicity of their expression in somatic precursors mirrors that of Notch signalling related molecules (Iso *et al.,* 2003a). Moreover, members of the Hes/HRT families have been shown to play an important role in vasculogenesis, for example, gridlock, the Zebrafish HRT-2 homologue, has been shown to be required for assembly of the aorta in Zebrafish. Studies by Campos *et al.,* (2002) have described HES and HRT proteins as being present and active within adult vascular smooth muscle cells however, their roles remain to be fully elucidated. It is the aim of this study to further elucidate the role of these Notch target genes within adult VSMC. In addition, if it is Notch IC which

activates the expression of these primary target genes of Notch signaling, a greater understanding of the signaling pathways which affect Notch signaling, will further help to bring greater understanding of the role of these target genes in cardiovascular disease.

Figure 13: An overview of the Notch Signalling Pathway

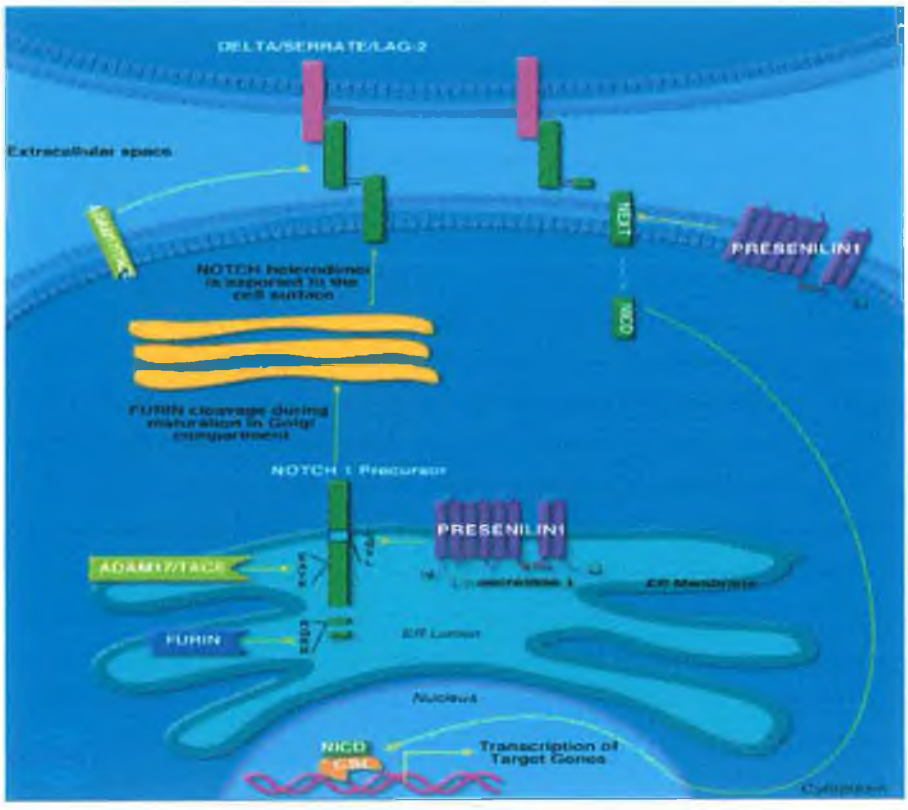


Figure 13: The Notch Signalling Pathway. Schematic diagrammatic representation of the Notch Signalling Pathway (Biocarta)

1.9: Hedgehog Signalling

1.9.1: Introduction

Hedgehog (Hh) is a secreted signaling molecule that serves multiple roles during embryonic development. The full-length protein is autocatalytically processed to produce the active amino peptide, which is modified by the addition of cholesterol and palmitoyl moieties (Ingham 2001). Release of the Hh signal from the sending cell is facilitated by the membrane protein Dispatched (Ma *et al.,* 2002), and a heparan sulphate proteoglycan is involved in receipt of the signal (Nybakken and Perrimon 2002). The secreted peptide then binds to its receptor, the 12-membrane pass protein Patched 1 (Ptc 1) (Chen and Struhl 1998). This serves to relieve the Ptc1-mediated repression of Smoothened (Smo) action. Although the precise manner in which Hh binding facilitates activation of the G-protein-like molecule Smo is unknown, downstream events focus on the transcription factor Cubitus interuptus (Ci) in *Drosophilia,* and its homologues, the Gli family, in vertebrates. The binding of Hh to Ptc1 inhibits the cleavage of Ci/Gli to its repressor form, permitting the full-length protein to promote expression of mediators of the Hh response. Regulation of Ci/Gli processing includes the action of Costal 2, Fused, and Suppressor of Fused, which form a scaffold that links Gli to microtubules. In the absence of the Hh signal, the kinases protein kinase A, glycogen synthase kinase 3, and casein kinase 1 phosphorylate Ci/Gli and mediate its degradation to the repressor form (Figure 14). It is also interesting to note that one of the genes expressed downstream of Hh signaling encodes the Hh receptor Ptc 1, thereby making Ptc 1 expression an indicator of Hh responsiveness.

Hh acts upon mesoderm in epithelial-mesenchymal interactions that are crucial to the formation of limb, lung, gut, hair follicles and bone (Johnson *et al.,* 1996; Pepicelli *et al.,* 1997; Ramalho-Santos *et al.,* 2000; St-Jacques *et al* 1999). There are three Hh genes in the mouse: *Sonic hedgehog (Shh), Indian hedgehog (Ihh)* and *Desert hedgehog (Dhh).* Sonic hedgehog is the most widely expressed during development and Shh deficiency in mice is embryonically lethal leading to multiple defects beginning in early to mid gestation (Chiang *et al.,* 1996). Indian hedgehog is less widely expressed and Ihh-deficient mice survive to late gestation with skeletal

and gut defects (Ramalho-Santos *et al.*, 2000). Desert hedgehog is expressed in the peripheral nerves, male gonads, as well as the endothelium of large vessels during development (Bitgood *et al.*, 1995). Dhh-deficient mice are viable but have periperal-nerve and male fertility defects (Parmantier *et al.*, 1996). The consensus is that the signals encoded by these three Hh genes all activate the same downstream signaling cascade, and that the presence of these three genes controlled by separate regulatory elements facilitate the expression of the signal at multiple sites and times during embryogenesis. Hh signaling is widely used throughout embryogenesis in many differentiating tissues to establish cell fate, promote cell proliferation and mediate apoptosis. This study will be the first to address these same processes, but in adult vascular smooth muscle cells

Figure 14: Hedgehog Signaling Pathway

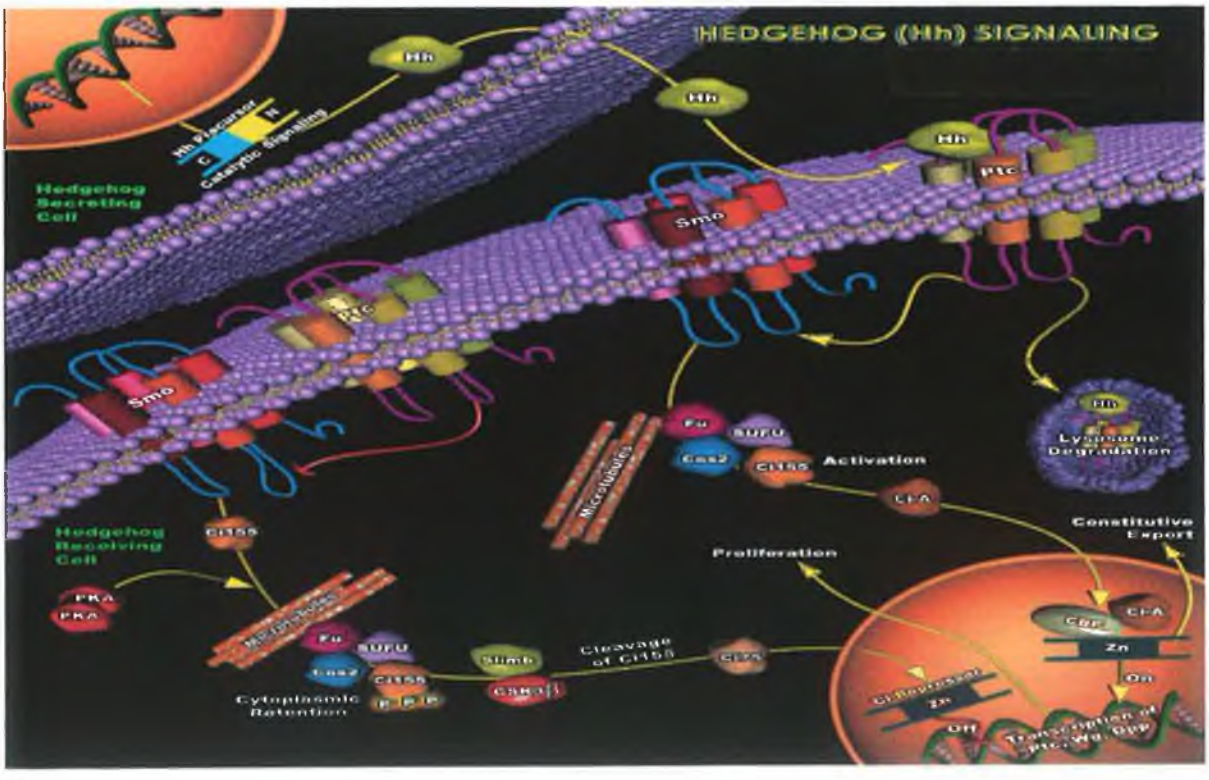


Figure 14: The Hedgehog Signalling Pathway. Schematic diagrammatic representation of the Hedgehog Signalling Pathway (Proteinlounge.com)

1.9.2: Hedgehog Signalling in the Vasculature

The evidence for Hedghog's role in vascular development came from the observations that zebrafish carrying mutations in components of the Hh signaling pathway carry defects in circulation and vascularization (Brown *et al.,* 2000, Lawson *et al.,* 2002). During normal development, two trunk axial vessels form the aorta and the posterior cardinal vein. Interestingly, the aorta develops immediately adjacent to a midline source of Shh. Moreover, the loss of Shh leads to a single large vessel that expresses only venous and no arterial markers (Lawson *et al.,* 2002). In a study by Vokes *et al.,* 2004, they presented work indicating that Shh is an important factor regulating blood vessel assembly and tubulogenesis. Firstly, Shh is expressed in endodermal tissues, immediately adjacent to the developing vascular network, as in the previous mentioned study, but not in the mesodermal tissue itself. This is consistent with previous studies indicating that a signal originating in the endoderm is required for vascular tube formation (Vokes and Krieg, 2002a). Furthermore, angioblasts, nascent endothelial tubes and cultured endothelial cells express Hedgehog transducing molecules, and are therefore capable of responding to Hedgehog signaling. In addition, the loss or inhibition of Hedgehog signaling results in a dramatic reduction in vascular assembly in the mouse, and completely eliminates vascular tube formation in avian embryos. Finally, they found that the implantation of beads containing Shh into endodermless avian embryos is sufficient to rescue vascular tube formation, and addition of Shh to cultured endothelial cells causes the formation of vascular network-like structures. Collectively, these findings provide compelling evidence in birds and mammals that Shh, which is produced by the endoderm, is an important regulator of vascular tube formation from specified angioblasts. In addition the role of Hedgehog signaling in vascular development is further strengthened by the evidence that murine embryoid bodies derived from ES cells lacking Smo initially express endothelial cell markers but fail to form endothelial enclosed blood islands (Byrd *et al.*, 2002), and an initial examination of Smo mutant embryos reported that extra-embryonic yolk sac vessels were poorly formed and greatly reduced in number (Byrd *et al.,* 2002). In other studies, Zebrafish Shh mutant embryos contain angioblasts but do not form vascular tubes in the trunk region of the embryo (Brown *et al.*, 2000). Furthermore, overexpression of Shh by injection of Shh mRNA, causes

the formation of lumenized ectopic vessels (Lawson et al., 2002). Transgenic mouse embryos ectopically expressing Shh in the neural tube display hypervascularization (Rowitch et al., 1999), and treatment of mouse embryonic neurectoderm explants with Ihh is reported to respecify explants to form tissues containing blood vessels (Dyer *et al.*, 2001). Finally, in culture, the addition of Shh ahs the effect of promoting endothelial cells to assemble into capillary networks (Kanda *et al.*, 2003).

These findings and others continue to add to the growing evidence of the fundamental importance of Hh signaling in regulating epithelial/mesenchymal interactions during embryonic development. However there is an appreciation that signaling pathways so fundamental to vascular development can be recapitulated postnatally in adult life. Pola *et al.*, 2001, reported that the Hedgehog-signalling pathway is present in adult cardiovasvascular tissues and can be activated *in vivo*. In juvenile and adult mice, they found that Ptc1 is normally expressed in cardiovascular tissues. In addition, they tested the potential for Shh to act upon the adult vasculature and protect against ischemic injury by administering Shh. A sharp increase in limb salvage was observed in mice treated with Shh as compared to vehicle controls. In parallel studies, Shh was also able to induce robust angiogenesis, characterized by distinct larger-diameter vessels.

To complement this study, the same group aimed to investigate whether the endogenous Hh pathway is physiologically involved in the revascularization of ischemic tissue in adults. For this study they used a murine model of muscle regeneration by inducing ischemia of the hindlimb. Following this, they then observed the expression pattern of different components of the Hh pathway, including Shh, Dhh, Ihh, and Ptc1, and studied the relationship between Hh activation, VEGF expression, and angiogenesis. They reported that Shh is activated in the regeneration after ischemia and that interstitial cells within the ischemic area strongly express Ptc1, indicating the postnatal activity of the Hh signalling pathway. They found that Ptc1 expression was associated with VEGF production and angiogenesis and that inhibition of Shh inhibits endogenous angiogenesis and VEGF production in the ischemic hindlimb.

The Hh pathway has been studied and characterized extensively during embryogenesis and the vast majority of these prenatal studies have focused on the role of Hh family members in the regulation of epithelial–mesenchymal interactions

crucial to limb, lung, gut, hair follicle, and bone formation, including a possible role during vascularization of certain embryonic tissues. In addition, the activation of components the Hh pathway during ischemia and the reduced angiogenesis observed after inhibition of Hh suggest a crucial role for these morphogens in the pathophysiology of muscle regeneration. Collectively, these results may open the possibility that members of the Hh family might play a role in the development of angiogenesis-related diseases, such as diabetic retinopathy or tumor angiogenesis. Therfore, a greater understanding on how Hh signalling interacts with other signalling pathways may have important implications for both proangiogenic and antiangiogenic therapeutic strategies.

1.9.3 Hedgehog and Notch - A Functional Relationship

To elucidate how the Hh signal interacts with other signalling pathways to play a role in vascular development, loss-of-function mutations of a number of genes implicated in angiogenesis, which resulted in vascular remodelling defects, were studied. One such example, included targeted mutation of the Ang1 or Tie2 genes resulting in severe yolk sac angiogenesis defects by midgestation which was reminiscent of the *Smo* homozygous mutant phenotype (Zhang *et al.*, 2001). This observation suggested that this angiogenesis growth factor and its receptor may act in the same pathway as Hh. This hypothesis was further supported by a study by Pola *et al.*, (2001) which reported that Shh treatment upregulates angiopoietins in adventitial fibroblast cells. This study which resulted in an upregulation of vascular endothelial growth factor (VEGF) as a result of Shh addition confirmed Hh as been placed upstream of these vascular-specific growth factors. Since the identification of VEGF a decade ago, its central role in developmental and pathologic vessel growth has been definitively elucidated (Ferrara 2000). Gene disruption studies revealed not only an absolute requirement for VEGF in vessel development, but also embryonic Lethality associated with haploinsufficiency, suggesting stringent dose dependency. Using genetic analysis coupled with ectopic gene expression to study development of the aorta in the zebrafish, Lawson *et al.*, (2001) placed VEGF downstream of Shh and upstream of the Notch pathway in determining the arterial fate of the dorsal aorta. Notch signalling has been implicated in blood vessel differentiation, and arrest at the

capillary plexus stage is observed in embryos deficient in Notch 1 (Krebs *et al.,* 2000). Delta- like-4 (Dll4), the likely vascular Notch receptor, is expressed in arteries but not veins, implying a role for this cascade in establishing vessel identity, a key step during vascular remodelling (Shutter *et al.,* 2000). Liu *et al.,* (2003), reported that expression of both Notch 1 and Dll4 are upregulated by VEGF in human arterial cells. Taken together, these data suggest a regulatory cascade that begins with Hh promoting VEGF expression, which in turn promotes Notch expression and signalling. In the study carried out by Lawson *et al.,* (2001), Zebrafish mutants defective in the Shh signalling pathway and a pharmacological approach to block Shh signalling were utilized to demonstrate a direct role of Shh in the induction of VEGF expression during vascular development. This study showed that ectopic expression of VEGF can rescue aortic differentiation in the Shh mutants, and that VEGF is sufficient to induce arterial markers expression in the aorta, independent of Shh signalling. However, on the other end of this proposed cascade of Hh-VEGF-Notch, disruption of either the Notch receptors or ligands lead to embryonic lethal vascular defects (Gridley 2001). Therefore, based on the work by Lawson on the Zebrafish, VEGF can restore normal arteriogenesis in the absence of Shh, but not in the absence of Notch function. Moreover, addition of Notch can compensate for the loss of VEGF activity. However it should be noted that these studies only offer a functional connection between Shh, VEGF and Notch in determining arterial fate. It is important to elucidate the molecular mechanisms by which Shh-mediated induction of VEGF expression can the direct role of Shh in the vasculature be established. Similarly, identification of the molecular mechanisms whereby VEGF mediates Notch activation. Elucidation of these different molecular pathways will prove a major challenge to vascular biologists for the years to follow.

Figure 15: Shh/VEGF/Notch in the Arterial Vasculature

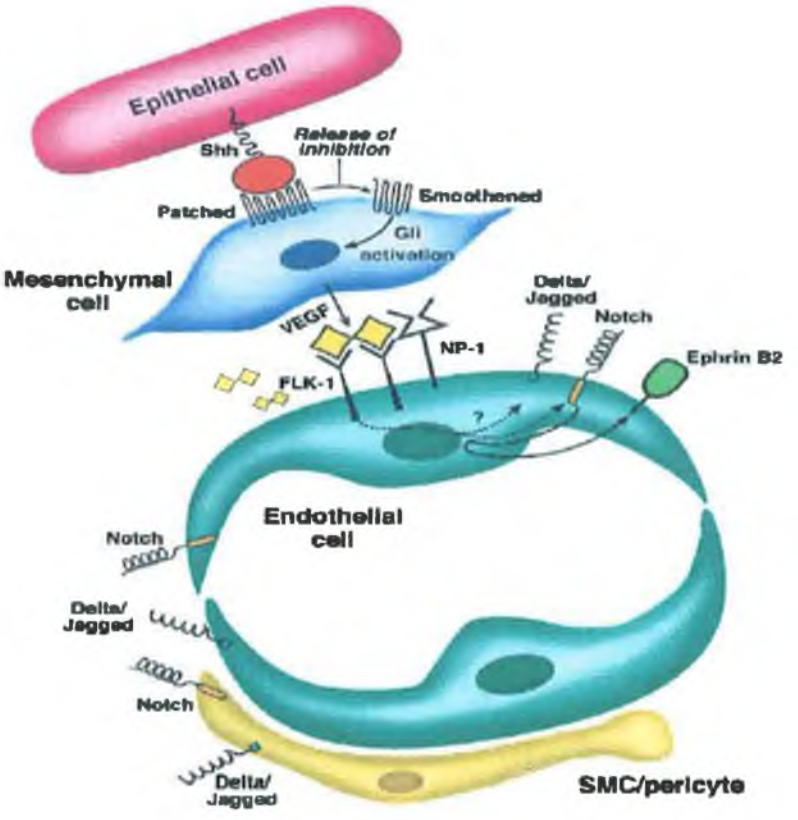


Figure 15: Shh/VEGF/Notch in the Arterial Vasculature (D'Amore 2002). Sonic hedgehog (Shh) binding to the receptor complex formed by Patched (Ptc) and Smoothened (Smo) can release the inhibition of Smo by Ptc. The derepressed Smo in turn activates the Gli family of transcription factors. Shh can upregulate the expression of VEGF by mesenchymal cells, but whether the Ptc/Smo/Gli pathway is involved in this Shh-mediated VEGF production is still unclear. VEGF acts on its specific receptors, includingFlk-1 and neuropilin-1(NP-1) and induces aterial – specific EphrinB2 expression on endothelial cells. VEGF-induced EphrinB2 expression is Notch dependent. Both the Notch receptor family and their ligand (Delta and Jagged) families are expressed by the EC and SMC/pericyte *in vivo*.

1.10 Relevance and Objectives of this study

Vascular smooth muscle cell fate decisions (proliferation, apoptosis, differentiation and migration) play an important role in neointimal formation during the pathogenesis of hypertension, atherosclerosis and in the arterial response to injury. With changes in vascular cell fate being apparent during vascular morphogenesis and modelling of the embryonic vasculature, the control of these cell fate decisions in adult life, may share similar signalling pathways. Therefore, since it is aberrant cell fate, which is a major cause of vascular disease, a better knowledge and understanding of the pathways that control these processes, could potentially lead to important therapeutical strategies.

Recent studies have demonstrated the importance of the Notch and Hedgehog signalling pathways during vascular development. One such example being Hh regulation of Notch signalling during arterial endothelial differentiation (Lawson *et al.,* 2002). Moreover, mutations in Hh and Notch signalling components result in embryonic lethality due to defects in both vasculogenesis and angiogenesis (Xue *et al.,* 1999; Schauerte *et al.,* 1998). Furthermore, the recent discovery of Hh and Notch signalling components in adult vascular tissue (Pola *et al.,* 2001; Wang *et al.,* 2003), combined with their known morphogenic functions in embryonic development, has led us to investigate whether these signalling pathways so fundamental to vascular development, may also co-ordinate vascular cell fate changes in adult tissues. In addition to this, is the well-documented signalling cascade of Hh-VEGF-Notch so integral in cell fate decisions during vascular development, recapitulated post-natally in controlling vascular SMC fate *in vitro*.

Therefore, the principal aims of this study are to establish the presence and activity of both the Notch and Hh signalling pathways within VSMC. Furthermore to establish the role, if any, of these pathways on SMC growth induced by serum stimulation or deprivation and following exposure to biomechanical forces both *in vitro* and *in vivo*. Moreover to elucidate the signalling order in which Notch and Hh form a cascade in determining these changes in cell fate. In conclusion, this study aims to provide an increased understanding into the regulation of VSMC growth both under physiological and pathological conditions, which will ultimately contribute to the future research of vascular disease diagnosis, prognosis and therapy.

Chapter 2

Materials and Methods

2.0 Material & Methods

All reagents used in this study were of the highest purity commercially available and were of cell culture standard when applicable.

2.1 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

Anti-goat 2^{o} antibody, HRP conjugated

ECL Hybond nitrocellulose membrane

ECL Hyperfilm

Rainbow molecular weight marker, broad range (6-175kDa)

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DMEM

dNTP's

DEPC-treated water

Trizol® reagent

BioRAD (Alpha Technologies, Dublin)

Iscript

BD Transduction Laboratories (Oxford, UK)

All apoptosis related 1° antibodies

Santa Cruz (Heidelberg, Germany)

HRT-1 1° antibodies

HRT-2 1° antibodies

HRT-3 1° antibodies

Patched-1 1° antibodies

Sonic 1° antibodies

Indian 1° antibodies

Upstate (Uk)

Notch 1 1° antibodies

Notch 3 1° antibodies

Calbiochem (San Diego, CA)

PD98059

Pertussis toxin

Dunn Labortechnik GmBH (Asbach, Germany)

6-well Bioflex® plates

Flexcell International Corp. (Hillsborough, NC)

Flexercell® Tension Plus™ FX-4000T™ system

Invitrogen (Groningen, The Netherlands)

Lipofectamine reagent

Lipofectamine 2000 reagent

Scientific Imaging Systems (Eastman Kodak Group, Rochester, NY)

Kodak 1D image analysis software

MWG Biotech (Milton Keynes, UK)

Notch 1 primer set

Notch 3 primer set

HRT 1 primer set

HRT 2 primer set

HRT 3 primer set

HES 1 primer set

HES 5 primer set

JAG 1 primer set

Shh primer set

Ihh primer set

Dhh primer set

Smo primer set

Ptc 1 primer set

Gli 2 primer set

Bax primer set

Bcl-Xl primer set

VEGF primer set

GAPDH primer set

HRT 1 siRNA duplex

HRT 2 siRNA duplex

HRT 3 siRNA duplex

VEGF siRNA duplex

Scrambled siRNA duplex

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemiluminescent substrate

Promega (Madison, WI)

Taq DNA Polymerase

MLV-RT

RNase H

Oligo dT

R and D Systems (Germany)

Recombinant Shh Protein

Sarstedt (Drinagh, Wexford, Ireland)

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

Sigma Chemical Company (Poole, Dorset, England)

β-glycerophosphate
β-mercaptoethanol
Acetic Acid
Acetone
Agarose
Ammonium Persulphate
Acrylamide/Bis-acrylamide
Bovine Serum Albumin
Brefeldin A
Brightline Haemocytometer
Chloroform
DMEM
DMSO
EDTA
EGTA
Methanol
Mineral oil
Monensin
Penicillin-Streptomycin (100x)
Ponceau S
Potassium Chloride
Potassium Iodide
p-Nitroaniline
RPMI-1640
Sodium Acetate
Sodium Chloride
Sodium Doecly Sulphate
Sodium Hydroxide
Sodium Orthovanadate
Sodium Phosphate

Ethidium Bromide
Eosin
Foetal Calf Serum
Glycerol
Glycine
Hanks Balanced Salt Solution
Hydrochloric acid
Isopropanol
Lauryl Sulphate
Syber Green
Temed
Tris Acetate
Tris Base
Tris Chloride
Triton X-100
Trypsin-EDTA solution(10x)
Tween 20

2.2 Cell Culture Methods

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.

2.2.1 Culture of Rat Vascular Smooth Muscle Cells (RVSMC)

The cell line used in this study were RVSMC, purchased from Cell Applications Inc. (CA, USA) cat no. R-354-05. RVSMC were maintained 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. Cells were cultured in 175 cm^2 or 75 cm^2 tissue culture flasks, or in 6-well plates. Only cells of passage number 3 to 20 were used in this study. Cells were passaged using a trypsinisation method which involved removal of the RPMI-1640 growth media from the cells, and two subsequent washes of the cells with Hanks Balanced Salt Solultion (HBSS). The cells were then incubated with 1x Trypsin/Ethlyenediamine Tetracetic

Acid (EDTA), diluted from the 10x stock solultion with HBSS. Typically 1 ml of 1x Trysin/EDTA was used per 25 cm^2 tissue culture flask area. The cells were then incubated at 37°C for 5 min, or until the cells had detached from the flask. RPMI-1640 growth media was then added to the flask to neutralize the trypsin/EDTA (an equal volume of RPMI-1640 growth media to trypsin/EDTA was typically added). The cell suspension was then removed from the flask, and centrifuged at 3,500 rpm for 5 min. The supernatant was subsequently removed, and the cells were re-suspended in fresh growth medium. For routine sub-culturing, a 1:2 to 1:4 dilution of cells was typically made.

2.2.2 Maintenance of RVSMC

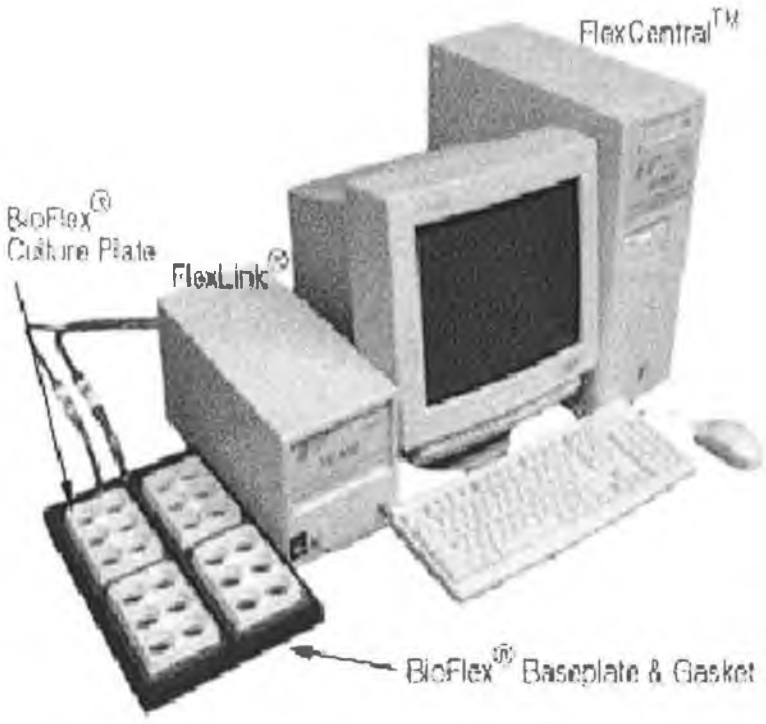
Cells were fed every 3-4 days with RPMI-1640 growth media, and routinely sub-cultured at 90-100% confluency, as described in 2.2.1.

2.2.3 Cyclic strain studies

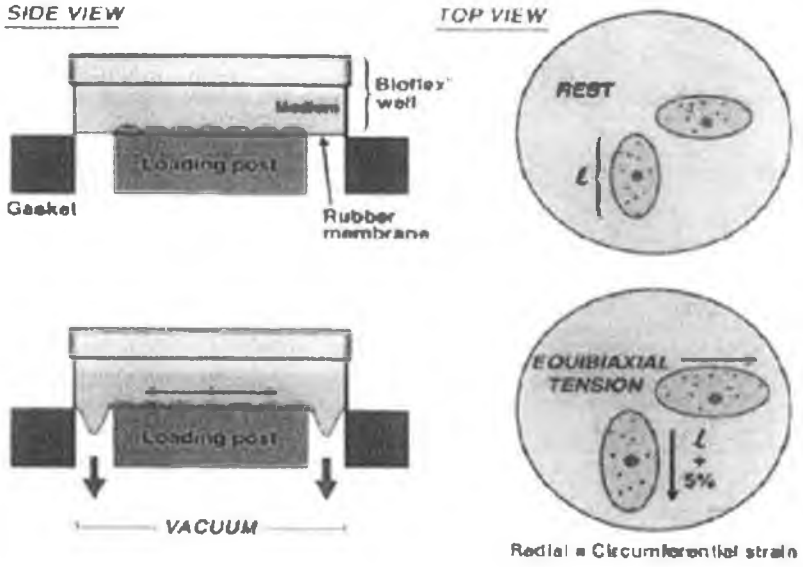
For cyclic strain studies, RVSMCs were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately $6X10^5$ cells/well, allowed to adhere for 24 hours and grown to confluency. After 24 hours, the media was removed and replaced with serum-free media and the cells exposed to varying levels of cyclic strain. Bioflex™ plates contain a pronectin-coated silicon membrane bottom that enables precise deformation of cultured cells by microprocessor-controlled vacuum (Banes *et al.,1995*). When cells had reached approximately 100% confluent, 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 (0%). Following strain, the cells were washed twice in Ix PBS, and harvested for either protein or RNA as described in sections 2.2.5 and 2.6.1 respectively.

Figure 1: Flexercell Tension Strain Unit



Left: Photograph of the computer-driven Flexercell Tension Strain Unit with baseplate which holds four BioFlex Culture plates.
Below: Schematic of the cyclic strain event occurring when SMC are subjected to deformation by the Flexercell unit.



2.2.4 Cryogenic Cell Storage and Recovery of Cells

For long-term storage of cells RVSMCs were maintained in liquid nitrogen in a cryofreezer unit. Cells to be stored were centrifuged following trypsinisation and the resultant pellet was resuspended in 20% (v/v) FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% (v/v). 1ml aliquots were transferred to sterile cryovials and frozen in a –80°C freezer at a rate of –1°C/min using a Nalgene cryo freezing container. Following overnight freezing at –80°C, the cryovials were transferred to a cyrofreeze unit (Thermoylen locator jr. cryostorage system). Cells were recovered from longterm storage by rapid thawing at 37°C and resuspension in 5ml of growth medium followed by centrifugation at 3500rpm for 5 min. The resultant cell pellet was resuspended in fresh medium and transferred to a culture flask. The following day the media was removed, the cells were washed in HBSS and fresh culture media added.

2.2.5 Preparation of Whole Cell Lysates

Following trypsinisation as described in section 2.1.1, the cell pellet was washed in 1X PBS to remove any trace levels of FBS. The cell suspension was then centrifuged at 3500rpm for 5 min. The PBS supernatant was removed and the cells were resuspended in 1X 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). The resulting lystaes were frozen and thawed three times followed by three cycles of ultrasonication for 5 sec on ice using a sonic disembrator (Vibra Cell, Sonics and materials Inc). Samples were stored at –20°C for short-term storage or –80°C for long-term storage.

2.2.6 Bicinchoninic Acid (BCA) Protein Microassay

The bicinchoninic acid protein microassay utilizes the biuret reaction, the reduction of Cu^{++} to Cu^{+} by protein under alkaline conditions, with the selective colourimetric detection of the cuprous cation (Cu^{+}) using a reagent containing bicinchoninic acid. 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 200 μl 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).

2.2.7 Preparation of Competent Cells

A modified Rhubidium chloride ($RbCl_2$) method was employed to prepare competent cells. The procedure is an adaptation of one described by Hanahan *et al.*, 1985 (Altered Sites Mutagenesis Kit Promega). An *E.coli* strain was streaked from a glycerol stock on to an LB agar plate and incubated at 37°C overnight. An isolated colony was then picked using a sterile inoculating loop and used to inoculate 5 ml of LB broth (1.0% tryptone, 0.5% yeast extract, 1.0% NaCL). This culture was incubated in a shaking incubator at 200 rpm overnight 37°C. The resulting culture (2.5 ml) was then used to inoculate 250 ml of sterile LB supplemented with 20mM MgSO4 and incubated in a 1L flask at 37°C until the O.D. of the culture at 640 mm was between 0.4 and 0.8 (approximately 4-5 hr). The cells were then transferred to two sterile 250 ml centrifuge tubes and pelleted by centrifugation at 4,500 x g, 4°C for 5 min. The resulting pellets were resuspended in 0.4 ml original volume ice cold buffer (TFB1) (100 ml for 250 ml culture- 50ml/centrifuge tube) and the two pellets

combined. Cells were kept on ice for all subsequent steps and pipettes tubes and flasks were chilled. The resuspendend cells were then incubated on ice at 4°C for 5 min and pelleted by centrifugation at 4500x g , 4°C for 5 minutes. Cells were then gently resuspended in 1/25 of the original volume of ice-cold buffer (TFB2) (For 250 ml subculture use 10 ml). Cells were then incubated on ice for 1hr, aliquoted at 100ul/tube for storage at -70°C. Prior to storage the aliquoted cells were snap frozen in a dry ice/isopropanol bath. JM109 competent cells prepared by this method are stable for 1 year.

2.3 DNA Manipulations

2.3.1 Expression and Reporter Plasmids

Plasmids used throughout this study.

PLASMID	*GIFT FROM*	*DESCRIPTION*
pCMV-ED1-HA *pCMV-ED4* *pHACS1*	Dr. Bettina Kempkes, GSF-Institute of Clinical Molecular Biology, Neuherberg, Germany.	pED1 expresses Notch 1 IC. pED4 expresses Notch 1 IC without it's RAM domain, rendering it unable to interact with CBF-1. pHACS-1 is the HA vector into which Notch 1 IC and Notch 1 IC-delta RAM were cloned.
pCMX-Notch 3 IC-HA	Dr. Urban Lendahl, Karolinska Institute, Stockholm, Sweden.	Notch 3 IC cDNA was cloned in pCMX-polylinker 2. The Notch 3 IC cDNA is followed by a DNA sequence encoding a HA immunotag.
pGa50-7 *pGa98-1-6*	Dr. Bettina Kempkes, 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).
pGL3 *pGL3-mHRT1* *pGL3-mHRT3*	Prof. Eric Olson, University of Texas, Southwestern Medical Centre, Dallas, Texas.	Plasmid expression constructs for HRT-1 and HRT-3 were prepared by inserting partial digestion fragments of mHRT-1 and –3 repsectively into pGL3 basic luciferase vector (Promega).
pTK-Hes 1-luc *pTK Hes-5-luc*	Dr. Urban Lendahl, Karolinska Institute, Stockholm.	Hes-1 and –5 promoter fragments were inserted into pTK-luc vector.

pcDNA3RPMS-I	Dr. Paul J Farrell, Ludwig Institute for Cancer Research, Imperial College School of Medicine London, U.K.	Contains the full length RPMS-I cDNA ORF cloned into the peDNA3HA vector (Invitrogen) (Smith, 2000)
pCMX-R218H-RBP-Jκ	Prof. Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.	pCMX-R218H-RBP-Jκ is a dominant-negative CBF-1 contstruct, with an arginine to histidine mutation at position 218.
pCMV	Dr. Celine Gelinas, University of Medicine and Dentistry of New Jersey. New Jersey, USA.	pCMV vector (Stratagene). (le Roux, 1994)
pTK-Gli-luc	Dr. Eileen Redmond Dept of Surgery. University of Rochester, Rochester, New York.	Gli promoter fragment was inserted into pTK-luc vector
pPGKpuro	Dr. Peter Laird, University of Southern California, Kerk School of Medicine, Los Angeles, California,USA.	Puromycin plasmid (Tucker et al., 1996)
mShh	Prof C. Chiang Vanderbilt University	Contains the full length mShh cDNA

2.3.2 Transformations

Two hundred microliters of competent cells were placed in a pre-chilled microcentifuge tube containing 10μl. The contents were mixed gently and incubated on ice for 30 min, during which time an aliquot of SOC was pre-heated at 42°C. After 30 min on ice the cells were heat-pulsed at 42°C for 90 sec followed by incubation on ice for a further 2 min. One mililiter of preheated SOC was then added to the cells and incubated at 37°C in a shaking incubator for 1 hr 10 min. The cells were concentrated by centrifugation following which 800 μl of supernatant was removed and discarded. The cells were resuspended in the remaining supernatant and

plated out with the appropriate controls on LB plates containing ampicillin (125ug/ml) or tetracycline (12.5ug/ml) and incubated overnight at 37°C. If the cells are transformed they become ampicillin/tetracycline resistant thus only transformed cells will yield colonies. These were used to prepare broth cultures by inoculating 5 ml of LB containing ampicillin (125ug/ml) or tetracycline (10ug/ml), and incubated overnight at 37°C and DNA mini preparations were carried out as described in section 2.3.3.

2.3.3 QIAGEN™ Plasmid DNA Purification Protocol

Plasmid DNA was purified using the QIAGEN-tip 100 solution system from Promega. A glycerol stock of the bacteria of interest was streaked out on LB ampicillin/tetracycline agar and incubated overnight at 37°C, an isolated colony from this plate was used to inoculate a 5 ml LB ampicillin/tetracycline starter culture and incubated in a shaking incubator at (300rpm) 37°C for 8 hrs. One millilitre of the starter culture was used to inoculate 25 ml of LB (containing the appropriate antibiotic) in a 250 ml sterile flask and incubated overnight in a shaking incubator at 37°C. The O.D. of the culture was monitored at 600 nm until cultures O.D were between 1-1.5. The following centrifugation steps were carried out using a JA-20 rotor in a Beckman centrifuge. The bacteria culture was transferred to a centrifuge tube and centrifuged by spinning at 6,000 x g for 15 min at 4°C. The supernatant was removed and the pellet was dried by inverting the tube on tissue paper and allowing the supernatant to drain off. The bacterial pellet was resuspended completely in 4 ml of Buffer PI (provide in kit) containing RnaseA (100μg/ml), 4 ml of freshly prepared Buffer P2 was added and incubated at room temperature for 5 min. Following incubation, 10 ml of prechilled Buffer P3 was added, mixed gently by inverting the tube 5-6 times then incubated on ice for 20 min. The mixture was then centrifuged for 1 hr at 20,000 x g at 4°C.

The Qiagen-tip 100 column was equilibrated by applying 4 ml of QBT buffer and allowing the column to empty by gravity. The column does not dry out at this stage as the flow of buffer will stop when the buffer reaches the upper filter. After the centrifugation step the supernatant was removed immediately from the tube without disturbing the pelleted material and applied to the column by filtering through 1mm

filter paper. The Qiagen-tip was washed with 2 x 10 ml of Buffer QC. DNA was then eluted with 5 ml of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol and centrifuged immediately at 15,000 x g for 30 min at 4°C and the supernatant was carefully removed. The resulting pellet was washed with 70% (v/v) ethanol, allowed to air dry for 5 min and re-dissolved in a suitable volume of TE or dH_2O. DNA was then quantified by spectrophotometric analysis as described in section 2.3.4.

2.3.4 DNA Quantitation and Storage

To determine the concentration of DNA in a sample obtained from the Qiagen plasmid midi kit, the sample was diluted 1:100 in 1x sterile TE buffer and spectrophotometric analysis carried out using the Shimadzu UV-160A dual spectrophotometer, blanked with TE. The sample was measured, usig a quartz cuvette, at wavelengths of 260 and 280 nm, and the concentration of the DNA in the sample was carried 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, ready for use in transient transfections.

2.4 Transient Transfections

Lipofectamine™ reagent is a polycationic liposome as such it is suitable for transfection of DNA into eukaryotic cells (Invitrogen-Groningen, Netherlands). The day prior to transfection, $1.5x10^6$ cells were plated on a T25cm^2 flask, and grown overnight in RPMI-1640 supplemented with serum and antibiotics.

When cells had reached approximately 70% confluency they were transfected with plasmid DNA. For transfection purposes plasmid DNA was diluted in 150 μl of DMEM without FCS or antibiotics such that there would be 1μg of DNA per 10cm^2 of surface area. In a separate tube 10μl of lipofectamine reagent (4μl per 10cm^2) was diluted in 150μl of DMEM without FCS or antibiotics. The diluted DNA was then

mixed with diluted lipofectamine reagent and incubated at room temperature for 30 minutes. This time permits the formation of DNA-liposome complexes.

While the DNA complexes were forming the cells were washed three times in HBSS followed by one wash in DMEM. This was to remove any antibiotics and serum from the flask, which may impede transfection efficiency. The DNA/lipofectamine mixture was made up to a final volume of 2ml, which is just enough media to cover the surface area of the flask. The contents of the tube were then added to the culture flask. The cells were incubated for 4 hr in transfection media, following this, the media was removed and replaced with normal RPMI-1640 growth media. The cells were allowed to recover overnight and subsequently they were exposed to experimental conditions. Cells were routinely co-transfected with either a Lac Z (see section 2.4.1) or green fluorescent protein (GFP) encoding plasmid as a means to determine approximate levels of transfection.

2.4.1 β-galactosidase assay

Lac Z a 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. Following trasnsfection and cell lysis, a 30μl sample was added to 3 μl of 100X Magnesium solution [0.1M $MgCl_2$ and 4.5M β mercaptoethanol], 66μl of 1X OPNG (o-nitrophenyl-β-D-galactopyranosidase)[4mg/ml ONPG in 0.1M sodium phosphate, pH 7.5] and 201μl of 0.1M sodium phosphate. The reaction was incubated for 4-6 hours at 37°C until a yellow colour developed. The reaction was subsequently stopped with 500 μl of Na_2CO_3, and optical density read at 420nm. Suitable positive and negative controls were included in this assay.

2.4.2 Luciferase Assay

To analyse transactivation of luciferase tagged reporter genes, cells were harvested 18-24 h post transfection. Cells were washed twice in 1x PBS, and incubated with 1x Reporter Lysis Buffer (Promega) for 10 min at 37°C in a

humidified atmosphere, 500 µl/well. Cells were then scraped, transferred to eppendorfs, and lysed by freeze-thawing once. The lysates were clarified by centrifugation at 3,000 rpm for 2 min, and the supernatants were saved in a fresh tube for analysis. Transactivation of the luciferase tagged reporter genes was then analysed by luciferase assay, using 40µl sample, and 50µl luciferase assay buffer at room temperature (Promega). Light emission was measured over a period of 60 sec, after a lag period of 10 sec. Briefly, the enzyme firefly luciferase, generated due to promoter activation on a luciferase tagged plasmid, catalyses the conversion of D-luciferin to oxyluciferin, with a concominant production of a photon of light, which is measured by the luminometer (Labsystems Luminoskan).

2.4.3 Puromycin Selection

Alternative to transfection with luciferase tagged reporter genes, cells were transfected with Notch 1 IC, Notch 3 IC or the Notch 1 IC mutant, and 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 100ug streptomycin, and 0.8 mg/ml puromycin for 48 h.

2.4.4 Transfection with siRNA

RNA interference (RNAi) is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA (Figure 2; step 1). In the cell, long dsRNAs are cleaved into short 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer (step 2). The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), unwinding in the process (step 3). Activated RISC then binds to complementary transcript by base pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved (step 4) and sequence specific degradation of mRNA results in gene silencing.

Figure 2: Mechanism of siRNA

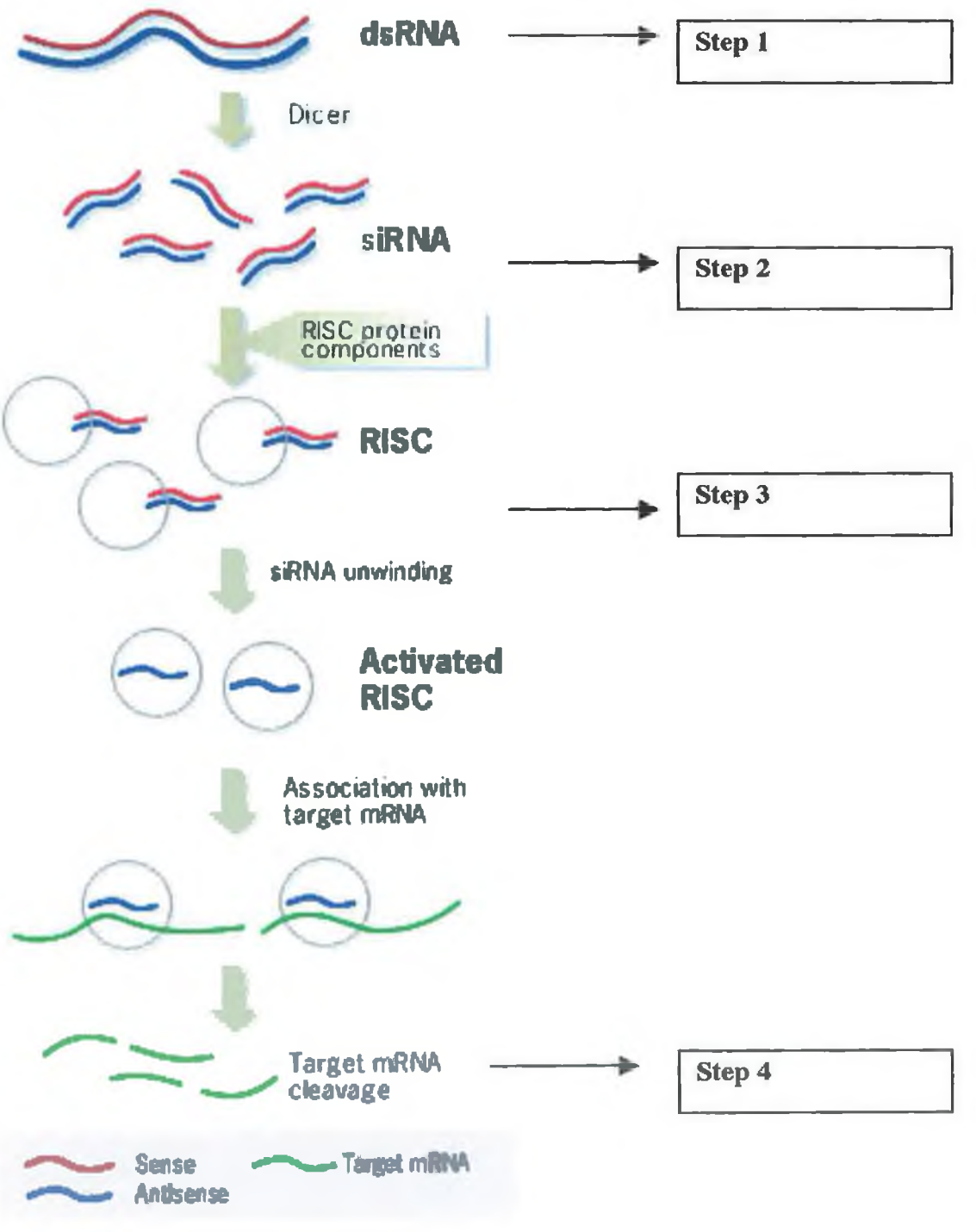


Figure 2: Mechanism of siRNA: Description on how transfected siRNA duplexes results in targeted mRNA cleavage.

2.4.5 Design of siRNA duplex

A sequence was selected in the open reading frame of the cDNA which is at least 75-100 bp downstream of the start codon. Untranslated regions near the start codon may be richer in regulatory protein binding sites, which may interfere with binding of the siRNP endonuclease complex. The first AA dimer is located and the next 19 nucleotides are recorded. The GC content of the AA-N_{19} base sequence is determined. Ideally the GC content must be greater than 30% and less than 70%. If the sequence does not meet these criteria, a sequence further downstream starting with an AA dimer is analysed. This is continued until a sequence is found which meets all of the above criteria. Subsequently the 21 base sequence is subjected to a blast search to ensure that only one gene is targeted.

Table 1: siRNA Sequences

Name	Sequence of SiRNA Duplex	GC Content	Mol Weight
HRT 1	**aa gacggagaggcaucaucga**	**52%**	**13204.2g/mol**
HRT 2	**aa ccaccucucagauuauggc**	**52%**	**13208.0g/mol**
HRT 3	**aa gcgcagagggaucauagag**	**55%**	**13372.0g/mol**
VEGF	**aa guucauggacgucuaccag**	**52%**	**13220.2g/mol**
Control	**Aa auucuaucacuagcgugac**	**42%**	**13373.0g/mol**

2.4.6 Transfection of siRNA Duplex:

siRNA was transfected into RVSMCs using lipofectamine™ 2000. siRNA was diluted in 5X universal buffer [200mM KCl; 30mM HEPES-KOH pH 7.5; 1mM $MgCl_2$] and RNase free water to a final concentration of 20μM (20pmoles/μl). The following procedure was utilised for transfection in a 6-well format. 8×10^5 cells were plated per well to achieve 70–80% confluency at time of transfection. siRNA/lipofectamine ™ 2000 complexes were prepared as follows:

- 5μl (100pmoles/10cm^2) of siRNA was diluted in 395μl of DME medium without serum or antibiotics, the presence of antibiotics during transfection causes cell death. The mixture was then gently mixed.
- Lipofectamine ™ 2000 was mixed prior to use and subsequently 5μl was diluted in 395μl of DME medium and incubated for 5 min at room temperature.
- The two mixtures were then combined and mixed gently. The mixture was incubated for 30 min at room temperature to allow the formation of siRNA/lipofectamine ™ 2000 complexes.
- The 800μl of siRNA/lipofectamine ™ 2000 complexes were added to the well and mixed gently by slowly rocking the plate back and forward to ensure complete coverage of the cells. Cells were incubated for 3 hr at 37°C in a CO_2 incubator.
- After the 3 hr incubation the media was removed and replaced with growth medium and cells were recovered overnight. Following recovery, media was replaced with fresh growth media and cells and media were harvested 24 hr later for analysis.

A non-specific control siRNA sequence (MWG-Biotech) was transfected into control cells.

2.5 SDS-PAGE and Western Blot Analysis

2.5.1 Western Blotting:

SDS–PAGE was performed as described by Laemmli using 10% polyacrylamide gels [Laemmli., 1970]. 10% resolving and 5% stacking gels were prepared as follows:

Resolving Gel:	1.5ml	1.5M Tris pH8.8
	1.5ml	40% acrylamide stock
	3ml	distilled water
	60μl	10% (w/v) SDS
	30μl	10% (w/v) ammonium persulphate
	7μl	TEMED
Stacking Gel:	0.75ml	0.5M Tris pH6.8
	0.375ml	40% acrylamide stock
	1.85ml	distilled water
	30μl	10% (w/v) SDS
	15μl	10% (w/v) ammonium persulphate
	7μl	TEMED

Cell lysate protein concentration was determined by BCA assay as previously described 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 resevior buffer (0.025M Tris pH 8.3; 0.192M Glycine; 0.1% (w/v) SDS) at 40 milliamps (mA) 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 16 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 30 min at 100V 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.

Membranes were blocked for 1 hr in blocking solution [5% (w/v) skimmed milk in Tris Buffered Saline [TBS];10mM Tris pH8.0; 150mM NaCl)]. Membranes were then incubated either overnight at 4°C or for 3-4 hr at room temperature, with primary antibody diluted according to manufacturers instructions in blocking solution. The blots were then vigorously washed in three changes of TBST (0.05% (v/v) Tween in TBS) and then incubated for 2 hr at room temperature with the appropriate HRP linked secondary antibody diluted in TBST. Following incubation in secondary antibody, the blots were again washed in three changes of TBST.

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 subsequently developed (Amersham Hyperprocessor Automatic Developer). Bands of interest were identified either by use of an antigenic positive control or based on molecular weight markers. Exposure times varied depending on the antibody being used but were typically between 1-5 min. Bands on a developed film were photographed using a Kodak DC290 digital camera. The image generated was then analysed using Kodak ID (version 3.5.4) densitometry imaging software. Briefly, a mean densitometric value was generated for each band, these values were then corrected using ponceau controls for each lane. The corrected values were then expressed as fold increase over negative control (where applicable) and graphically expressed using prism.

2.5.2 Table 2: Antibody Dilutions

Primary and secondary antibodies were diluted to the concentrations outlined in the table.

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 Notch 3 (Santa Cruz)	1:800	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti Jagged (Santa Cruz)	1:800	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti HRT-1 (Santa Cruz)	1:500	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti HRT-2 (Santa Cruz)	1:500	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti HRT-3 (Santa Cruz)	1:800	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti Hes-1 (Santa Cruz)	1:800	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti Hes-5 (Santa Cruz)	1:800	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti Bcl-X_L (Santa Cruz)	1:500	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:2000
Anti Bax (Upstate)	1:800	HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences)	1:1000
Anti Shh (Santa Cruz)	1:1000	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti Ihh (Santa Cruz)	1:1000	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000
Anti Ptc (Santa Cruz)	1:1000	HRP-Conjugated Anti-goat IgG (Amersham Biosciences)	1:1000

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Preparation of Total RNA

Total RNA was isolated from RVSMC according to the method of Chomczynski and Sacchi (1987) using Trizol® reagent, a mono-phasic solution of phenol and guanidine isothiocyanate. Trizol reagent maintains the integrity of RNA while disrupting cells and dissolving cell components. Growth media was removed and cells were washed with HBSS 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 falcone tube and incubated for 5 min at 15°C to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of trizol was then added, the tube was then shaken vigorously for 15 sec and then spun down at 12,000 x g for 15 min at 2 to 8°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°C to 30 °C for 10 min and spun down at 12,000 x g for 10 min at 2 to 8 °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. All total RNA preparations were stored at -80 °C.

2.6.2 Quantification of Total RNA in Samples

To determine the amount of total RNA in samples obtained in section 2.6.1, the sample was diluted 1:500 in sterile water and spectrophotometric analysis carried out using the Shimadzu UV-160A dual spectrophotometer, blanked with RNase free water. The sample was measured, using a quartz cuvette, at wavelengths of 260 and 280 nm, and the concentration of the RNA in the sample was carried out as follows;

Abs_{260nm} x dilution factor x 40 = concentration of RNA (µg/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 or a dirty cuvette. 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.

2.6.3 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. 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 and are listed in table 3.

Table 3: Primer Sequences

Target Gene	Primer Sequence	Annealling Temp (°c)	product size (Base pairs)
Notch1 (rat)	for 5' GAGTCACCCCATGGCTAC 3' rev 5' GTGGCTGCACCTGCTGG 3'	55 55	550
Notch3 (rat)	for 5' GACCGTGTGGCCTCTTTCT 3' rev 5' GCAGCTGAAGCCATTGACTCT 3'	55 55	390
Hrt1 (rat)	for 5' GTCTGAGCTGAGAAGGCTGGT 3' rev 5' GGAATGTGTCCGAGGCCC 3'	55 55	290
Hrt2 (rat)	for 5' CATCAGAGTCAACGCCATGT 3' rev 5' GACACTGATAACGGTGGGCT 3'	55 55	330
Hrt3 (rat)	for 5' GTGGCACAGGGTTCTTTGAT 3' rev 5' GCTGAGATAGGGTAAGGGGG 3'	55 55	340
Hes1 (rat)	for 5' GAGAAAAATTCCTCGTCCCC 3' rev 5' TGATCTGGGTCATGCAGTTG 3'	55 55	280
Hes5 (rat)	for 5' GCTCAGTCCCAAGGAGAAAA 3' rev 5' GTCGGGGTCTCCTTGACAG 3'	55 55	380
Jagged1 (rat)	for 5' AACAGAACACAGGGATTGCC 3' rev 5' AGGTTTTGTTGCCATTCTGG 3'	55 55	147
Bax (rat)	for 5' TTGCCCTCTTCTACTTTGCT 3' rev 5' CAAAGATGGTCACTGTCTGC 3'	60 60	207
Bcl-X_L (rat)	for 5' TGAAACCACCTAGAGCCTTG 3' rev 5' AATGACCACCACCTAGAGCCT 3'	60 60	170
Shh (rat)	for 5' GGCCATCATTCAGAGGAGTC 3' rev 5' CCACGGAGTTCTCTGCTTTC 3'	60 60	210
Ihh (rat)	for 5' TGGAGAAGAGTCCCCTGAGA 3' rev 5' GCGGCCCTCATAGTGTAAAG 3'	60 60	230
Dhh (rat)	for 5' GACCTCGTCCCCAACTACAA 3' rev 5' TAGAGCATTCACCCGCTCTT 3'	60 60	180
Smo (rat)	for 5' AATTGGCCTGGTGCTTATTG 3' rev 5' CTGAAGGTGATGAGCACGAA 3'	60 60	230
Ptc1 (rat)	for 5' GCTGGAGGAGAACAAGCAAC 3' rev 5' CCAGGAGTTTGTAAGCGAGG 3'	60 60	164
Gli2 (rat)	for 5' CGCCTGGAGAACTTGAAGAC 3' rev 5 TTCTCATTGGAGTGAGTGCG 3'	60 60	168
GAPDH (rat)	for 5' TGCTGAGTATGTCGTGGAGT 3' rev 5' GCATTGCTGACAATCTTGAG 3'	60 60	350

2.6.4 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 Promega Murine Leukemia Virus Reverse Transcripase (MLV-RT) as follows. The initial amount of RNA used for each primer set was determined empirically to ensure a semi quantitative analysis section. For the purpose of this protocol 2 μg of RNA was transferred to an RNase free microcentrifuge tube. To this 1 μl of Promega Oligo dT primer was added. This ratio of amount of RNA to Oligo dT remained constant for each primer set used. The volume was then made up 12 μl with RNase free water. The tube was heated to 70°C for 5 min to melt secondary structure within the template. The tube was cooled immediately to prevent secondary structure from reforming. The following cocktail was made up and added to each reaction:

MLV 5x Reaction buffer	5 μl
dATP, 10 mM	1.25 μl
dCTP, 10 mM	1.25 μl
dGTP, 10 mM	1.25 μl
dTTP, 10 mM	1.25 μl
MLV-RT	1 μl
RNase free water	2 μl

The components were mixed by gently flicking the tube. The mix was then spun down in a microfuge and incubated for 60 min at 42°C, followed by a 15 min incubation at 70°C. 1 μl of RNaseH (2 units/μl) was then added per reaction, and was incubated for 20 min at 37°C. A negative control was also carried out with every RT reaction. The control contained no reverse transcriptase, any amplification by RT PCR from the negative control was indicative of genomic DNA contamination. All RT samples were stored at -80°C until needed for PCR.

2.6.5 Polymerase Chain Reaction (PCR)

PCR was carried out using Promega Taq DNA Polymerase. A PCR mix was made up for each sample to be amplified. The mix was made up as follows:

RNase free water	36.5 µl
Buffer 10x	5.0 µl
dNTP 10mM	1.0 µl
$MgCl_2$ 25mM	3.0 µl
Forward primer 10 µM	1.0 µl
Reverse primer 10 µM	1.0 µl
Taq Polymerase 2.5u/µl	0.5 µl
RT sample	2.0 µl
Mineral oil	50.0 µl

When the reaction mixture was made up it was placed in the PCR thermocycler. All PCR were intiallly carried out in a Hybaid PCR thermocycler (SPRT 001), used to optimize conditions for PCR. The program used was optimised for each primer set, with the annealing phase being 55°C for the Notch signalling pathway primers, and 60°C for the apoptosis primers. For the purpose of this protocol the program was as follows:

Denaturing Phase: 94^0C – 4 minutes
Annealing Phase: 55^0C/60^0C – 2 minutes
Elongation Phase: 72^0C – 3 minutes
} 40 cycles

4^0C – Hold

When finished the samples were stored at –80°C until needed for agarose gel electrophoresis.

2.6.6 Agarose gel electrophoresis

All DNA gel electrophoresis was carried out using a Gibco BRL 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 12.5 g of agarose in 500 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. 100 ml of the liquid agarose was then transferred to a fresh glass beaker. To this 250 µl of 200 µg/ml of Ethidium Bromide (EtBr) solution was added and mixed thoroughly to give a final concentration of 0.5 µg/ml EtBr. 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 buffer. The samples were prepared as follows: 13 µl of PCR product + 5 µl of 4x loading dye. 8 µl was loaded 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. When finished the gel was placed on an Ultra Violet Products UV transilluminator for visualization. A picture was taken using a Kodak DC290 digital camera for documentation. The image generated was then analysed using Kodak ID (version 3.5.4) densitometry imaging software. Briefly, a mean densitometric value was generated for each band, these values were then corrected using housekeeping gene controls (gapdh) for each lane. The corrected values were then expressed as fold increase over negative control (where applicable) and graphically expressed using prism. The gel was then disposed of in the appropriate EtBr waste container.

2.6.7 Real-Time PCR

Quantitative PCR was also carried out using a Real time Rotor-GeneRG-3000™ lightcycler (Corbett Research). The principle of real time amplification detection is that the amount of fluorescence is directly 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	12.5 µl
RNAse free water	8.5 µl
cDNA	2.0 µl
Forward primer 10uM	1.0 ul
Reverse primer 10uM	1.0 ul

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

Denaturing Phase: 95^{0}C - 20 s
Annealing Phase: 55-60^{0}C - 30s (Notch/hh primers)
57 -60^{0}C- 45s (Apoptosis primers)
Elongation Phase: 72^{0}C - 30 s

} 55 cycles

The Comparative Ct method (comparing cycle times where DNA amplification begins) was used for quantiatative analysis while Melt Curve analysis was carried out for qualitative analysis.

2.7. Cell Growth Assays

2.7.1 RVSMC Proliferation

To analyze RVSMC proliferation, cells were seeded equally at a density of 1×10^3 cells per well after serum deprivation for 48hr. Cells were then counted using a Brightline Haemacytometer at 3 day intervals 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. Concurrently cells were fixed using isopropanol and stained using Haematoxylin and Eosin for visualization using the Olympus C40 microscope for comparative cell proliferation analysis.

2.7.2 RVSMC Apoptosis

2.7.2.1 Acridine Orange/Ethidium Bromide Cell Staining

The staining of RVSMCs with the Acridine Orange/Ethidium Bromide (AO/EtBr) dual stain (10 μg/ml, Sigma) allows determination of viable, apoptotic and necrotic cells concurrently. Acridine Orange is a metachromatic dye which differentially stains double stranded (ds) and single stranded (ss) nucleic acids. When acridine orange intercalates into the dsDNA of healthy cells it emits a green fluorescence upon excitation at 480-490 nm. However, this stain emits an orange fluorescence when intercalated with the ssDNA of apoptotic cells. Ethidium bromide is a nucleic acid stain which does not permeate viable or apoptotic cells. It does, however, penetrate necrotic cells due to plasma membrane disruption and stains the nucleus red. Therefore, viable cells appear to have a bright green nucleus with intact structure, while apoptotic cells exhibit a bright green nucleus showing condensation of chromatin as dense green areas. Late apoptotic cells have an orange nucleus showing condensation of chromatin, while necrotic cells display a red nucleus with intact structure. Briefly, cells were washed twice in 1x PBS, and fixed in ice-cold isopropanol for 5 min. Cells are then rehydrated in 1x PBS for 7-8 min, and stained with the AO/EtBr dual nuclear stain for 5 min, rinsed with PBS, and visualized using an Olympus DP-50 fluorescent microscope (excitation 460-490 nm, emission 515-565 nm).

2.8 *In Vivo* Studies

2.8.1 Carotid Artery Ligation

The left carotid arteries of male Sprague-Dawley rats (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 (inhalational). Following induction of anesthesia, the animal was positioned on a clean operating table, with a 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 28 days post-ligation for RNA isolation (4 vessels per preparation) and protein isolation (2 vessels required per preparation). Terminal surgery was carried out by halothane inhalation and cervical dislocation, followed by harvesting of the carotid arteries.

2.8.2 Portal Vein Ligation

This procedure involved the portal vein ligation (PVL) of male Sprague-Dawley rats (Charles River Laboratories, Massachusetts, USA). 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 (inhalational). Following induction of anesthesia, the animal was positioned on a clean operating table, with a body pad to maintain body temperature. The animal was clipped and the surgical site prepped using betadine solution and alcohol. An appropriate incision was made, and, with the aid of a dissecting microscope, the portal vein was isolated, and a stenosis was created by means of a single ligature placed around the portal vein using a blunt-edged 20-gauge needle. The immediate removal of the needle allowed for the portal vein to expand to the limit imposed by the ligature (PVL). 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 portal vein ligation. Vessels were harvested from sham operated and ligated animals at 2, 4, 6, 9 and 15 days post-ligation for RNA isolation (4 vessels per preparation) and protein isolation (2 vessels required per preparation). Terminal surgery was carried out by halothane inhalation and cervical dislocation, followed by harvesting of the portal vein (Hou *et al.*, 1998).

2.9 Data Analysis

Results are expressed as mean ± SEM. Comparison between control versus treated cells were made by (i) Student's unpaired t test and (ii) Wilcoxon matched-pairs signed-rank test, with statistical significance established at $p \leq 0.05$.

Chapter 3

Results

Notch Receptor Signalling Modulates VSMC Growth via a CBF-1/RBP-Jκ-dependent Pathway

3.1 Introduction

The determination of VSMC fate (growth, migration, differentiation and apoptosis) is fundamental to the pathogenesis of vascular disease (Swhwartz and Henry, 2002). Pathological changes in vessel structure are induced, in part, by signalling pathways that govern SMC growth, death, differentiation and migration (Ferguson and Patterson, 2003). This change in SMC fate is believed to play an important role in neointimal formation during the pathogenesis of cardiovascular disease, including atherosclerosis and hypertension, and in the arterial response to injury. However, how these cell fate decisions are regulated and controlled remains poorly understood. Because changes in vascular cell fate are also apparent during vascular morphogenesis and modelling of the embryonic vasculature (Majesky 2003), the control of these cell fate decisions in adult cells may share similar signalling patterns. A number of pathways have been implicated in the control of vasculogenesis and angiogenesis; these include the VEGF, PDGF, and ephrin/Eph receptor pathways (Kojika and Griffin 2001). Recent studies have added the Notch signalling pathway to this list. Notch receptor-ligand interactions are a highly conserved mechanism, originally described in developmental studies using *Drosophila* that regulates intercellular communication and direct individual cell fate decisions (Iso *et al.*, 2003). Notch receptors and ligands are transmembrane proteins that have been identified in mammalian cells (Notch receptors: 1 to 4; Notch ligands: Delta, Serrate, and Jagged). Studies using constitutively activated Notch receptors missing their extracellular domains Notch intracellular (IC) have shown that Notch signalling determines proliferation, differentiation, and, more recently, apoptosis in several mammalian cell types. Following cleavage by presenilin, Notch IC is translocated to the nucleus where it interacts with the CSL family of transcription factors (CBF-1/RBP-Jk, Su (h) and LAG-1). CSL factors alone or as a complex with Notch are believed to become transcriptional activators that can then modulate the expression of Notch target genes that regulate cell fate decisions (Lai, 2002). These include the hairy enhancer of split (*hes*) gene and HES-related transcription factors (HRTs) that are critically involved in mammalian cell differentiation (Iso, 2003).

Recent studies report that Notch-receptor ligand interactions are prevalent during fibroblast growth factor-induced angiogenesis, suggesting a major role for this

signalling mechanism in regulating vascular development (Zimrin *et al.,* 1996). In addition, Notch receptors and downstream effectors (HRTs) play a critical role in cell fate determination during vascular ontogeny (Iso 2003). Mutations of Notch signalling pathway components in mice, for example, results in embryonic lethality due to defects in the formation of the vascular system. Mice homozygous for null mutations in Jagged 1 and Notch 1 die *in utero* due to defects in vascular morphogenesis and angiogenic vascular remodelling (Han *et al.,* 2000). In addition, mutations in Notch target genes also highlight the importance of Notch-receptor ligand interactions in vascular development. Zebrafish embryos harbouring a mutation in the gridlock gene, a hrt-2 orthalogue, show impaired vascular formation due to aortic coarctation (Zhong *et al* 2000). While the role of the Notch signalling pathway in development is reasonably well established its functional role in adult VSMC remains to be fully elucidated. However if the adult SMC phenotype is not terminally differentiated and is similar in both morphology and gene expression to embryonic SMC (Gittenberger-de Groot *et al.,* 1999), it is highly probable and conceivable that Notch signalling could be both present and playing an equally important functional role in adult VSMC.

Dysregulation of the Notch signaling pathway underlies a number of adult disorders with associated vascular pathologies, such as CADASIL and AGS (Loomes *et al.,* 1999; Brulin *et al.,* 2002). The human CADASIL syndrome of premature stroke and dementia is a heritable arteriopathy with alterations in VSMC, which results from mutations within the Notch 3 receptor (Kalaria *et al., 2002*). It is now becoming clear that the well-described modulation of SMC fate following stimulation involves a coordinate regulation of the Notch and HRT pathways in several cell types (Wang 2002). Indeed, recent studies demonstrate that Notch receptors and *hrt* genes are coordinately up-regulated in neointimal cells but down-regulated in medial cells following vascular injury, an effect that is mimicked by addition of serum mitogens (PDGF) to cultured cells *in vitro* (Wang *et al.,* 2002). Furthermore, initial evidence that Notch 3 signalling may be a critical determinant of SMC survival and vascular structure by modulating the expression of downstream mediators of apoptosis via signalling cross-talk with the ERK/MAPK pathway has been presented (Wang *et al.,* 2003).

In most cell types examined, expression of Notch 1 IC leads to increased CBF-

1/RBP-Jk-dependent gene expression. However, increasing evidence indicates that Notch signalling may occur independent of CBF-1/RBP-Jκ gene expression. In 1996, Shawber *et al.,* provided the first *in vitro* evidence of a Notch-activated CBF-1-independent pathway whereby Notch 1 prevented the differentiation of muscle cells (C2C12) upon serum withdrawal, without the interaction with CBF-1 or up-regulation of endogenous Hes-1 expression. This present study examined the endogenous role of Notch 1 and 3 receptors in controlling the modulation of SMC growth in response to growth factor stimulation. In addition we demonstrate that *hes* and *hrt* are direct downstream target genes of Notch 1 and 3 signalling in SMC and that a CBF-1/RBP-Jκ dependent mechanism is essential for this regulation. Moreover, transient overexpression of constitutively active Notch 1 and 3 IC or inhibition of endogenous Notch signalling and target gene expression resulted in a modulation of CBF-1/RPB-Jk dependent promoter activity concomitant with changes in SMC growth. Furthermore we examined the role of individual hrt genes in controlling the modulation of SMC growth *in vitro.* Hrt gene knockdown by siRNA resulted in a significant inhibition of SMC growth while concurrently increasing SMC apoptosis. An increased understanding in the regulation of SMC fate in the vasculature, has the potential to lead to future therapeutic benefits, as it is aberrant SMC fate, which is a major cause of vascular disease.

The aim of this chapter was to determine the role Notch 1 and 3 receptors in controlling the modulation of SMC growth and in addition, to examine the individual role of downstream notch target genes, hrt 1, 2 and 3 in determining SMC growth in vitro.

3.2 Results

3.2.1 Notch Receptors and Downstream Target Genes in SMC

The presence of Notch 1 and 3 receptors in serum-stimulated adult RVSMC was examined *in vitro*. Immunohistochemical analysis revealed the presence of Notch 1 and 3 IC receptors in these cells with significant nuclear and cytoplasmic staining (**Fig 3.1**). The presence of Notch 1 and 3 receptors was further confirmed by Western blot analysis and RT-PCR (**Fig 3.1**). Notch 1 and 3 IC domains were present in cycling cells as a protein with an apparent molecular weight of 120 and 90 kDa, respectively. In a similar manner, Notch target genes, *hes* (*hes -1* and *-5*), and HRTs (*hrt-1*, *-2*, and *-3*) were present in these cycling cells (**Fig 3.1B**) and expressed at the protein and mRNA level and by immunohistochemical analysis.

3.2.2 Notch 1 and 3 IC Receptors Activate Notch Target Genes in a CBF-1-Dependent Manner.

We confirmed that Notch 1 and 3 receptors were significantly overexpressed in RVSMC at both the mRNA and protein level following constitutive expression of Notch 1 and 3 IC (**Fig 3.2 A and B**). In addition, the specific over-expression of Notch 1 and 3 IC proteins was confirmed using anti-HA antibodies specific for both Notch 1 and 3 IC proteins Moreover, in cells co-transfected with the luciferase reporter plasmids, pGa981-6, which contains a CBF-1-regulated enhancer linked to the β-globin minimal promoter or the corresponding enhancerless control vector, pGa50-7, we found a significant increase in CBF-1/RBP-Jk dependent promoter activity following constitutive expression of Notch 1 and 3 IC, 24±5.09 and 15.72±3.8 respectively (**Fig. 3.3 A and B**). In addition, Notch 1 and Notch 3 IC dependent reporter gene expression was significantly inhibited following co-expression with the mutant Notch 1 IC receptor (mNotch 1 IC), and by using a selective inhibitor of the CBF-1/SKIP interaction by 71±2.0% and 52.29±19.1% respectively. In addition, the Epstein Barr virus encoded gene product RPMS-1, resulted in a 71.38±4.1% decrease in Notch 1 IC dependent reporter gene expression and a 79.95±8.27% in Notch 3 IC reporter gene expression. Following treatment with

Brefeldin A, a pharmacological inhibitor of Notch receptor trafficking from the Golgi apparatus, a marked decrease in CBF-1 transactivation of 78.75±3.75% and 57.86±4.56% was observed for Notch 1 and 3 IC respectively (**Fig 3.3 A and B**). In a similar manner, constitutive expression of Notch 1 and 3 IC significantly increased the transactivation of a native Notch target gene promoter *hrt-1* by 19.51±4.17 and 4.96±1.22 respectively (**Fig 3.4A**). Moreover, constitutive expression of mNotch IC significantly inhibited Notch 1 IC dependent transactivation of the *hrt-1* promoter by a decrease of 77.25±7.89% (**Fig. 3.4 B**). We used quantitative Real-Time RT-PCR (QRTPCR) to determine the effects of inhibiting CBF-1/RBP-Jκ regulated promoter activity on Notch target gene mRNA levels in these cells. Using QRTPCR analysis, constitutive expression of Notch 1 IC revealed a significant increase in Notch target gene mRNA levels (*hrt-1*, *hrt-2*, and *hes-5*) by 2.98±0.81, 1.51±0.52 and 2.19±0.05, an effect that was significantly inhibited following co-expression of RPMS-1 by 71.48±0.33%, 79.48±3.31% and 90.42±1.14% respectively (**Fig 3.5 A**). RPMS-1 has previously been shown to bind directly to the CBF-1 nuclear complex and inhibit Notch IC CBF-1/RBP-Jκ dependent promoter activity (Smith *et al.,* 2001; Zhang *et al.,* 2001). Similarly, constitutive expression of Notch 3 IC resulted in a significant increase in mRNA levels of Notch target genes of 3.55±0.5, 1.62±0.41 and 1.52±0.39 for *hrt-1, 2 and 3* respectively, an effect that was also significantly attenuated following inhibition of CBF-1/RBP-Jk regulated transcriptional activity by co-expression of RPMS-1 by 80.0±0.28%, 53.1±4.3%, and 53.95±3.2% (**Fig 3.5 B**).

3.2.3 Serum-Stimulated Notch Target Gene Expression in SMC *in vitro*

In cells transfected with pGa981-6, a significant temporal increase was reported in luciferase activity following serum addition. The increase in pGa981-6 promoter activity post serum was 17.9±5.4, 26.6±2.3 and 11.0±0 at 4, 8 and 24 hr respectively (**Fig 3.6 A**). Co-transfection with a vector encoding a non-DNA binding mutant of CBF-1/RBP-Jκ (R218H), which inhibits CBF-1/RBP-Jκ regulated gene expression in SMC (Kato *et al.,* 1997), inhibited serum stimulated CBF-1/RBP-Jκ regulated promoter activity in these cells at 4 h by 41.57±12.34%, and a significant attenuation at 8 h and 24 h by 76.32±3.64% and 70.37±2.36% respectively, as compared to serum stimulated CBF-1 luciferase activity at each time point (**Fig 3.6 A**). Co-expression of

RPMS-1 significantly decreased serum-stimulated CBF-1/RBP-Jκ-regulated promoter activity in these cells by 47.21±1.9%, 56.43±18.34% and 28.82±8.0% at 4 h, 8 h, and 24 h post-serum stimulation (**Fig 3.6 A**). In a similar manner, the effect of co-transfection of mNotch 1 IC, and the pharmacological inhibitors, Brefeldin A and Monensin, both of which attenuated serum-stimulated increases in CBF-1 luciferase activity, was investigated (**Fig 3.6 B**). All three inhibitors resulted in a significant attenuation of the serum-induced increase in CBF-1 luciferase activity at 8 h post-serum stimulation. Cells treated with Brefeldin A and Monensin exhibited a 69.73±10.72% and 81.61±7.85% decrease in luciferase activity respectively, and cells co-transfected with mNotch 1 IC exhibited a 83.91±11.87% decrease in CBF-1 luciferase activity, as compared to that at 8 h post-serum stimulation. An attenuation in the serum-stimulated increase in CBF-1 luciferase activity was also seen at 10 h post-serum addition, with decreases of 61.6±16.54%, 69.07±10.79% and 84.9±3.59% by Brefeldin A, Monensin and mutant Notch 1 IC respectively. In parallel studies, a significant temporal increase was found in transactivation of *hrt-1* promoter following serum stimulation compared with mock control The effect of serum stimulation on *hrt-1* promoter activity was investigated over a period of 24 h (**Fig 3.7**). Serum stimulation resulted in a significant fold increase (24.6±6.4) in *hrt-1* luciferase activity at 8 h post-serum addition. Similarly, fold increases in *hrt-1* luciferase activity of 13.6±4.8 and 6.0±2.2 were evident at 10 h and 24 h post-serum addition. Co-transfection of the Notch inhibitor, RPMS-1, resulted in an attenuated fold increase in luciferase activity due to serum stimulation (**Fig 3.7**). The fold increase was significantly attenuated by 82.65±10.97% and 81.62±6.6% at 8 h and 10 h post-serum addition respectively, and by 56.5±21.6% at 24 h post-serum addition, as compared to serum stimulated CBF-1 luciferase activity at each time point.

Using semiquantitative RT-PCR, we demonstrated a significant temporal increase in Notch target gene mRNA levels in quiesced cells following serum stimulation (**Fig 3.8).** Serum-stimulated increases in *hrt-1* and *hrt-2* mRNA levels were maximal after 5–10 h (**3.8 A and B**). Subsequent analysis using QRTPCR, similarly showed that serum stimulation for 8 h resulted in an increase in Notch target gene mRNA as compared to un-stimulated control cells (**Fig 3.8 C**). The fold increases for *hrt-1*, *hrt-2*, *hrt-3*, *hes-1* and *hes-5* being 1.65±0.17, 1.67±0.1, 1.55±0.29, 2.12±.31 and 1.75±0.01 respectively. To further verify the involvement of

the CBF-1-dependent Notch signalling pathway in serum-stimulated increases in Notch target gene mRNA expression, cells were mock-transfected, or transfected with the CBF-1-dependent Notch signalling pathway inhibitor, RPMS-I (**Fig 3.9A**). Quiesced cells were serum stimulated for 8 h, and assayed for Notch target gene mRNA expression. Control levels of the Notch target genes in mock-transfected cells were arbitrarily assigned a value of 1. *Hrt-2* and *hrt-3* mRNA expression levels were significantly decreased by 58.0±1.0% and 65.0±5.0% respectively, similarly, *hrt-1* and *hes-5* mRNA expression levels were also decreased by 58.0±12.0% and 67.0±13.0% respectively due to the presence of RPMS-I. Serum-stimulated *hes*-5 mRNA levels after 8 h were significantly attenuated following co-expression of RPMS-1 (**Fig 3.9 B**). Subsequent quantitative analysis of Notch receptors by QRTPCR confirmed that serum stimulation increased Notch 1 and 3 receptor mRNA levels after 8 h (**Fig 3.10 A).** Furthermore, treatment of quiesced cells with 10% FCS resulted in a significant increase in Notch 1 IC protein expression (**Fig 3.10 B**).

3.2.4 Notch Receptor Signaling and Serum-Stimulated SMC Proliferation

A significant temporal decrease was found in serum-stimulated SMC proliferation following inhibition of CBF-1/RBP-Jκ activity with RPMS-1 compared with mock controls (**Fig 3.11 A**). Furthermore, constitutive expression of Notch 1 and 3 IC significantly increased SMC proliferation compared with mock controls (**Fig 3.11 B**), an effect that was significantly inhibited by co-expression with RPMS-1 (**Fig 3.11 C**). This increase in serumstimulated SMC proliferation was mirrored in clonal proliferation assays where constitutive expression of Notch 3 IC promoted clonal proliferation, an effect that was also inhibited by coexpression with RPMS-1 (**Fig 3.11 D**). In addition, the expression of proliferating cell nuclear antigen (pCNA), a marker for cell proliferation in SMC, was enhanced in cells following constitutive expression of Notch 1 IC compared with mock control. This increase in pCNA expression was reversed by co-expression with RPMS-1 (**Fig. 3.11 D**).

3.2.5 Notch Target Gene Silencing by siRNA

The presence of Notch target genes *hrt1, hrt2* and *hrt3* in serum-stimulated adult RVSMC was examined *in vitro*. QRTPCR analysis revealed the relative expression levels of hrt1-*3* as compared to GAPDH levels in RVSMC (**Fig 3.12 A**) and the subsequent gene knockdown by transfection of siRNA directed against these Notch target genes as compared to a scrambled control (**Fig 3.12 B**). SiRNA interference resulted in a 69.0±7.7%, 63.0±12.2% and 64.0±7% decrease in *hrt1, 2* and *3* mRNA expression respectively.

3.2.6 Notch Target Gene silencing and Serum Stimulated RVSMC Proliferation

The functional importance of Notch signalling on SMC growth was confirmed by determining the effect of Notch target gene knockdown on SMC growth (balance between proliferation and apoptosis) by selective siRNA directed against Notch target genes, *hrt1-3*. In quiesced cells, a significant decrease was found in serum-stimulated SMC proliferation following *hrt* gene silencing by siRNA as compared with scrambled controls (**Fig.3.13 A**). *Hrt* gene knockdown resulted in a significant decrease in pCNA expression of 55.0±11%, 44.0±8.8% and 33.0±3.7% for *hrt1, 2* and *3* respectively (**Fig 3.13 B**). Furthermore, in parallel cultures, a significant temporal decrease was found in serum-stimulated SMC proliferation following *hrt* gene knockdown whereby the most significant decrease in RVSMC proliferation was observed by *hrt1* gene knockdown.

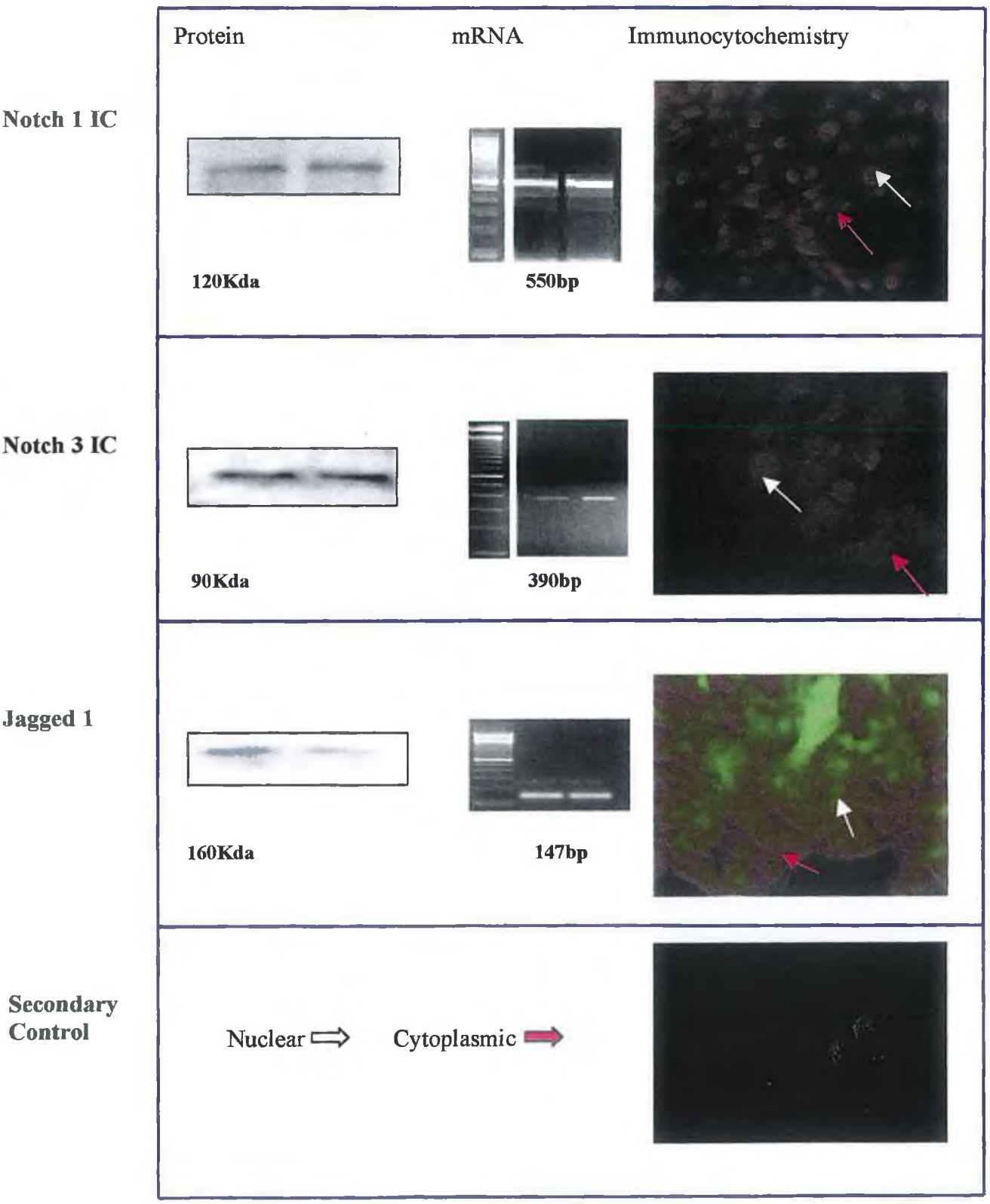
3.2.7 Notch Target Gene Silencing and RVSMC Apoptosis

To further examine the functional importance of the *hrt* genes on SMC growth, we determined the effects of *hrt* gene knockdown on SMC apoptosis. Hrt gene knockdown resulted in a significant increase in pro-apoptotic *Bax* mRNA and protein levels, a decrease in *Bcl-*X_L mRNA and protein levels concomitant with a significant decrease in the number of apoptotic nuclei. An increase in *Bax* expression of 1.7±.386, 4.1±.87 and 11.1±3.56 for mRNA and a subsequent increase in protein expression of 1.67±.08, 1.6±.08 and 1.7±.09 was observed for gene knockdown of

hrt1, *hrt2* and *hrt3* respectively (**Fig 3.15**). In parallel cultures a significant decrease in *Bcl-X_L* expression was observed following *hrt* gene knockdown. 48.0±17%, 58.0±10.6% and 15.0±48.4% inhibition of mRNA was observed while an inhibition in Bcl-X_L protein expression of 48.0±12.2%, 45.0±13.2% and 20.0±3.4% was observed for *hrt1, 2* and *3* knockdown respectively (**Fig 3.16**). Concomitant with the subsequent increase in *Bax* and decrease in *Bcl-X_L* expression an increase in apoptotic nuclei was observed. *Hrt1, 2* and *3* gene knockdown resulted in a 8.1±.05, 4.0±.05 and 12.0±.05 increase in apoptosis respectively (**Fig 3.16**).

Figure 3.1 The Presence of Notch Signaling in Adult VSMC

Protein and mRNA is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. Immunocytochemistry showing protein expression at 20x magnification, and a secondary control. N=3



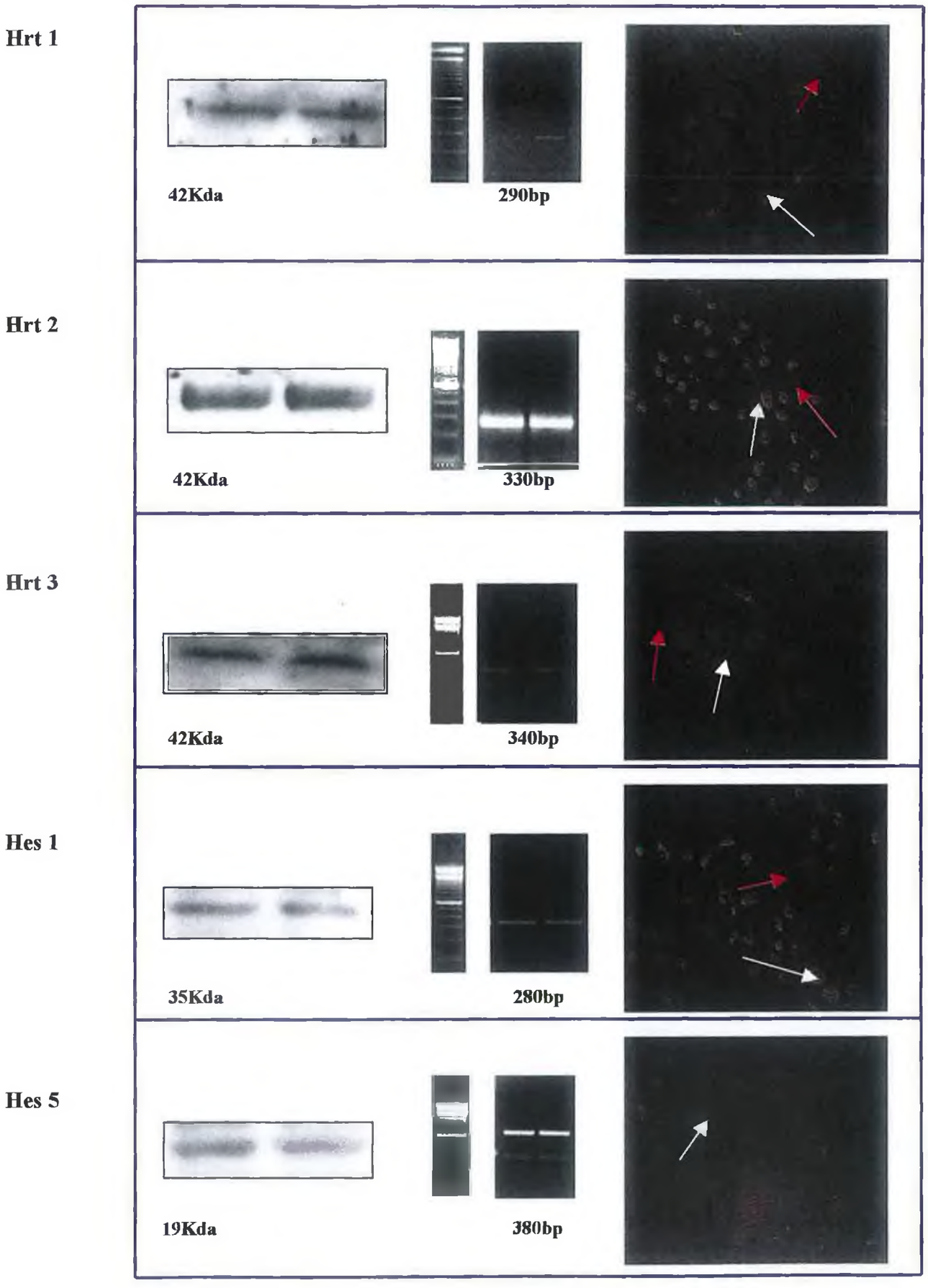

Hrt 1
42Kda
290bp
Hrt 2
42Kda
330bp
Hrt 3
42Kda
340bp
Hes 1
35Kda
280bp
Hes 5
19Kda
380bp

Figure 3.2 Up-regulation of Notch 1 IC and Notch 3 IC in Transfected RVSMC

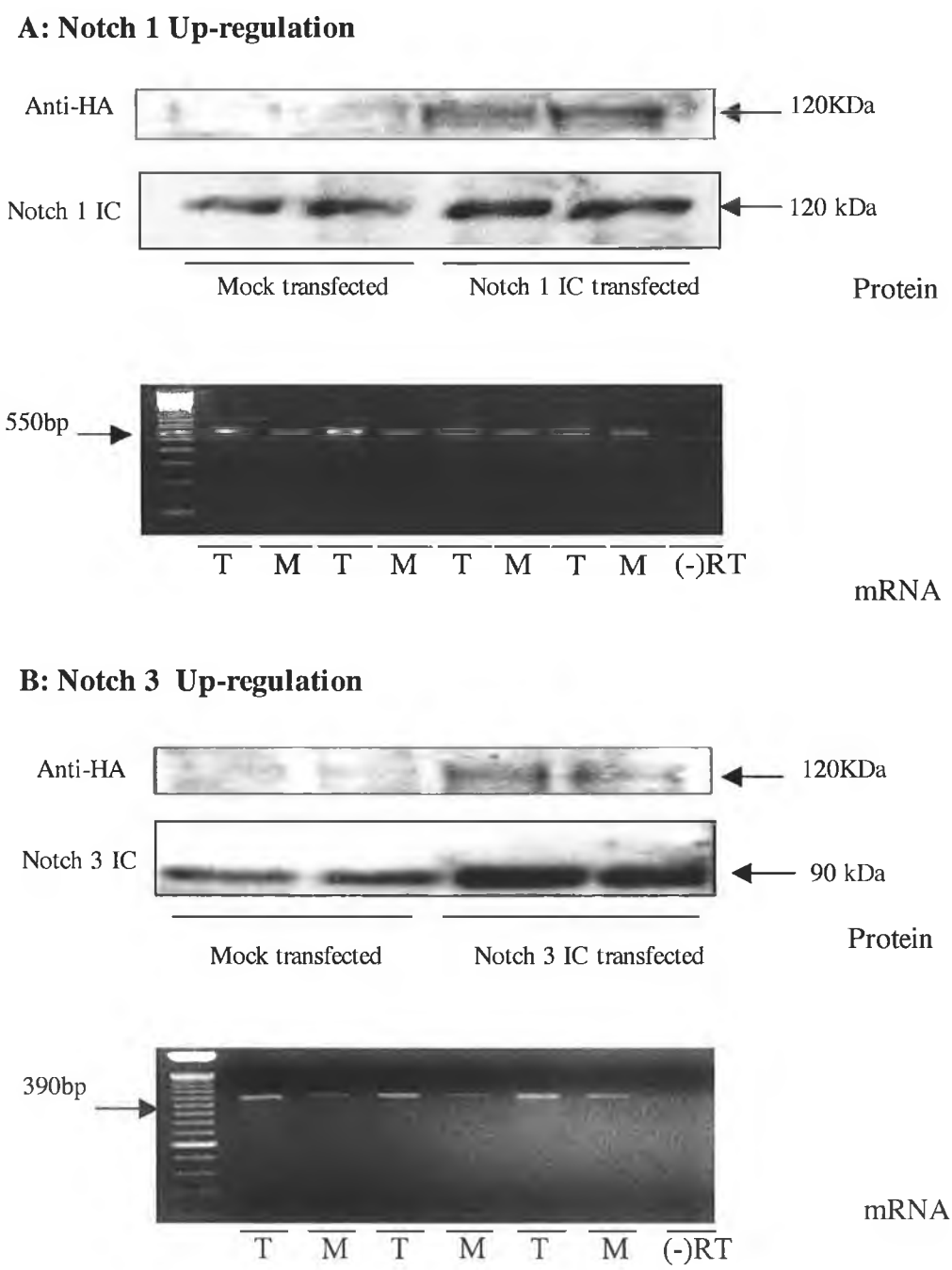


Figure 3.2 Up-regulation of Notch 1 IC and Notch 3 IC in RVSMC. Up-regulation of Notch 1 protein and mRNA (A) expression following transfection with Notch 1 IC. Up-regulation of Notch 3 protein and mRNA (B) expression following transfection with Notch 3 IC. M=mock transfected with p7pcmv empty vector, T=Notch 1 IC/Notch 3 IC transfected. N=3

Figure 3.3 Activation of the Notch Signalling Pathway in RVSMC

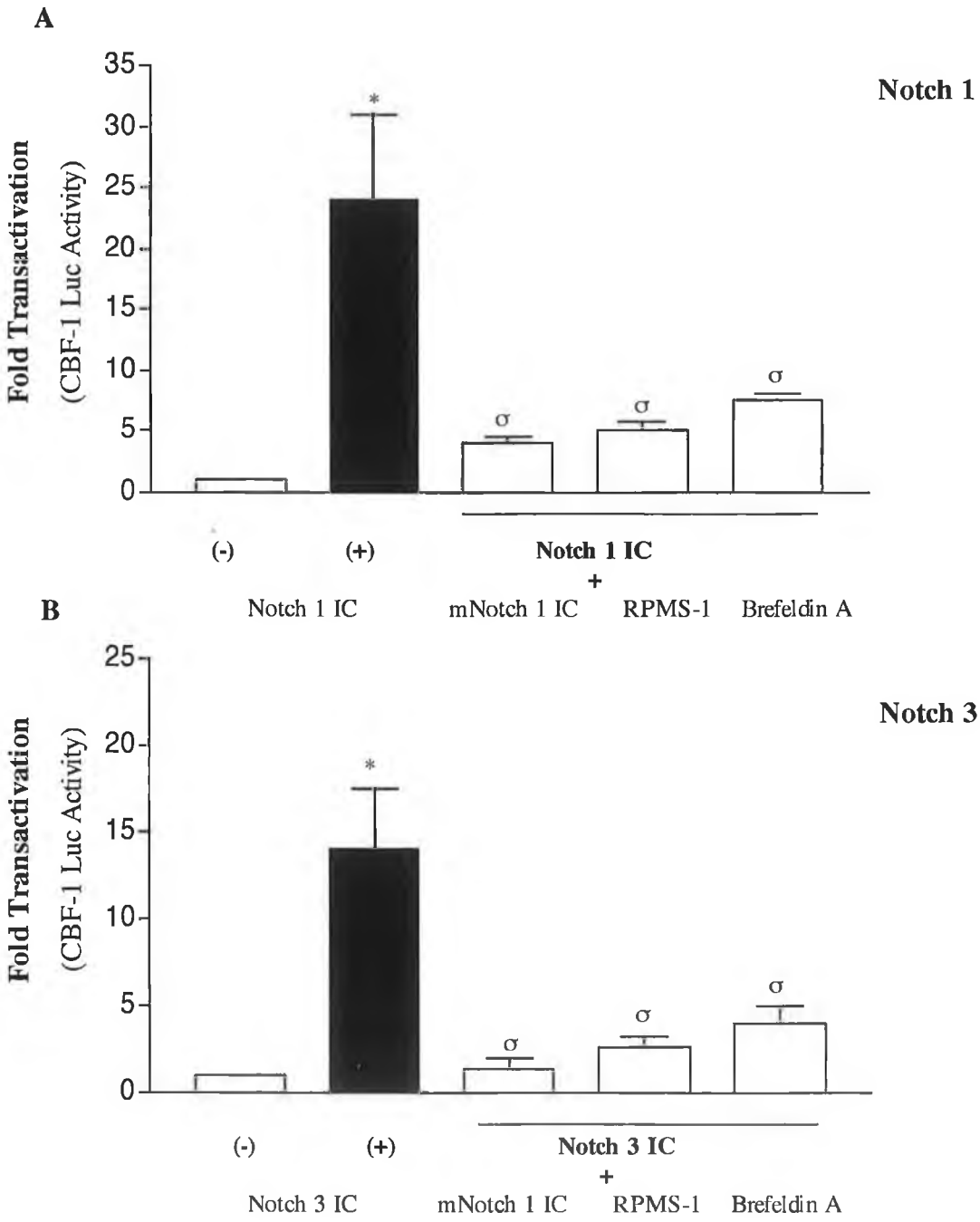


Figure 3.3 Notch 1 and Notch 3 activates CBF-1 activity in RVSMC. RVSMC were transiently transfected with the CBF-1 luciferase-tagged reporter plasmid and co-transfected with Notch 1 IC (A) or Notch 3 IC (B). Additionally, cells were co-transfected with inhibitors of the Notch signalling pathway, mNotch 1 IC, RPMS-I and treated with the pharmacological inhibitor Brefeldin A (0.1μg/ml, 24 h). Luciferase assays were normalized to β-galactosidase activities and protein levels, n= at least 3, and expressed as fold increase over control (the value obtained with pGA98-1-6 transfected cells arbitrarily assigned a value of 1). * $p<0.05$, (rank test) as compared to mock transfected control, $^{\sigma}$ $p<0.05$ as compared to Notch transfected cells (rank test).

Figure 3.4 Activation of Notch Target Genes in RVSMC

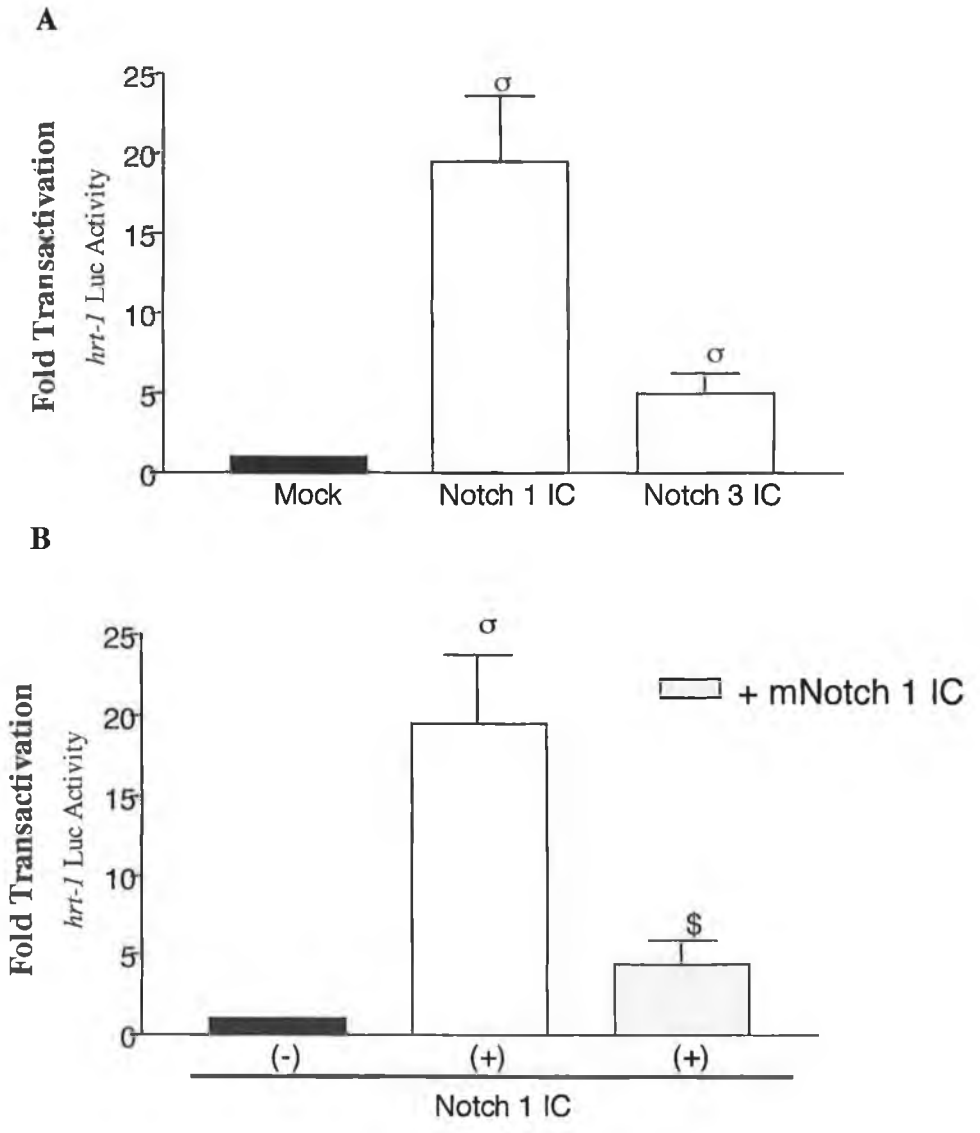


Figure 3.4 Over-expression of Notch 1 and Notch 3 activates notch target genes in RVSMC.
(A) RVSMC were transiently transfected with the HRT-1 reporter plasmid and co-transfected with either Notch 1 IC or Notch 3 IC. B) RVSMC were transiently transfected with the HRT-1 reporter plasmid and co-transfected with Notch 1 IC in the presence and absence of the mutant Notch 1 IC plasmid. Luciferase assays were normalized to β-galactosidase activities and protein levels, n=3, and expressed as fold increase over control (= the value obtained with HRT-1 reporter plasmid transfected cells arbitrarily assigned a value of 1). $^{\sigma}$ $p<0.05$ as compared to mock transfected control cells, $^{\$}$ $p<0.05$ as compared to Notch transfected cells (student's t test).

Figure 3.5 Upregulation of Notch Target Gene mRNA Expression in RVSMC

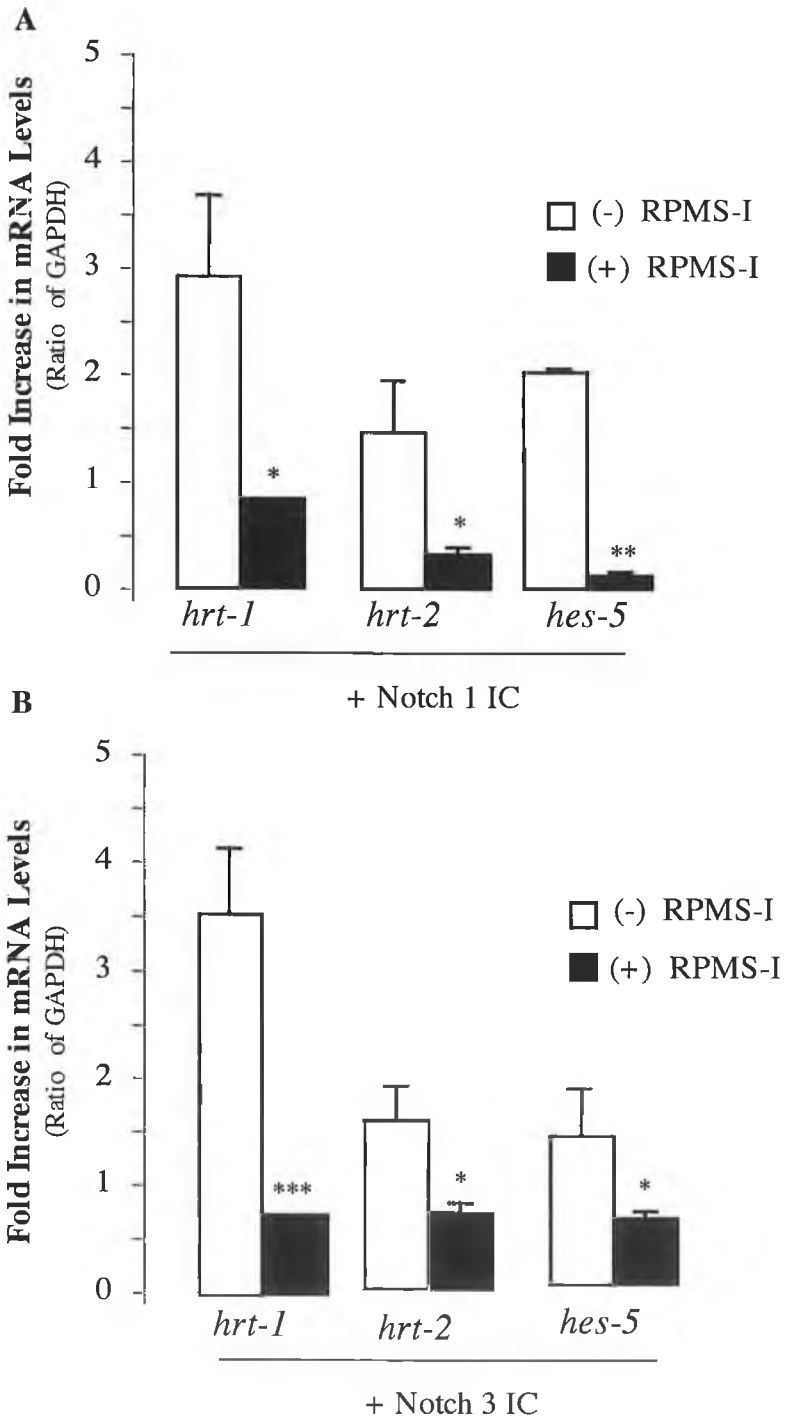


Figure 3.5 Notch 1 and Notch 3 up-regulate Notch Target Gene mRNA Expression,. RVSMC were transiently transfected with Notch 1 IC (A) and Notch 3 IC (B) in the presence and absence of the Notch inhibitor, RPMS-I. mRNA levels were measured using quantitative RT-PCR, and expressed as fold increase over control (aq the levels of target gene present in untransfected cells (not shown)), n=3. *p<0.05, ** p<0.005, *** p<0.005 as compared to mock transfected control (student's t test).

Figure 3.6 Serum Stimulation of the Notch Signalling Pathway in RVSMC

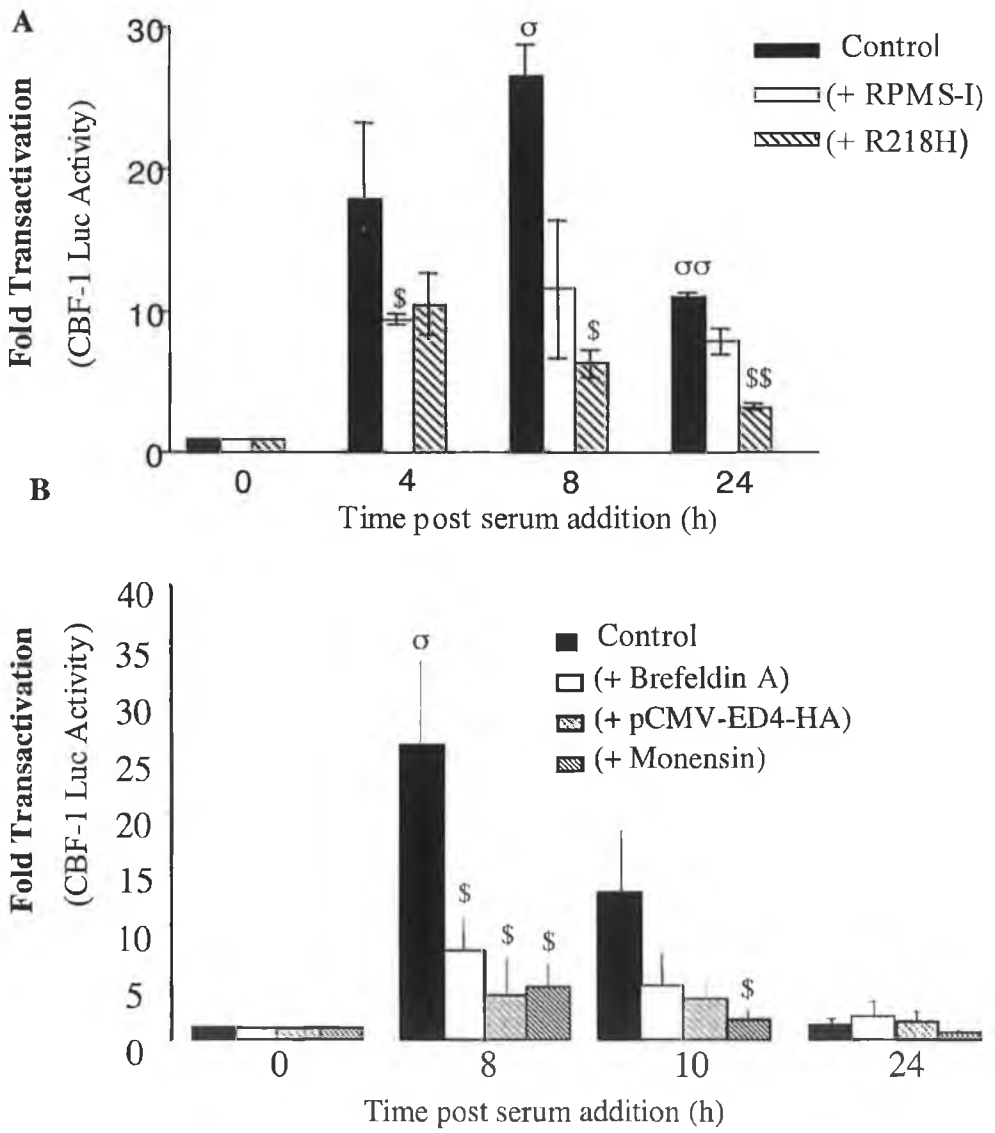


Figure 3.6 Serum stimulates CBF-1 activity in adult VSMC in a time-dependent manner, which can be inhibited by inhibitors of the Notch signaling pathway. A) RVSMC were transiently transfected with the CBF-1 reporter plasmid, pGA98-1-6, and co-transfected with the Notch pathway inhibitors, RPMS-I and R218H as indicated. Following overnight recovery from transfection, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h. Samples were taken over a period of 0–24 h, and assayed for luciferase activity. B) RVSMC were transiently transfected with the CBF-1 reporter plasmid, pGA98-1-6, and co-transfected with the Notch 1 mutant plasmid, pCMV-ED4-HA. Following overnight recovery from transfection, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h, in either the presence or absence of the pharmacological inhibitors Brefeldin A (0.1μg/ml) or Monensin (0.25 μg/ml) as indicated. Luciferase assays were normalized to β-galactosidase activities and protein levels, n=3, and expressed as fold increase over control (= the value obtained with relevant reporter plasmid transfected cells at 0 h arbitrarily assigned a value of 1). ^{s} $p<0.05$, ^{ss} $p<0.05$ as compared to 0 h serum control, $^{\$}$ $p<0.05$ as compared to serum control at that timepoint (student's t test).

Figure 3.7 Serum Stimulation of Notch Target Genes in RVSMC

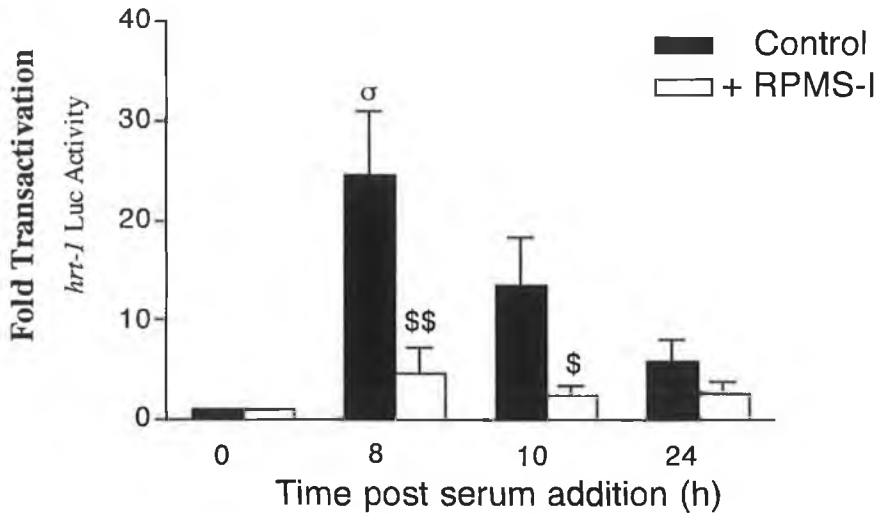


Figure 3.7 Serum stimulates Notch target gene activity in RVSMC in a time-dependent manner. RVSMC were transiently transfected with the HRT-1 reporter plasmid, and co-transfected with the Notch inhibitor RPMS-I. Following overnight recovery from transfection, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h. Samples were taken over a period of 0–24 h, 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 relevant reporter plasmid transfected cells at 0 h arbitrarily assigned a value of 1). $^{\$}$ $p<0.05$, $^{\$\$}$ $p<0.005$ as compared to 0 h serum control, $^{\$\$}$ $p<0.005$ as compared to serum control at that timepoint (student's t test).

Figure 3.8 Serum Stimulation of Notch Target Gene mRNA Expression in RVSMC

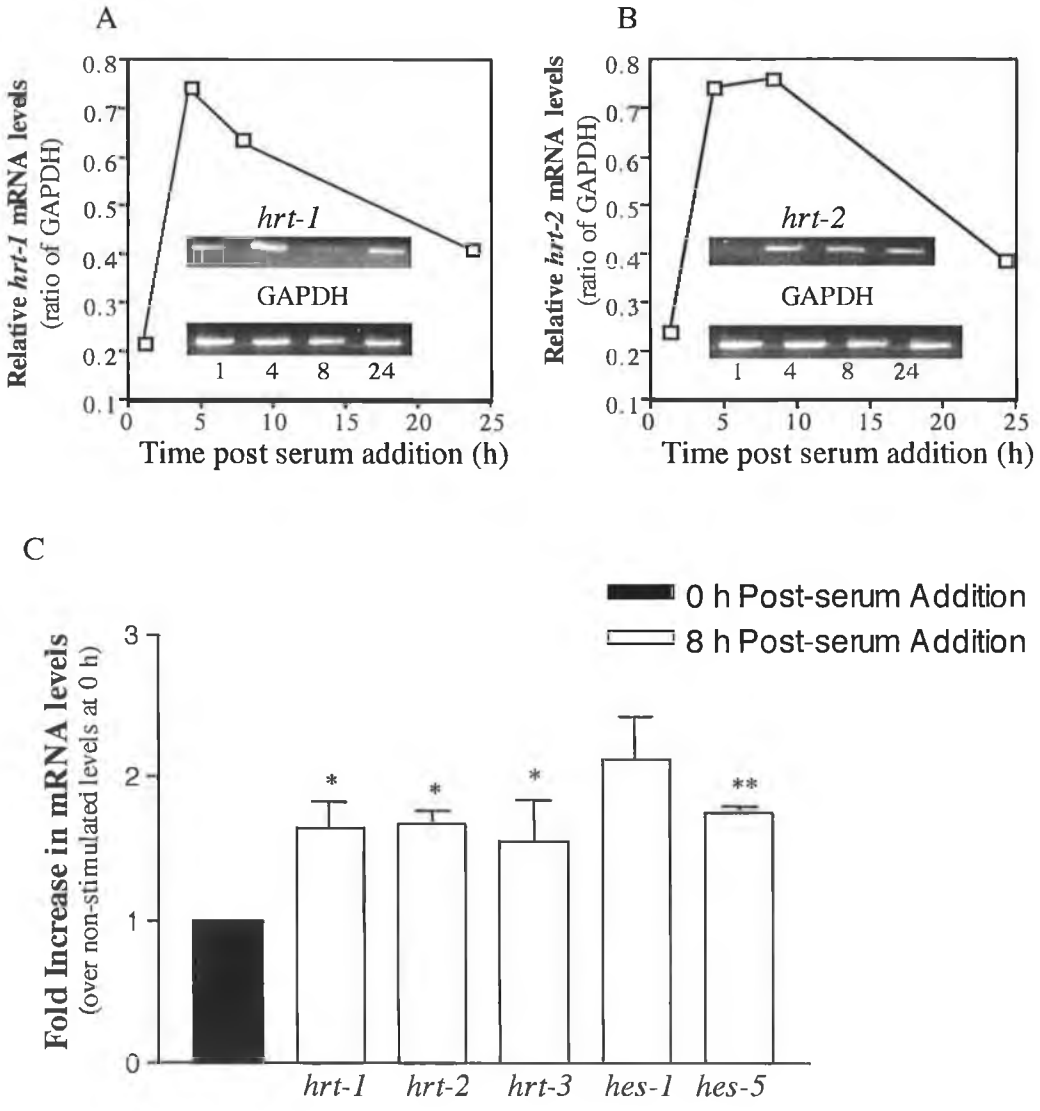


Figure 3.8 Serum stimulates Notch target gene activity in RVSMC in a time-dependent manner. A & B) RVSMC were quiesced for 48 h and stimulated with media containing 10% FBS at 0 h. Samples were taken over a period of 0–24 h, and subsequently assayed using semi-quantitative PCR. C) RVSMC were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h. Samples were isolated at 8 h post-serum addition and subsequently assayed using quantitative PCR. All values were normalized to GAPDH levels, and expressed as a ratio of GAPDH (A & B) or as fold increase over control (the value obtained with serum depleted mRNA levels at 8 h arbitrarily assigned a value of 1) (C), n=3. * $p<0.05$, ** $p<0.005$ as compared to 0 h serum control (student's t test).

Figure 3.9 Inhibition of Serum Stimulated Notch Target Gene mRNA Expression in RVSMC

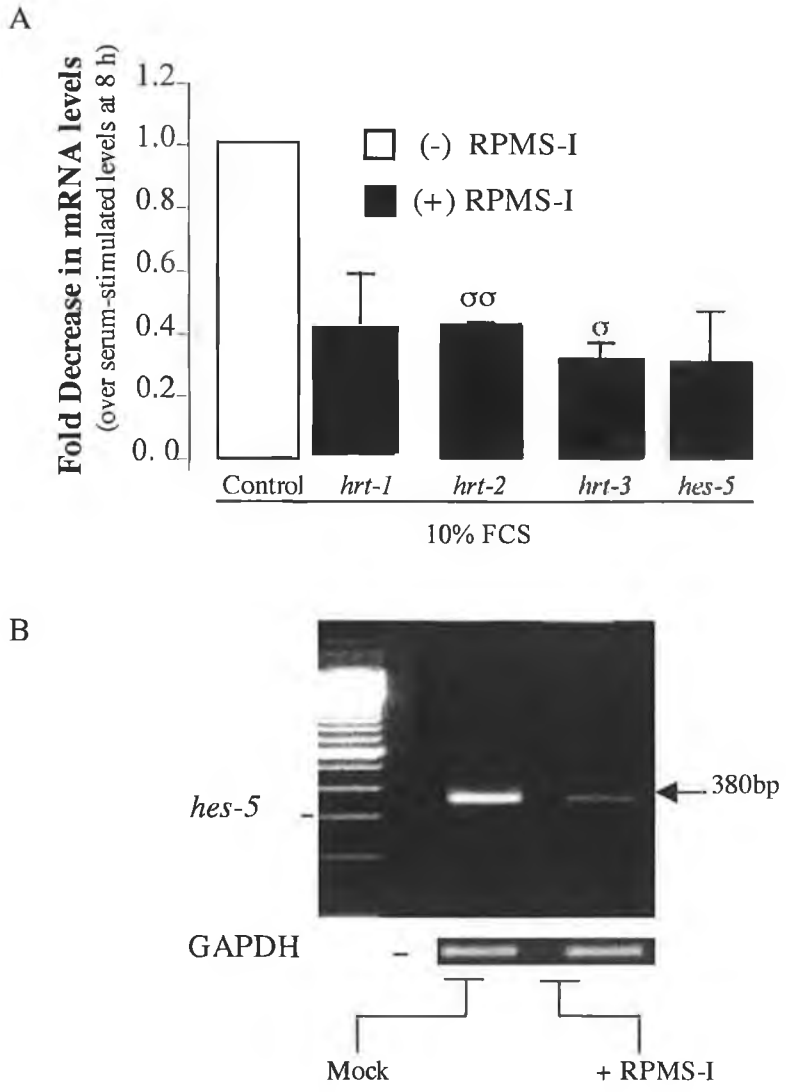
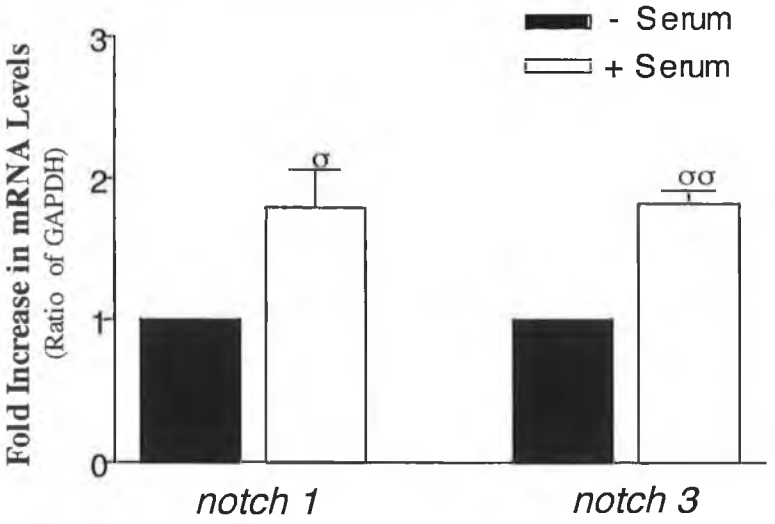


Figure 3.9 Inhibition of serum stimulated Notch target gene mRNA expression in RVSMC A) RVSMC were transfected with RPMS-1 or the empty empty vector (-RPMS-I). Following recovery overnight, cells were quiesced for 48 h and stimulated with media containing 10% FBS at 0 h. Samples were isolated at 8 h post-serum addition and subsequently assayed using quantitative PCR for Notch target gene mRNA expression. All values were normalized to GAPDH levels, and expressed as a fold decrease over control (the value obtained with serum stimulated mRNA levels at 8 h in the absence of the Notch inhibitor, arbitrarily assigned a value of 1), n=3. B) Representative semi-quantitative PCR gel for *hes-5*, and GAPDH in both the presence and absence of RPMS-I. ^{s} $p<0.05$, ^{ss} $p<0.005$ as compared to mock transfected control (student's t test).

Figure 3.10 Serum Stimulation of Notch Receptor Expression in RVSMC

A: Notch Receptor mRNA Expression



B: Notch 1 Protein Expression

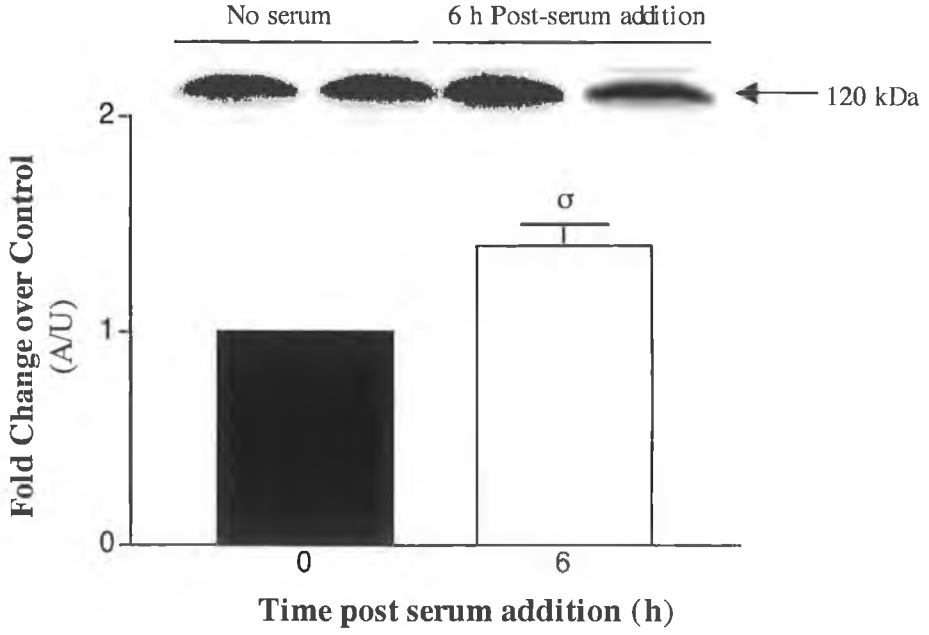


Figure 3.10 Serum increases Notch protein expression in RVSMC. B) RVSMC were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h as indicated. Samples were taken at 6 h post-serum addition in serum containing and non-serum containing samples, and subsequently assayed using for Notch 1 protein expression. Samples are expressed as fold increase over non-serum stimulated control (arbitrarily assigned a value of 1). A) RVSMC were quiesced for 48 h, and stimulated with media containing 10% or 0% FBS at 0 h. Samples were isolated at 8 h post-serum addition and subsequently assayed using quantitative PCR. All values were normalized to GAPDH levels, and expressed as fold increase over control (the value obtained with serum depleted mRNA levels at 8 h arbitrarily assigned a value of 1), n=3. ^{s} $p<0.05$, ^{ss} $p<0.005$ as compared to 0 h serum control (student's t test).

Figure 3.11 Serum Stimulated Cell Proliferation

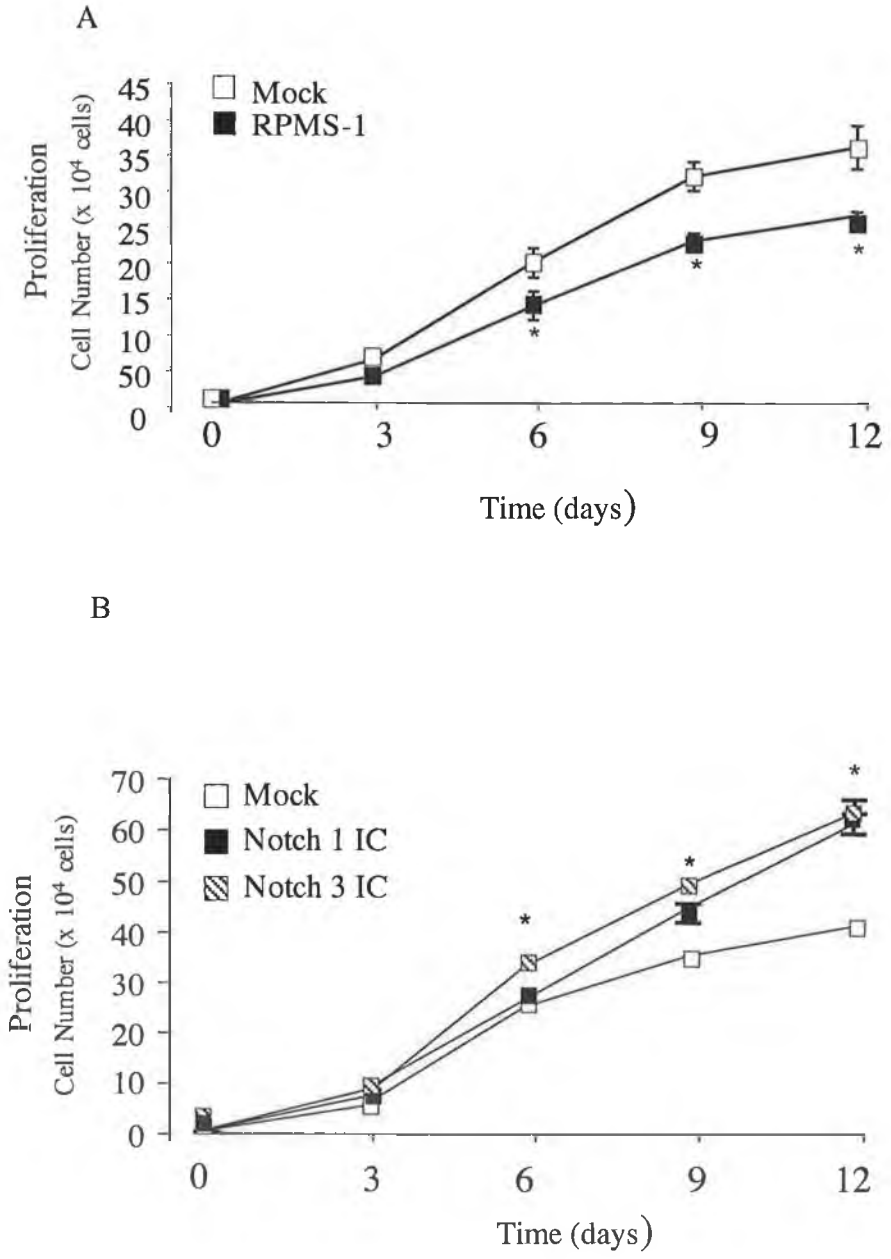


Figure 3.11 Serum Stimulated RVSMC Proliferation A) RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro and co-transfected with the empty vector p7pCMV or with RPMS-1. Following recovery overnight, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h and seeded at equal densities of 1×10^3 cells. Cells were then counted at 3 day intervals as described in section 2.7.1. B) RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro and co-transfected with the empty vector p7pCMV , with Notch1 IC or with Notch3 IC. Following recovery overnight, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h and seeded at equal densities of 1×10^3 cells. Cells were then counted at 3 day intervals as described in section 2.7.1. N=3. $^*p<0.05$

Figure 3.11 Serum Stimulated Cell Proliferation

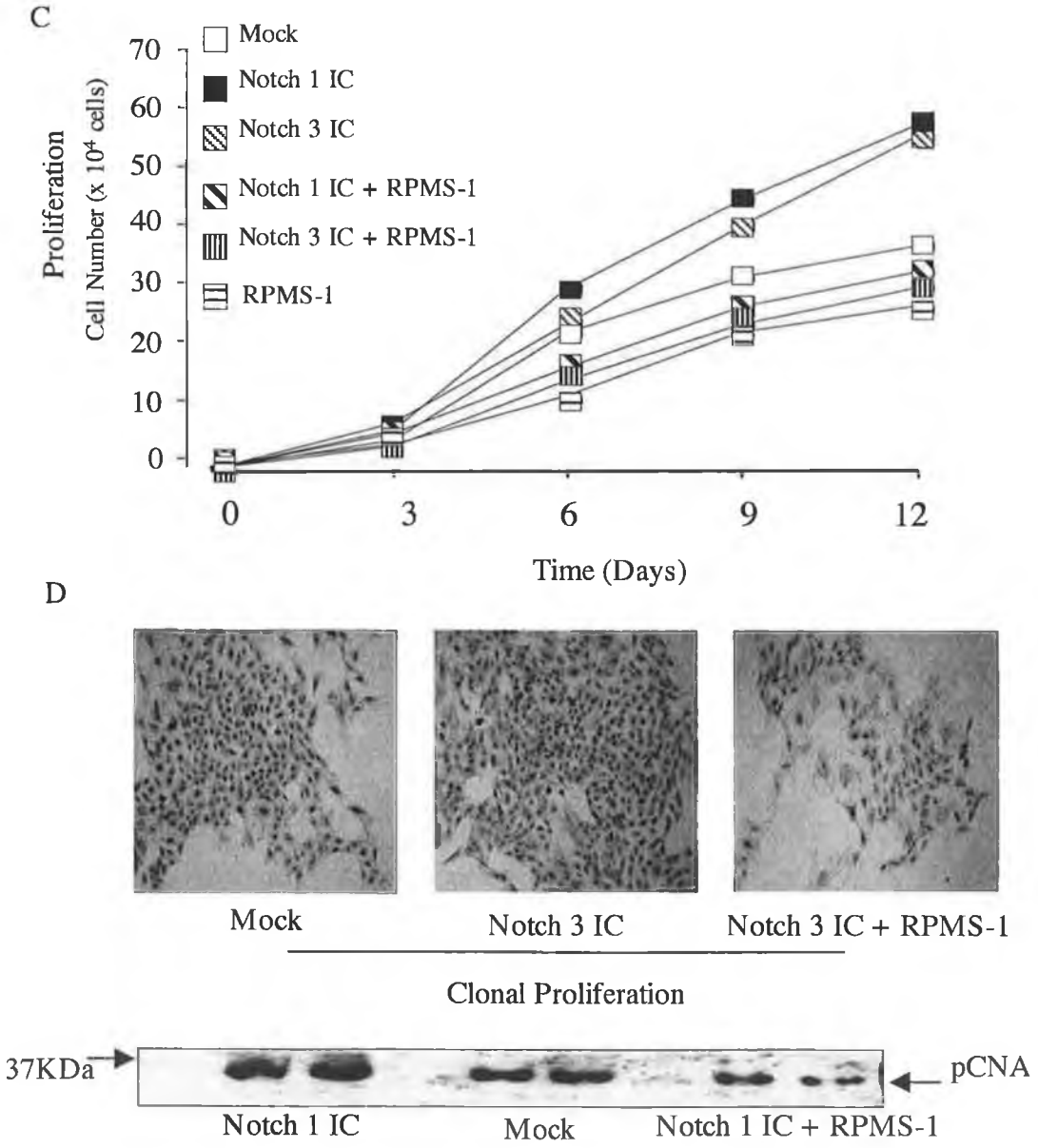


Figure 3.12 Serum stimulated RVSMC proliferation C) RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro and co-transfected with the empty vector p7pCMV,with Notch 1 IC or with Notch 3 IC or with RPMS-I. Following recovery overnight, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h and seeded at equal densities of 1×10^3 cells. Cells were then counted at 3 day intervals as described in section 2.7.1. D) In Parallel Studies RVSMCs transfected with the empty vector p7pCMV or with Notch3 IC or Notch1 IC with or without RPMS-1 were fixed and stained using Haematoxylin and Eosin. representative pictures of clonal proliferation assay. In addition, pCNA expression analysis wasp erformed on RVSMC transfected with the empty vector p7pCMV , with Notch 1 IC with or without RPMS-1. Representative western blot. N=3.

Figure 3.13 The Effect of siRNA on Notch Target Gene mRNA Expression

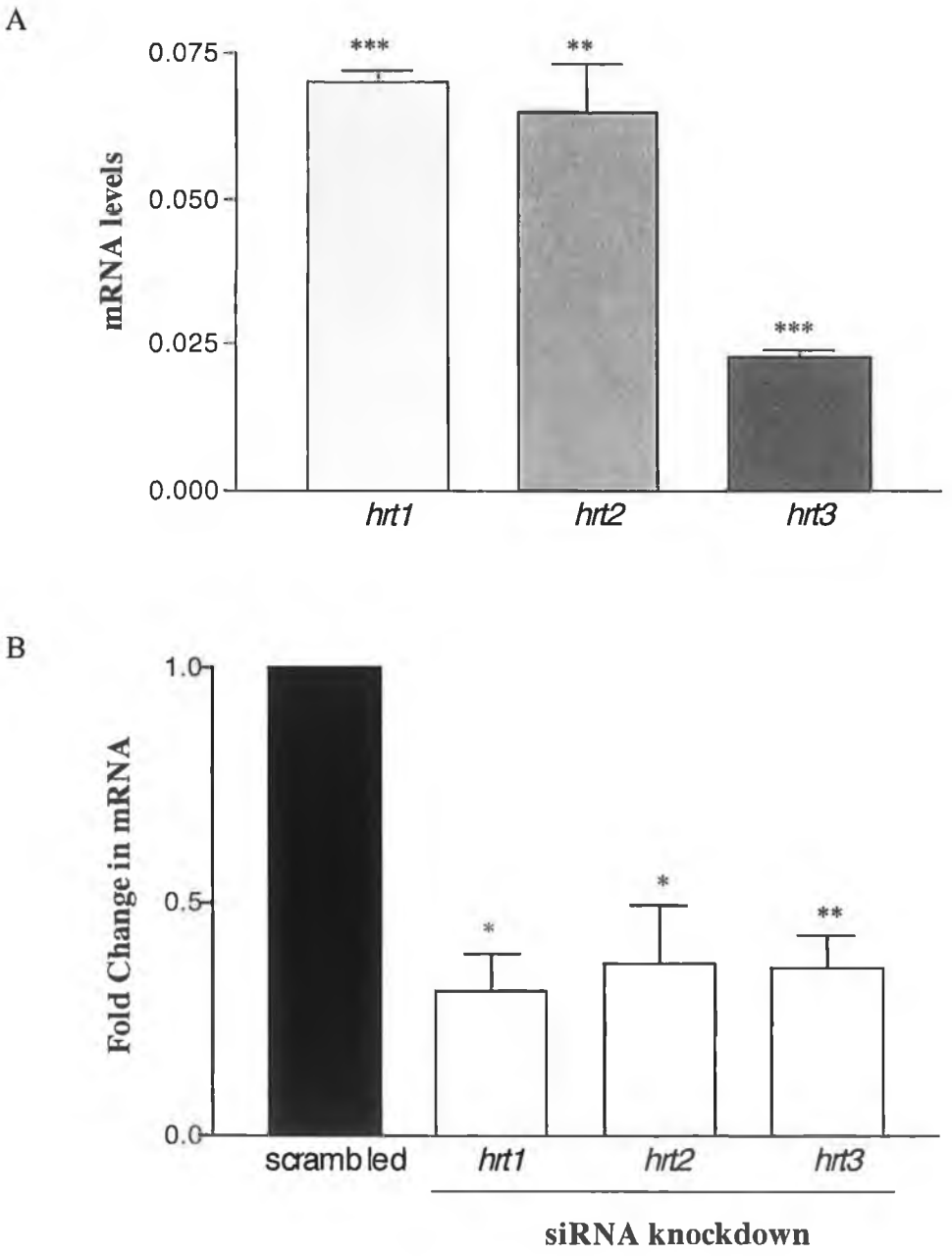


Figure 3.13 The Effect of siRNA on Notch Target Gene mRNA Expression in RVSMCs. A) *hrt1*, *hrt2* and *hrt3* mRNA expression levels were compared using quantitative real-time PCR. B) RVSMCs were transfected with siRNA directed against *hrt1*, *hrt2*, *hrt3* or a scrambled control. Following recovery overnight RNA was extracted and *hrt* mRNA expression levels were determined using QRTPCR. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ as compared to control (rank test). N=4

Figure 3.14 The Effect of Notch Target Gene Silencing on RVSMC Proliferation

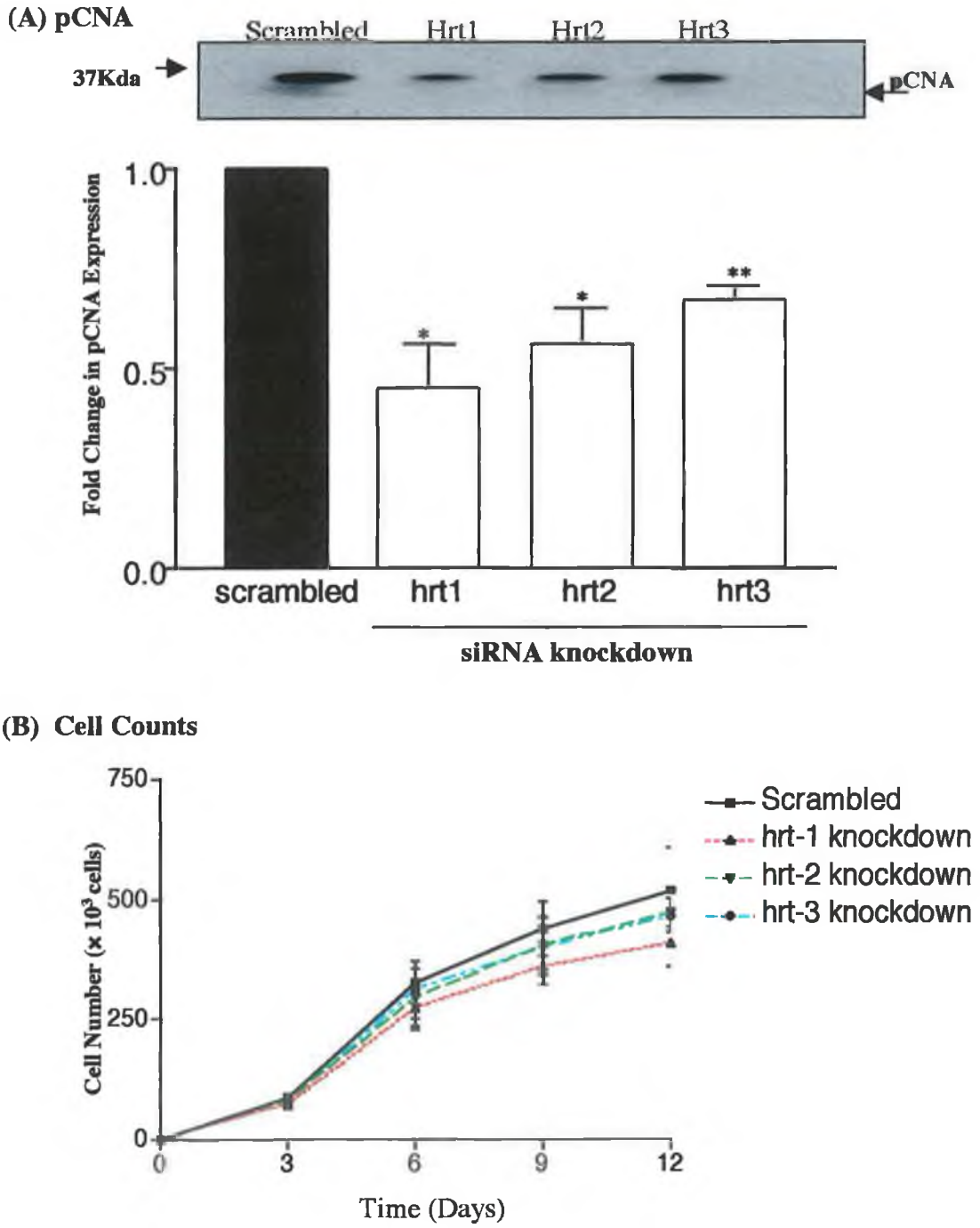


Figure 3.14 The Effect of Notch Target Gene Silencing on RVSMC Proliferation. RVSMCs were transfected with siRNA directed against Notch target genes *hrt1,2* and *3* and a scrambled control. Following recovery overnight, cells were serum starved for 48h and stimulated with media containing 10% serum at 0 h. (A) At 8 h protein was extracted and pCNA expression levels were determined.(B) In parallel cultures cells treated as above were seeded at equal low densities and were counted at 3 day intervals as describe in section 2.7.1. * $p<0.05$, ** $p<0.005$, as compared to Scrambled control (student's t test). N=4

Figure 3.16 The Effect of Notch Target Gene Silencing on Bax Expression

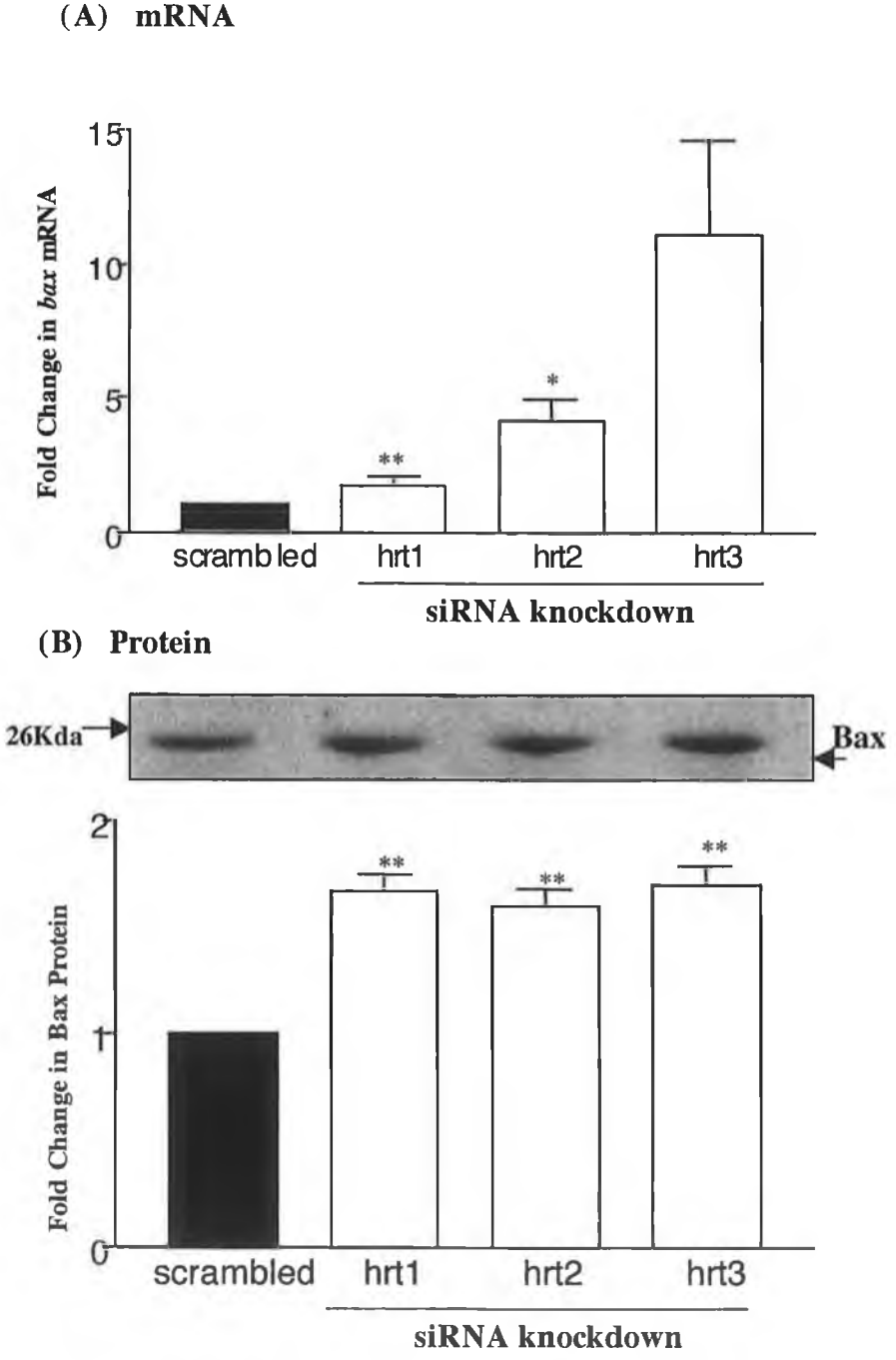


Figure 3.16 The Effect of Notch Target Gene Silencing on Bax Expression. RVSMCs were transfected with siRNA directed against Notch target genes *hrt1,2* and *3* and a scrambled control. Following recovery overnight protein and RNA was extracted. (A) *Bax* mRNA expression was determined by QRTPCR. (B) Total Bax protein expression was determined by western blot analysis). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ as compared to Scrambled control (student's t test). N=3

Figure 3.17 The Effect of Notch Target Gene Silencing on Bcl-X_L Expression

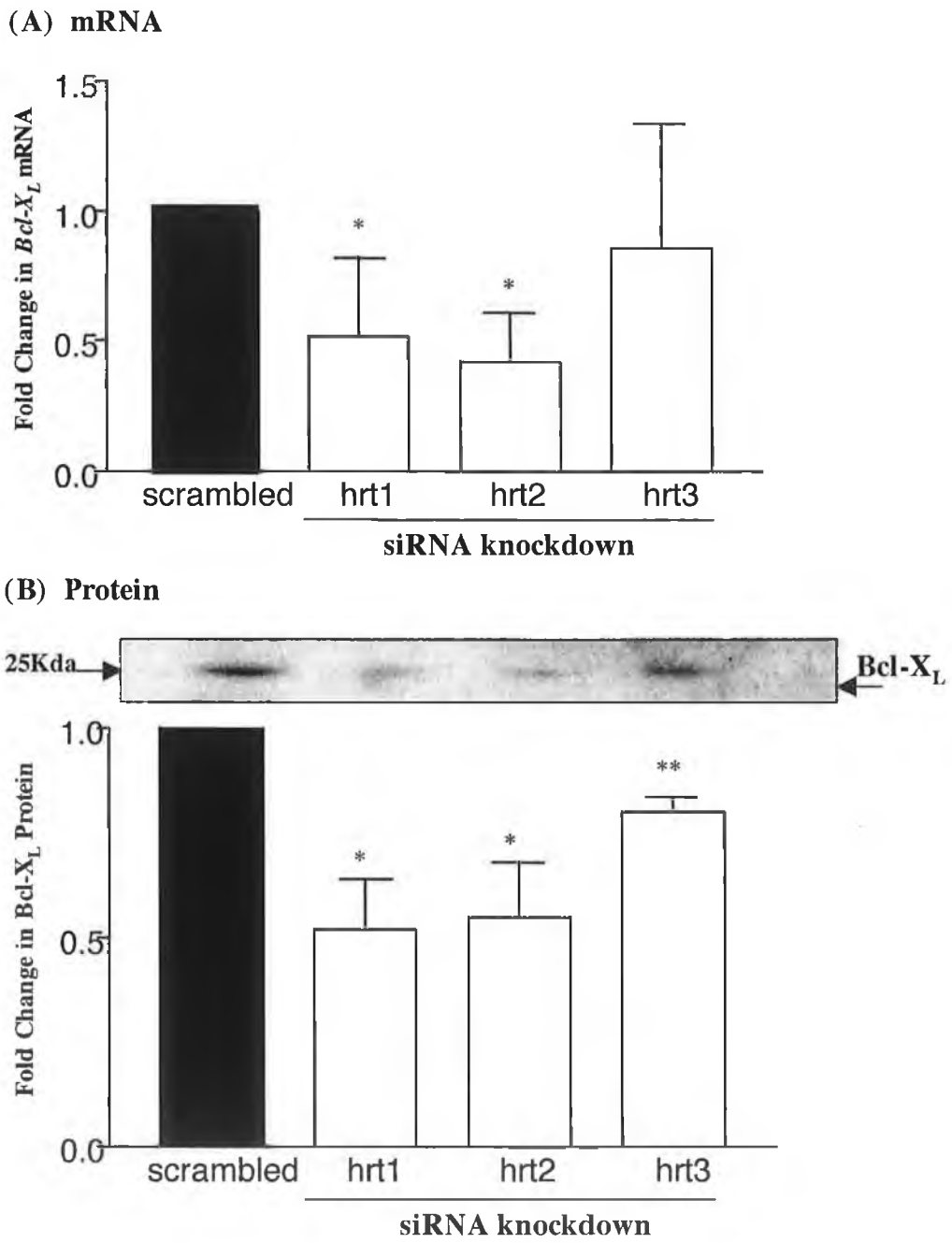


Figure 3.17 The Effect of Notch Target Gene Silencing on Bcl-X_L Expression. RVSMCs were transfected with siRNA directed against Notch target genes hrt1,2 and 3 and a scrambled control. Following recovery overnight protein and RNA was extracted.(A) *Bcl-X_L* mRNA expression was determined by QRTPCR. (B) Total Bcl-X_L protein expression was determined by western blot analysis . * $p<0.05$, ** $p<0.005$ as compared to Scrambled control (student's t test). N=3.

Figure 3.18 The Effect of Notch Target Gene Silencing on RVSMC Apoptosis

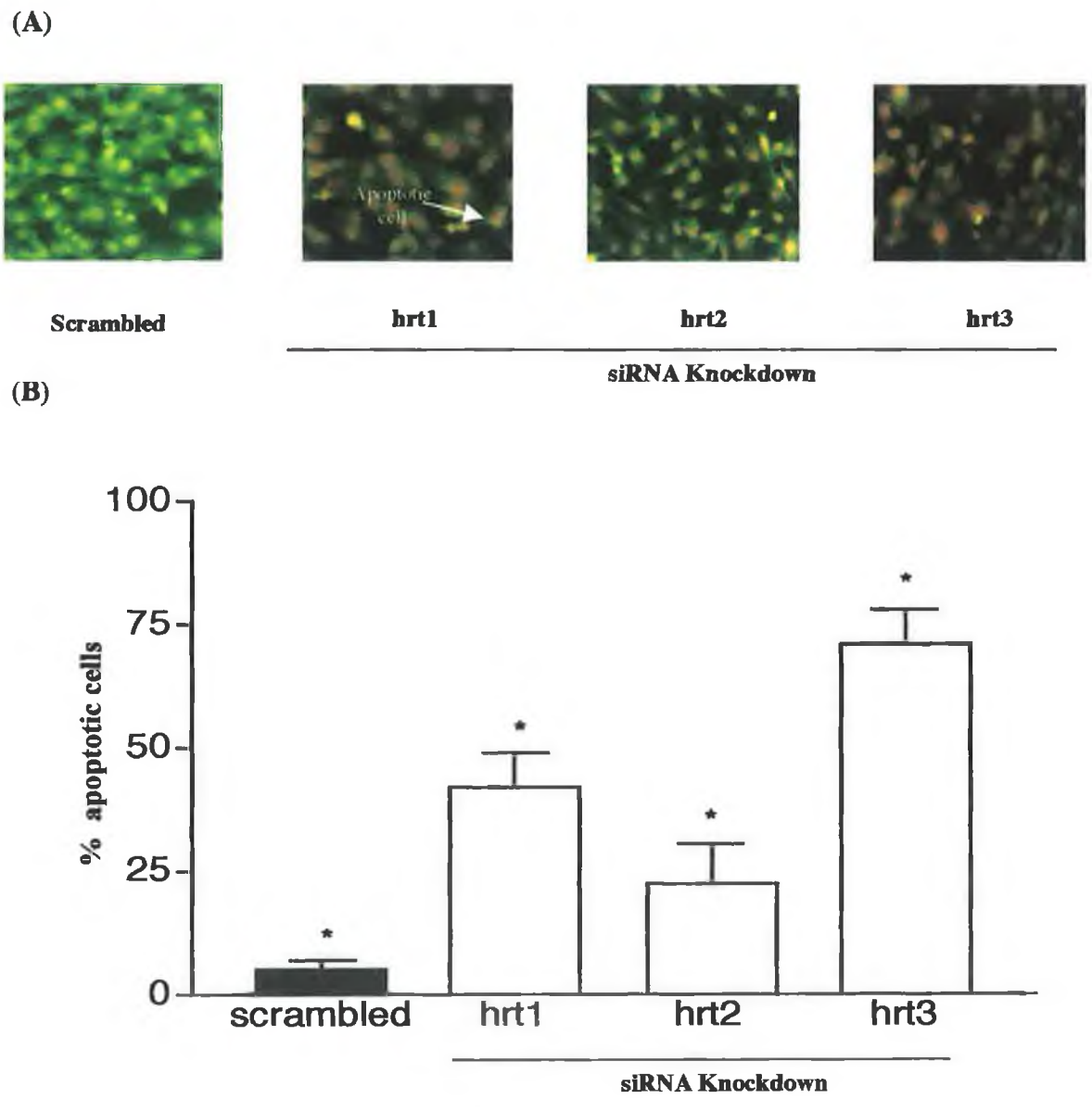


Figure 3.18 The Effect of Notch Target Gene Knockdown on RVSMC Apoptosis. RVSMCs were transfected with siRNA directed against Notch target genes *hrt1,2* and *3* and a scrambled contol. Following recovery overnight, cells were stained with the Acridine Orange/ Ethidium Bromide dual stain and viewed under a fluorescent microcope, n=3. A) Representative images. B) Graph showing fold change in apoptosis. * $p<0.05$, as compared to scrambled control (student's t test). N=3

Discussion

This study has clearly established the effect of the Notch signalling pathway on VSMC growth and that it is at least in part, a CBF-1/RBP-Jκ dependent event. The functional role of CBF-1/RBP-Jκ activation of Notch target genes as a primary downstream effector system for Notch signalling in adult VSMC, has only recently been addressed (Wang *et al.,* 2002). Furthermore there has been recent evidence to suggest that the Notch pathway is involved in multiple aspects of vascular development and remodelling (Iso *et al.,* 2003), including proliferation (Wang *et al.,* 2003), apoptosis (Matsumoto *et al.,* 2002; Wang *et al.,* 2002), endothelial migration (Favre *et al.,* 2003), SMC differentiation (Shawber *et al.,* 1996) and angiogenic processes (Mailhos *et al.,* 2001). However, recent studies have also focussed on the role of a CBF-1/RBP-Jκ independent signalling in mediating the response of Notch receptor activation in several cell systems (Nofziger *et al.,* 1996; Iso *et al.,* 2003). This study describes for the first time, the regulation of endogenous signalling following inhibition of CBF-1/RBP-Jκ dependent Notch signalling or constitutive expression of functionally active Notch IC, which results in fundamental changes in VSMC growth *in vitro.* In addition, this study describes the regulation of VSMC growth by individual Notch target genes *hrt1*, *2* and *3* following selective knockdown of these genes by targeted siRNA.

There have been several studies, which have reported the expression of Notch signalling pathway components in adult vascular tissue and cells (Lindner *et al.,* 2001; Wang *et al.,* 2002). This study indicated that constitutive expression of Notch 1 and 3 IC resulted in a significant increase in the expression of both Notch 1 and 3 IC protein and Notch receptor mRNA levels concomitant with a significant increase in Notch target gene promoter activity and mRNA levels in RVSMC. In addition, by inhibiting CBF-1/RBP-Jκ-dependent signalling using both pharmacological inhibitors Brefeldin A and Monensin, in addition to molecular interventions, we reported a significant inhibition of Notch 1 and 3 IC-dependent Notch target gene promoter activity and receptor mRNA levels. This data would suggest that expression of constitutively active Notch 1 and 3 IC requires golgi trafficking mechanisms to translocate to the nucleus as Brefeldin A and Monensin, which disassemble the golgi

apparatus and causes it's collapse into the endoplasmic reticulum, both inhibited Notch IC signalling events.

The inhibition of the Notch signalling pathway clearly decreased RVSMC growth in cycling VSMC suggesting that Notch exerts a net pro-proliferative, anti-apoptotic effect in these cells. This would concur with the pro-survival role of the Notch signalling pathway, which has been established in many other cell types (Kaneta *et al.,* 2000; Mackenzie *et al.,* 2004). Studies by Wang *et al,* 2002, reported that Notch 3 IC can promote SMC proliferation by inhibiting the expression of p27KIP1, a critical cell cycle inhibitor and can promote survival through induction of c-flip, a well-established anti-apoptotic mediator. However, in contrast to this study, they reported that Notch 3 inhibits VSMC growth in a CBF-1/RBP-Jκ-independent manner, whereas we have established this anti-apoptotic effect of Notch to be a CBF-1/RBP-Jk dependent event. However, it is very likely that Notch can promote VSMC growth in both a CBF-1/RBP-Jκ-dependent and -independent manner. In fact, recent studies by Mackenzie *et al,* 2004 reported details that Notch 4 inhibits apoptosis in both a CBF-1/RBP-Jκ-independent and -dependent manner.

This study demonstrates that serum stimulation promotes endogenous Notch signalling through a CBF-1/RBP-Jκ dependent pathway and induces proliferation while in contrast, serum deprivation downregulates Notch signalling while inducing apoptosis. A similar anti-apoptotic effect of Notch 1 has been recently demonstrated in serum deprived arterial EC (Liu *et al.,* 2003). This study reported that both Notch 1 IC and Hes-1 conferred a strong resistance to serum deprivation-induced apoptosis in EC. This indicated that the Notch signalling pathway could play a significant role in regulating EC survival. In contrast to our data, Wang and co-workers, using cultured SMC reported that angiotensin II and PDGF markedly downregulated Notch 3 and Jagged –1 through ERK-dependent signalling mechanisms. Moreover, this down regulation of Jagged-1 and Notch 3 was associated with a decrease in CBF-1/RBP-Jk-mediated gene transcription and a decrease in the mRNA levels of *Hrt-1.* However, one possible explanation for this contrasting report is that Wang and colleagues utilised a clonal rat embryonic wild-type and stably transfected Notch 3 IC cell line that exhibits a de-differentiated phenotype. Moreover, stimulation of SMC growth *in vivo* following injury was associated with an increase in the expression of Notch 3 IC and Notch signalling components (Wag *et al.,* 2002). This again reinforced the

concept that stimulation of differentiated SMC growth is associated with enhanced Notch signalling (Campos *et al.*, 2002; Lindner *et al.*, 2001). Though this study clearly demonstrates that serum stimulation activates Notch signalling and subsequently promotes VSMC growth, the stimulus foe Notch IC activation following serum stimulation remains to be elucidated. There have been several serum mitogens implicated in modulating Notch receptor expression and signalling in several cell systems (Wang *et al.*, 2002; Bongarzone *et al.*, 2000). Vascular endothelial growth factor (VEGF) has been reported to induce gene expression of Notch1 and its ligand Delta-like 4 (Dll4) in human arterial endothelial cell (Liu *et al.*, 2003). In this study, 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. This study will later demonstrate that VEGF can induce the upregulation of Notch target genes *hrt-1, 2* and *3* and subsequently promote cell growth through a signalling cascade involving the Hedgehog and Notch signalling pathways.

The mechanisms on how endogenous Notch IC signalling through a CBF-1/RBP-Jk-dependent pathway promotes a pro-survival and an anti-apoptotic effect remains unclear. In an attempt to elucidate the mechanisms through which the Notch signalling pathway exerts its pro-survival, anti-apoptotic effect in VSMC, we examined the effect of the Notch signalling pathway on the Bcl-2 family of apoptotic genes. The Notch signalling pathway has been shown to regulate members of the Bcl-2 family in both vascular and non-vascular cells. Notch 1 and Notch 4 have been reported to upregulate Bcl-2 expression in T-cells and EC respectively (Deftos *et al.*, 1998; Mackenzie *et al.*, 2004). As the Bcl-2 family is central to the regulation of apoptosis in many cell types and changes in Bcl-2 family expression are often observed during the pathogenesis of vascular disease, it is likely that the Notch signalling pathway may regulate some Bcl-2 family members. Therefore, this study examined the effect of the Notch signalling pathway on the expression of both pro-apoptotic Bax and anti-apoptotic Bcl-X_L. We observed following inhibition of Notch IC, that there was a significant increase in Bax while a concurrent decrease in Bcl-X_L was reported. Furthermore, by selective knockdown of *hrt-1, 2* and *3* by siRNA, there was a significant increase in Bax expression while concurrent decrease in Bcl-X_L was observed. In addition, serum deprivation resulted in an increase in Bax and a decrease

in Bcl-X_L, further suggesting a Notch-mediated regulation of Bcl-2 family members in VSMC. Moreover, using bioinformatics this study examined whether any Notch target gene promoter sites were present on either the Bax or Bcl-X_L genes. Nakagawa *et al,* reported that the Hrt family of genes preferentially bind to an E box motif CACGTG, in addition to CAACTG, CACCTG, CACTTG and CATCTG to regulate transcription of other genes. Therefore, through sequence alignment of cloned Bax and Bcl-X_L promoters (Grillot *et al.,* 1997; Igata *et al.,* 1999), we hypothesised that the Hrt genes could bind and possibly regulate both Bax and Bcl-X_L. However, though this would require confirmation with mutational analysis, the significant changes in expression of both bax and Bcl-X_L by *Hrt* gene knockdown by siRNA, certainly adds weight to this argument.

In conclusion, this study has provided further evidence that both Notch 1 and 3 receptors acting through a CBF-1/RBP-Jκ dependent signalling pathway are an important determinant of SMC growth *in vitro*. Furthermore that Notch target genes *hrt-1, 2* and *3* are important modulators of VSMC growth by varying degrees with *hrt-1* having the most significant effect on VSMC proliferation, while *hrt-3* exerts the most significant effect on VSMC apoptosis *in vitro*. Notch receptor expression is also upregulated within the vasculature of animal models following vascular injury (Leong *et al.,* 2002; Campos *et al.,* 2002). Therefore greater knowledge of the regulation of Notch receptors and the downstream activation of Notch target genes in SMC may provide new insights into the molecular mechanisms underlying changes in vascular cell fate that underlie vascular proliferative disease.

Chapter 4

Results

Sonic Hedgehog Regulates VSMC Fate *in vitro* Through VEGF Activation of Notch Signalling

4.1 Introduction

In the previous chapter we clearly established a role for the Notch signaling pathway in directing individual vascular cell fate decisions. Notch signaling may therefore be a critical determinant of SMC survival by modulating the downstream expression of downstream mediators of apoptosis such as Bax and Bcl-X_L. However the mechanism of how this signaling pathway may be regulating cell fate remains to be fully elucidated. This study, for example has clearly shown that the Notch signalling pathway is activated by serum. Serum stimulation increases Notch signaling component activity and CBF-1-RBPJκ dependent activity while serum deprivation conversely decreases expression and CBF-1- RBPJκ dependent activity of this pathway. One of the possible mechanisms of serum regulation of the Notch signaling pathway is the presence of growth factors within the serum. Recent studies have shown that VEGF alters the expression of Notch signaling pathway components in vascular and other cell types (Lawson *et al.*, 2002; Wang *et al.*, 2002). Gene knockout studies have revealed that VEGF plays a critical role in developmental vasculogenesis and angiogenesis, in that mice with disrupted VEGF-R1 expression die embryonically due to severe vascular defects. Furthermore, in a similar manner, disruption of the Notch signaling pathway also leads to embryonic lethality due to defects in vasculogenesis and angiogenesis (Xue *et al.*, 1999; Smith *et al.*, 2000). In addition both the Notch and VEGF signaling pathways regulate arterial-venous differentiation in development (Lawson *et al.*, 2002), further highlighting the functional relationship between these two pathways. Recent studies have shown that VEGF acts upstream of the Notch signaling pathway to determine arterial cell fate and that Notch is required to mediate this VEGF-induced arterial differentiation (Lawson *et al.*, 2002).

As VEGF has been shown to be an important factor in regulating the Notch signalling pathway, it therefore proves both an important and interesting study to consider what factors are mediating VEGF expression.

Recent studies have demonstrated that Hh and Notch signaling pathways regulate cell division during development of the *Drosophilia* (Amin *et al.,* 2004). In additional studies, Shh was shown to upregulate the expression of Notch target genes during arterial differentiation, suggesting a possible role for Shh in maintaining cell proliferation through the Notch signaling pathway (Lawson *et al.,* 2002)

Sonic hedgehog is a member of a family of closely related proteins consisting of Shh, Indian hedgehog (Ihh) and Desert hedgehog (Dhh). These proteins are known to regulate the morphology of many kinds of tissues (Ingham *et al.,* 2001). Hh transduces signals via its receptor Patched (Ptc). Ptc forms a complex with a seven-pass membrane protein, Smoothened (Smo), which exists as an inactive form. Hedgehog-bound Ptc dissociates Smo, resulting in the activation. Activated Smo allows the entry of a transcription factor, *Cubitus interuptus* (Ci) into nuclei, which induces the expression of a panel of downstream target molecules (Ingham *et al.,* 1998). Vertebrate homologs of Ci are Gli1, 2 and 3. The downstream target targets of the Gli gene products include both Ptc and Gli themselves; thus Ptc and Gli are both components and targets of the Hh signalling pathway. Several recent studies have highlighted the involvement of Hh in the development of embryonic vascular tissues, including hypervascularization of neuro ectoderm following overexpression of Shh (Rowithch *et al.,* 1999). Disorganization of endothelial precursors in Shh-deficient zebrafish (Brown *et al.,* 2000) and poor vascularization of the developing lung in Shh-deficient mice (Pepicelli *et al.,* 1998). In addition Vokes *et al.,* (2004) showed that endodermally-derived Shh is both necessary and sufficient for vascular tube formation in avian embryos. They also showed that Hh signalling is required for vascular tube formation in mouse embryos, and for vascular cord formation in cultured mouse endothelial cells. These results demonstrate a previously uncharacterized role for Hh signaling in vascular development and identify Hh signalling as an important component of the molecular pathway leading to vascular tube formation.

Moreover, more recently, it has been shown that Shh signaling is present in adult cardiovascular tissues and can be activated *in vivo* to induce robust angiogenesis (Pola *et al.,* 2001). With the discovery that Hh is preferentially expressed in vascular tissue (Gering *et al.,* 2005; Colnot *et al.,* 2005), combined with Hh known morphogenic

functions in embryonic development, it is therefore reasonable to hypothesise that Shh may co-ordinate VSMC changes in adult tissue.

The aim of this chapter was to examine whether Hh components were present on adult VSMC and if activation of this signalling pathway controls cell fate. Since this study has clearly demonstrated that Notch signaling controls cell fate, we investigated the role of the Hh signaling pathway in determining cell fate through VEGF activation of the Notch signaling pathway in vitro.

4.2 Results

4.2.1 Expression of Hedgehog Components in RVSMC

Analysis of RVSMC revealed the presence of Hh signaling pathway components. We confirmed the presence of these components by immunocytochemical analysis, RT-PCR and western blot analysis in cultured RVSMC (**fig. 4.1**). By immunocytochemical staining we confirmed that Shh, Ihh and Ptc1 was expressed with significant staining within the cytoplasm and membrane. In addition the expression of the Hh signaling components was further confirmed by western blot analysis. Shh, Ihh and Ptc1 protein expression was confirmed using commercially available antibodies from Santa Cruz Biotechnology (Heidelberg, Germany). The expression of components of the Hh signaling pathway was also further confirmed by RR-PCR (**fig. 4.1**). Shh, Ihh, Ptc1, Smo and Gli2 mRNA transcripts were all detected in RVSMCs. Furthermore gli-promoter activity was also confirmed by luciferase reporter assays and was significantly increased in quieced RVSMC following serum stimulation (**fig. 4.8**). In all cases the appropriate primary and secondary controls were performed in parallel with each experiment.

4.2.2 Activation of Hedgehog Signaling in RVSMC

After the successful detection of Hedgehog signalling components in RVSMC we next investigated whether we could constitutively activate the Hh signaling pathway in these cells. To examine the activation of Hh signaling recombinant Shh protein was used which was commercially available from R and D systems (UK). Treatment of RVSMC with recombinant Shh resulted in 6.68±.91 fold increase in Gli2 mRNA expression as compared to a BSA control. In parallel studies, treatment of cells with the Hh inhibitor cyclopamine, resulted in a significant reduction of 39.0±2% in baseline *gli-2* mRNA expression (**Fig. 4.2**). To further examine the activation the Hh signalling pathway in these cells we transiently transfected an expression vector encoding full-length mouse Shh. In order to maximize the percentage of cells expressing Shh, we puromycin selected by co-transfecting a puromycin resistant plasmid and treating with puromycin containing media (0.8ug/ml) for 48h. Shh over

expression resulted in a significant 4.5±.05 fold increase in *gli-2* mRNA expression over mock-transfected control. Furthermore by co-transfecting with RPMS-1, hence inhibiting CBF-1/RBP-Jκ-dependent Notch signaling, we could significantly attenuate this Shh- induced upregulation of *gli-2* mRNA expression by 56.0±5% (**Fig. 4.4**).

4.2.3 Hedgehog Activation of Notch Target Gene Expression in RVSMC

With the evidence that Hh signalling was both present and that it could be successfully activated in RVSMC we next aimed to elucidate whether Hh activation could activate the Notch signaling pathway in these same cells. Treatment of RVSMC with recombinant Shh protein resulted in a significant upregulation in Notch target gene expression. *Hrt-1*, *2* and *3* mRNA expression increased by 1.78±.21, 3.06±.37 and 3.92±.84 fold respectively over control (**Fig. 4.3 A**). In parallel experiments Hrt-1 and Hrt-2 protein expression was increased by a fold of 2.1±.1 and 1.9±.05 respectively following treatment with recombinant Shh protein (**Fig. 4.3 A**). Moreover Hh inhibition with cyclopamine resulted in a significant decrease in baseline Notch target gene expression. *Hrt-1*, *2* and *3* mRNA expression decreased by 51.5±17.6%, 51.5±17.6% and (29.0±8.0%) respectively while concurrently decreasing Hrt-1 and hrt-2 protein expression by 44.0±9.0% and 52.0±11.0% (**Fig. 4.3 B**). To further confirm that Shh- mediated increases in Notch target gene expression, we transiently transfected full-length mouse Shh. Shh overexpression resulted in a significant increase of 2.2±.1, 2.4±.12 and 3.5±.05 in *Hrt-1, 2* and *3* mRNA expression respectively, an effect that was significantly attenuated following inhibition of CBF-1/RBP-Jk-dependent Notch signalling by co-expression with RPMS-1 to 60±9.0%, 45.4±8.0% and 57.2±5.0% for Hrt 1, 2 and 3 respectively (**Fig. 4.5**).

4.2.4 Notch Activation of Hedgehog Signalling in RVSMC

With confirmation that Hedgehog signalling activates Notch signalling in RVSMCs *in-vitro,* we now investigated the effect of Notch IC over-expression on Hh signalling. We examined this through the transient transfection of RVSMC with the active (IC)

portion of either the Notch 1 or Notch 3 receptor, co-transfected with the Notch inhibitor RPMS-1 and a puromycin resistant plasmid as previously described. Over-expression of Notch IC resulted in a marked increase in Hh signalling which was attenuated by RPMS-1 confirming that Notch mediated increases in Hh signaling is occurring, at least in part, in a CBF-1-dependent manner. Overexpression of Notch 1 IC resulted in a significant increase of 1.3±.05 and 1.4±.07 fold increase in Shh and Ihh protein expression in respect to a mock transfected control, an increase which was attenuated following co-transfection with RPMS-1 by 53.9±9.0% and 50.0±7% respectively (**Fig. 4.6**). Furthermore over-expression of Notch 3 IC showed no significant increase in Notch 3 protein expression while resulting in a 1.35±.05 increase in protein expression. Although no significant increase in Shh protein expression was observed following over-expression of Notch 3 IC, co-transfection with Notch inhibitor did result in a significant decrease of 42.0±6% in Shh protein expression while concurrently attenuating the Notch 3-mediated increase in Ihh protein expression by 40.7±5% (**Fig. 4.6**). In parallel studies we investigated the effect of Notch IC on Smo expression. Over-expression of Notch 1 IC resulted in a 17.5±.41 increase in smo mRNA levels over mock-transfected controls, while co-transfection with RPMS-1 attenuated this increase by 40.0±4% (**Fig. 4.7**). Furthermore, we investigated the effect of Notch 3 IC on Gli promoter activity and subsequently on Gli2 mRNA expression. Over-expression of Notch 3 IC served to significantly increase both Gli promoter activity and *gli-2* mRNA levels by 2.3±.05 and 1.5±.04 respectively (**Fig. 4.7 B**). As in the previous experiments, we confirmed that these Notch-mediated increases in Hh signaling were occurring to some extent in a CBF-1-dependent manner, since co-transfection with RPMS-1 resulted in an attenuation in Notch IC mediated increase in Gli-promoter activity and Gli2 mRNA expression by 39.0±5.% and 26.7±6% respectively (**Fig. 4.7 B**).

□□

4.2.5 The Effect of Shh on RVSMC Proliferation

In the previous chapter we established the functional role of the Notch signalling pathway on VSMC fate and confirmed this by determining the effects of Notch signalling on VSMC growth. The present study has demonstrated the interaction of

the Notch and Hh signalling pathways, therefore we aimed to determine the effect of Hh signalling on VSMC growth. In quiesced cells there was a significant temporal increase in serum- stimulated proliferation in cultures treated with recombinant Shh. 3.5 μg of Shh significantly increased RVSMC proliferation after 9 days as compared to control (**Fig. 4.9** C). In addition, serum stimulated pCNA expression, a marker for cell cycle dependent SMC proliferation increased by 2.1±.1 after 24h (**Fig. 4.9 A**). Furthermore, in parallel cultures Shh inhibition by cyclopamine resulted in a significant decrease in RVSMC proliferation after 9 days (**Fig. 4.9 C**). This was mirrored by a 48±8% decrease in pCNA expression after 24h post serum addition (**Fig. 4.9 B**). To further address the effect of Shh on RVSMC proliferation, we transiently transfected cells with full-length mouse Shh. Overexpression of Shh resulted in a significant increase in RVSMC clonal proliferation after 12 days in culture (**Fig. 4.10 B** and **C**). Furthermore, serum-stimulated pCNA expression increased by 4.0±0.4 fold over mock-transfected controls. The functional importance of the Notch-Hh signaling interaction in determining VSMC fate was highlighted by the fact that co-transfection of the Notch inhibitor RPMS-1 resulted in a marked decrease in Shh-mediated increase in proliferation. Clonal proliferation was attenuated to control levels (**Fig. 4.10 B** and **C**), while Shh-mediated increased pCNA expression was inhibited by 68.0±10% (**Fig. 4.10 A**).

4.2.6 The Effect of Shh on RVSMC Apoptosis

To further elucidate the functional importance of Shh on RVSMC fate, we determined the effect of Shh on VSMC apoptosis. As in the previous chapter, we investigated the ratio of expression of the pro-apoptotic Bax against the expression of the anti-apoptotic Bcl-X_L while concurrently observing the number of apoptotic nuclei by staining cells with the dual stain of acridine orange/ethidium bromide as a measure of the degree of apoptosis within the cells. Treatment of RVSMC with recombinant Shh protein resulted in a marked decrease in *Bax* mRNA and protein expression of 35.0±6% and 64.0±11% respectively in comparison to mock treated control. Furthermore, this was paralleled with a significant increase in anti-apoptotic Bcl-X_L expression, with a 1.63±0.1 fold increase in mRNA and an increase of 1.8±0.9 in

protein expression over control. In quiesced cells, Shh treatment resulted in a 88.0%±16% inhibition in serum starved-induced apoptosis. In contrast to this, RVSMCs treated with the Hh inhibitor cyclopamine showed a marked increase in apoptosis. Hh inhibition resulted in a 2.4±0.05 fold increase in *Bax* mRNA while increasing Bax protein expression by a fold of 1.8±0.14 (**Fig. 4.12 A**). Concurrent with an increase in Bax expression, Bcl-X_L expression was significantly decreased by 67.0±5% for mRNA and a subsequent 71.0±11% decrease in protein expression (**Fig. 4.12 A**). Concomitant with these changes in Bax and Bcl-X_L expression levels, a significant increase in the number of apoptotic nuclei was observed. Hh inhibition resulted in an 8.4±0.1 fold increase in apoptosis over control (**Fig. 4.12 B**). As with our investigation into the functional importance of Hh signaling in the promotion of RVSMC proliferation, we determined the effect of Shh over-expression and Notch inhibition on RVSMC apoptosis. Constitutive over-expression of Shh in puromycin-pooled cells resulted in a significant decrease in RVSMC apoptosis. Shh over-expression resulted in a 70.0±11% and 61.0±12% decrease in *Bax* mRNA and protein respectively compared to mock transfected controls (Fig 4.13) while concurrently increasing Bcl-X_L mRNA and protein expression by a significant fold increase of 2.0±0.05 and 2.2±0.07 (**Fig. 4.14**). Concomitant with these Shh induced changes in Bax and Bcl-X_L expression a significant decrease in apoptotic nuclei was observed following serum deprived induced apoptosis. Shh over-expression resulted in a 59.0±16% decrease in apoptosis compared to mock-transfected control (**Fig. 4.15**). Furthermore the effect of inhibiting CBF-1RBP-Jκ- dependent Notch signalling on Shh-mediated inhibition of RVSMC apoptosis was examined. Co-transfection of RPMS-1 significantly reversed the Shh-mediated decrease in *Bax* mRNA and protein expression to control levels while concurrently attenuating the Shh-mediated increase in *Bcl-X_L* mRNA and protein expression to mock control levels. This effect was mirrored by reversing the decrease in apoptotic nuclei in Shh transfected cells by inhibiting CBF-1/RBP-Jκ-dependent Notch signaling in these cells (**Fig. 4.15**).

4.2.7 Hedgehog Stimulates RVSMC Growth Through VEGF Activation of Notch Signalling

Since it is well documented that VEGF acts downstream of Hh in activating Notch signalling during arterial differentiation (Lawson *et al.*, 2002), we investigated the effect of Shh on VEGF expression in RVSMCs and the subsequent effect on Notch signaling. Treatment of the cells with recombinant Shh resulted in a significant fold increase of 2.3±0.1 in VEGF protein expression as compared to control (**Fig. 4.16**). Furthermore, *VEGF* mRNA expression was significantly increased by 4.2±0.2 fold over mock control following treatment with recombinant Shh (**Fig. 4.17**). In addition, Shh inhibition with cyclopamine resulted in a significant decrease of 74.0±16% in *VEGF* mRNA expression (Fig 4.18). We then addressed the effect of recombinant VEGF (25ng) on Notch target gene mRNA expression. Recombinant VEGF treatment resulted in a 2.64±0.7, 5.1±1.0 and 2.5±0.8 fold increase in *hrt-1, hrt-2* and *hrt-3* mRNA expression respectively (**Fig. 4.19**). Selective knockdown of VEGF with a targeted siRNA was confirmed at the mRNA level with a significant knockdown of 76.0±2% *VEGF* mRNA as compared to scrambled controls (**Fig. 4.20**). In parallel cultures, Shh induced increases in *hrt-1* and *hrt-3* mRNA expression by 2.6±0.4 and 4.4±0.1 fold respectively over control. Inhibition of VEGF expression with the siRNA 76.0±2% significantly resulted in a 84.0±26% and a 78.0±9% inhibition in the Shh-mediated increase in *hrt-1* and *hrt-3* respectively as compared to scrambled controls (**Fig. 4.20**).

Figure 4.1 The Presence of Hedgehog Signalling in RVSMC

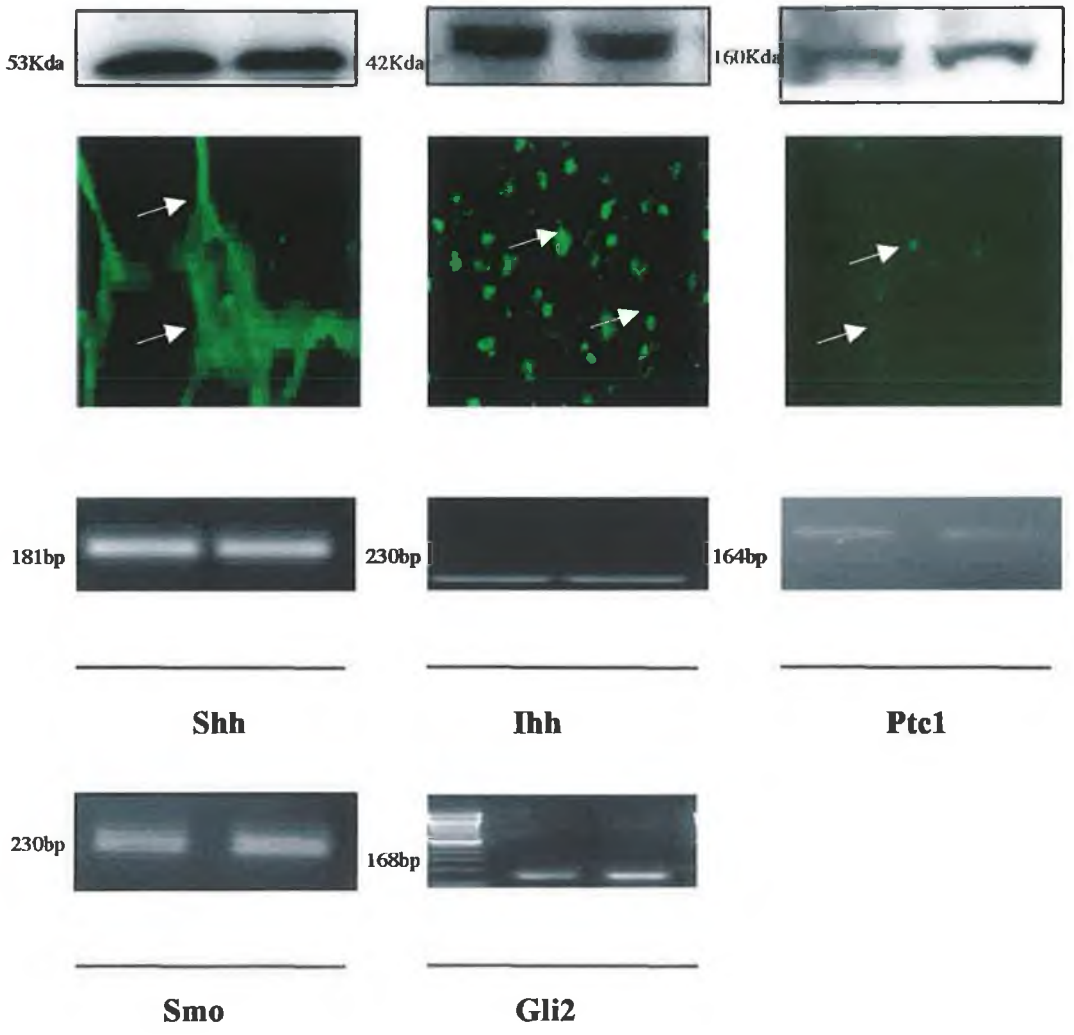


Figure 4.1 The presence of Hedgehog signaling in RVSMC. Shh, Ihh and Ptc1 are expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR r. Immunocytochemistry showing Shh, Ihh and Ptc1 expression at 20X magnification. Smo and Gli2 is expressed in RVSMC as determined by semi-quantitative PCR.Gels and immunocytochemistry pictures are representative experiments. n=3.

Figure 4.2 Activation of Hedgehog signalling in RVSMCs

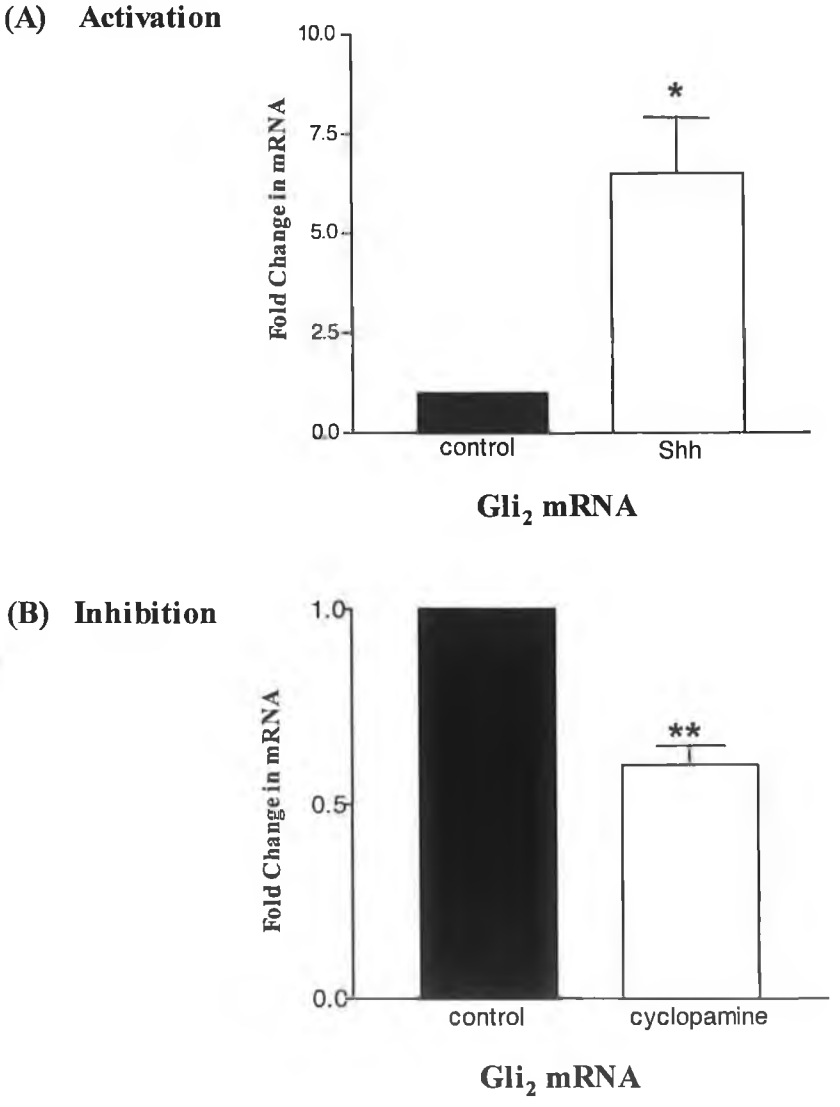


Figure 4.2. Activation of Hedgehog Signaling in RVSMC. A) RVSMCs were treated with recombinant Shh protein (3.5μg) for 48 h and the effect on gli_2 mRNA expression was determined QRTPCR was performed for expression levels of gli_2 in Shh treated RVSMCs compared to BSA control. B) The effect of Hh inhibition with Cyclopamine (40μM) for 24 h on gli_2 mRNA expression as determined by QRTPCR. All values were normalized to GAPDH levels, and expressed as fold increase over control. * $p<0.05$, ** $p<0.005$ as compared to control (student's t test).n=3

Figure 4.3 Sonic Hedgehog Activates Notch Target Gene Expresson in RVSMCs

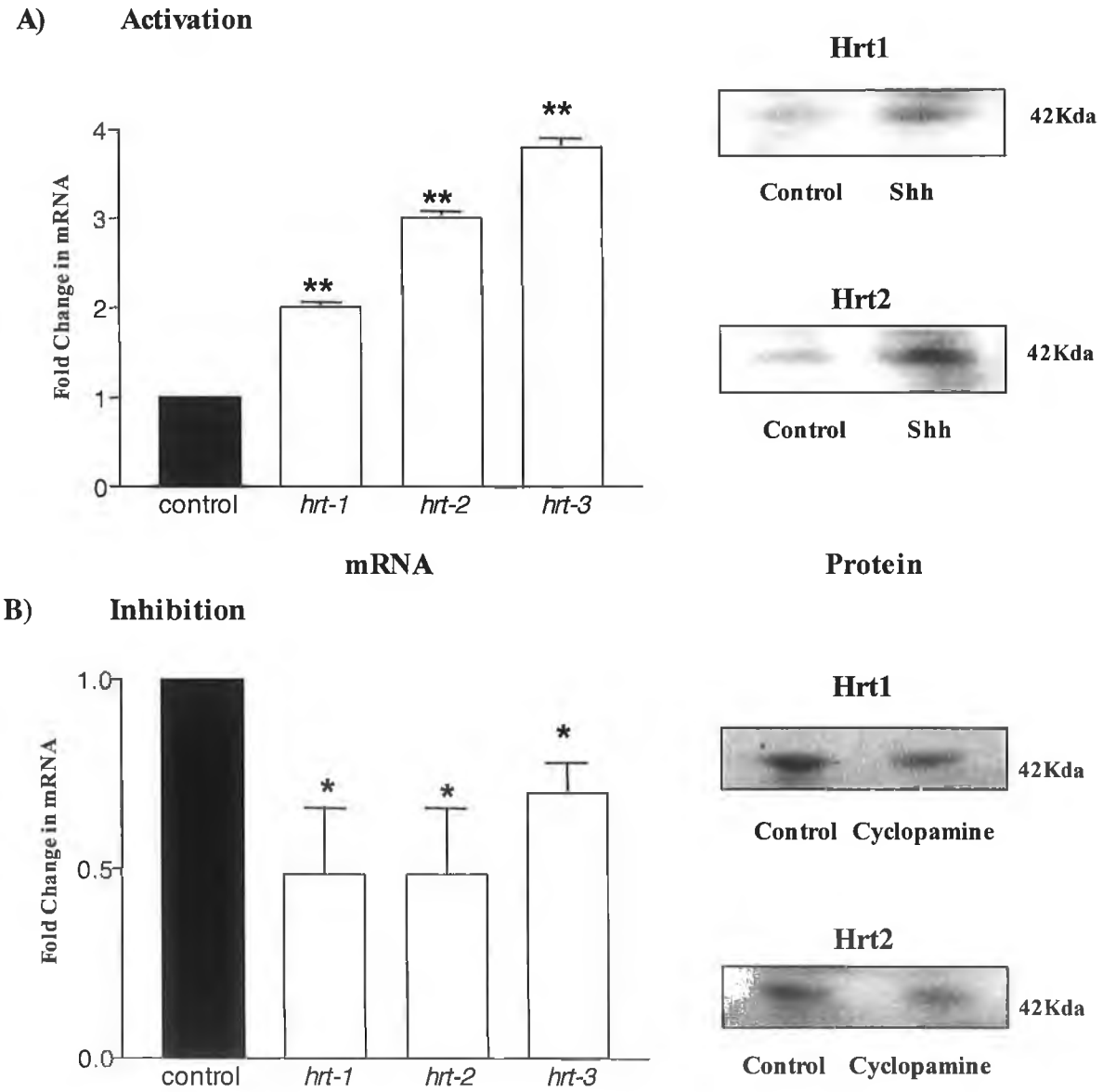


Figure 4.3 Sonic Hedgehog activates Notch target gene expresson in RVSMCs. A) RVSMCs were treated with recombinant Shh (3.5μg) for 48 h and the effect on Notch target gene mRNA and protein expression was determined using QRTPCR and western blotting. The data was normalised to GAPDH and compared to a BSA control. B) The effect of Hh inhibition with cyclopamine (40μM) for 24 h on Notch target gene mRNA and protein expression was determined using QRTPR and western blot analysis. The cumulative data was normalised to GAPDH and compared to a DMF control. * $p<0.05$, ** $p<0.005$ as compared to control (rank test).n=3.

Figure 4.4 The Effect of Notch Inhibition on Hedgehog Target Gene Expression

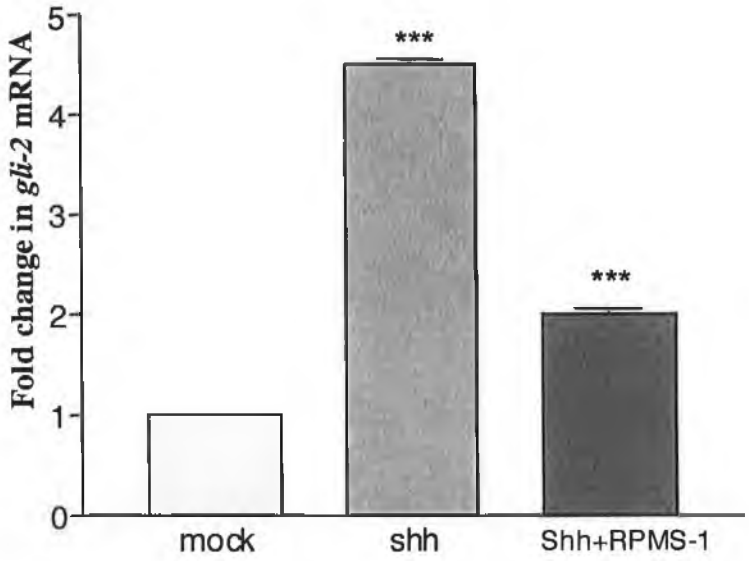


Figure 4.4 The Effect of Notch Inhibition on Hedgehog target gene expression. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the mock vector p7pCMV, Shh or the Notch inhibitor RPMS-1. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8ug/ml, 48h). Samples were isolated and *gli-2* mRNA expression was determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over mock control, *** $p<0.0005$ as compared to control (rank test).n=3.

Figure 4.5 The Effect of Shh Overexpression on Notch Target Gene Expression

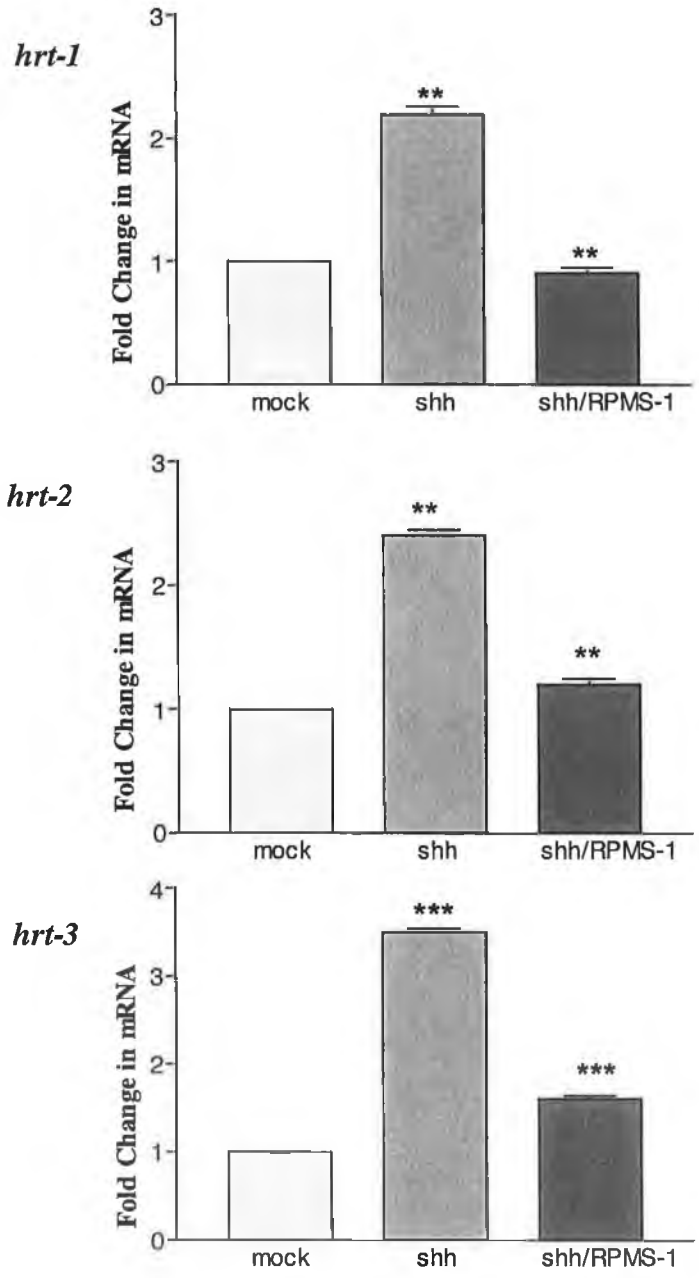


Figure 4.5 The Effect of Shh Overexpression on Notch Target Gene Expression. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co- transfected with the mock vector p7pCMV alone or with Shh or the Notch inhibitor RPMS-1. Samples were isolated and *hrt-1, hrt-2* and *hrt-3* mRNA expression was determined using QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over mock control, *** $p<0.0005$, ** $p<0.005$ as compared to control (rank test). n=3.

Figure 4.6 The Effect of Notch IC on Shh and Ihh Expression

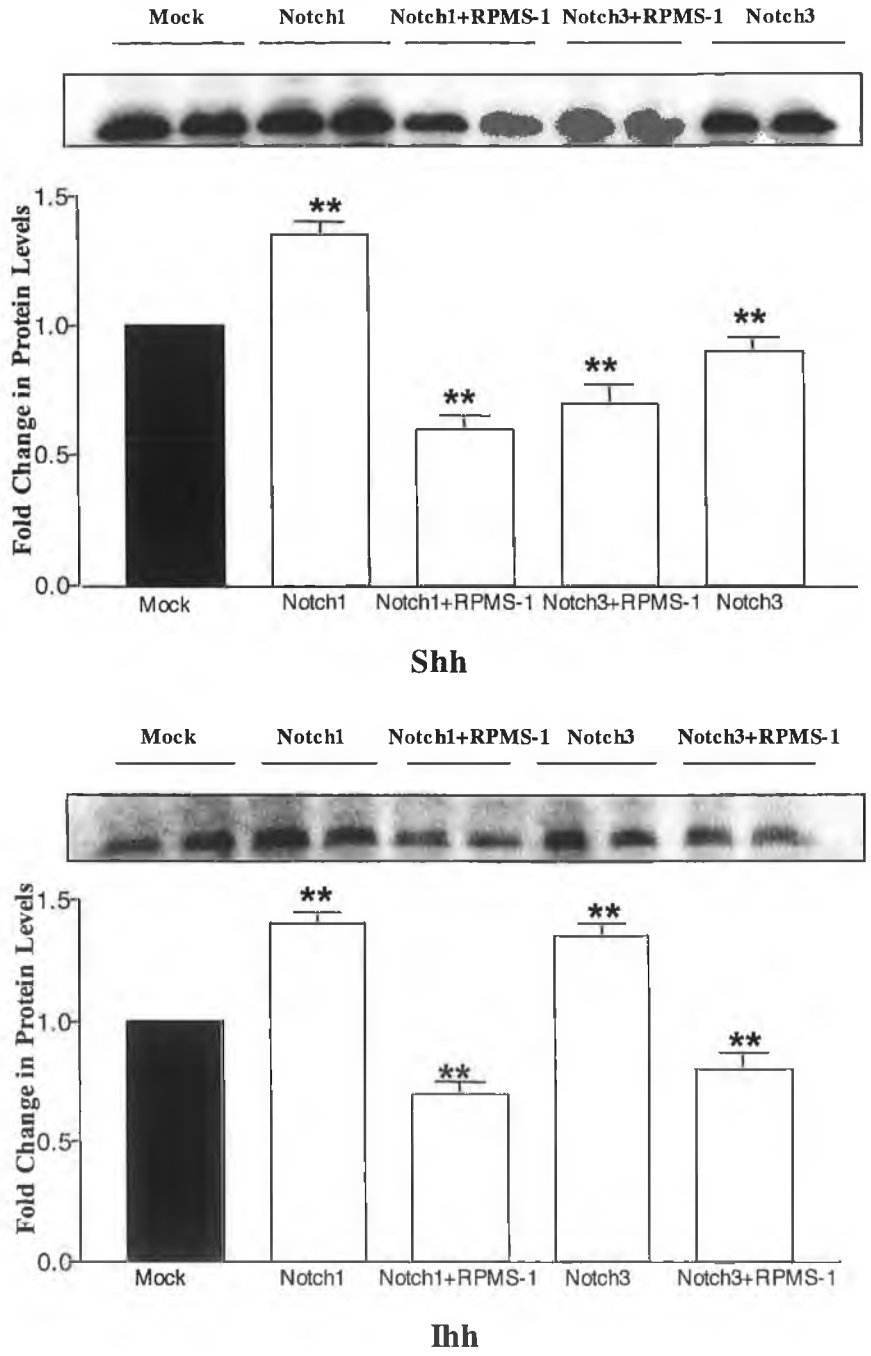


Figure 4.6 The Effect of Notch IC on Hedgehog Signaling. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, Notch 1 IC, Notch 3 IC plus or minus the Notch inhibitor RPMS-1. Protein samples were isolated and western blot analysis was carried out for Shh and Ihh protein expression. Values are expressed as fold change over mock control. ** $p<0.005$ as compared to control (student's t test). Western blots are representative. n=3.

Figure 4.7 The Effect of Notch IC on Smo and Gli_2 mRNA and Promoter Activity

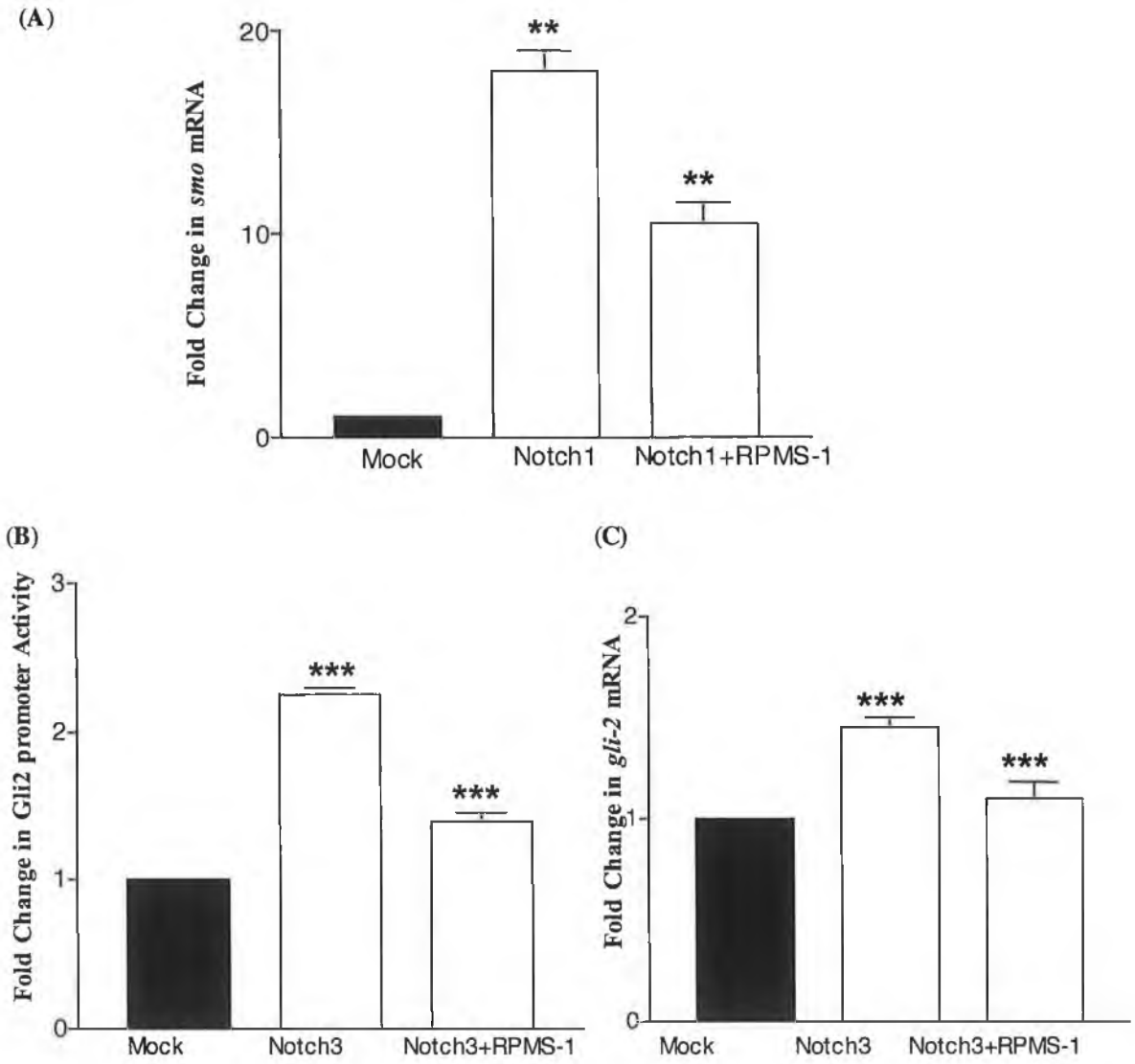


Figure 4. 7 The Effect of Notch IC on Hedgehog Signaling. A) RVSMCs were transiently transfected with the puromycin resistance plasmid pGK3puro and co-transfected with the mock vector p7pCMV, Notch 1 IC plus or minus the Notch inhibitor RPMS-1. QRTPCR analysis was then carried out for *smo* mRNA expression. All data was normalised to GAPDH levels and expressed as fold change over mock control B) RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, the gli-luciferase reporter plasmid and co-transfected with the mock vector p7pCMV, Notch 3 IC, plus or minus the Notch inhibitor RPMS-1. Gli-promoter activity was determined as fold increase over mock control. C) RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co- transfected with the mock vector p7pCMV, Notch 3 IC plus or minus the Notch inhibitor RPMS-1. QRTPCR analysis was then carried out for *gli-2* mRNA expression. All data was normalised to GAPDH levels and expressed as fold change over mock control. *** $p<0.0005$, ** $p<0.005$ as compared to control (rank test). n=3.

Figure 4.8 The Effect of Serum Stimulation on Gli-Promoter Activity

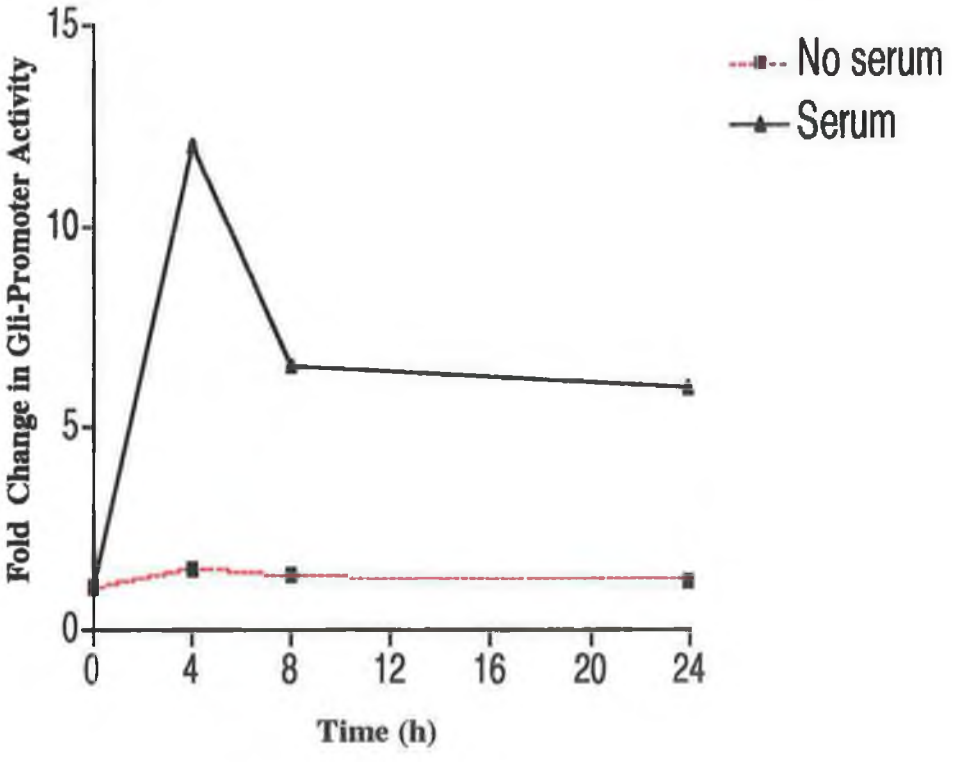


Figure 4.8 The Effect of Serum Stimulation on Gli-Promoter Activity. RVSMCs were transiently transfected with a luciferase tagged Gli-promoter. Following recovery overnight cells were quiesced for 48 h and the effect of serum stimulation on gli-promoter activity was observed. Data represents the mean of 3 independent experiments and values are expressed as fold change over no serum control.

Figure 4.9 The Effect of Shh on RVSMC Proliferation

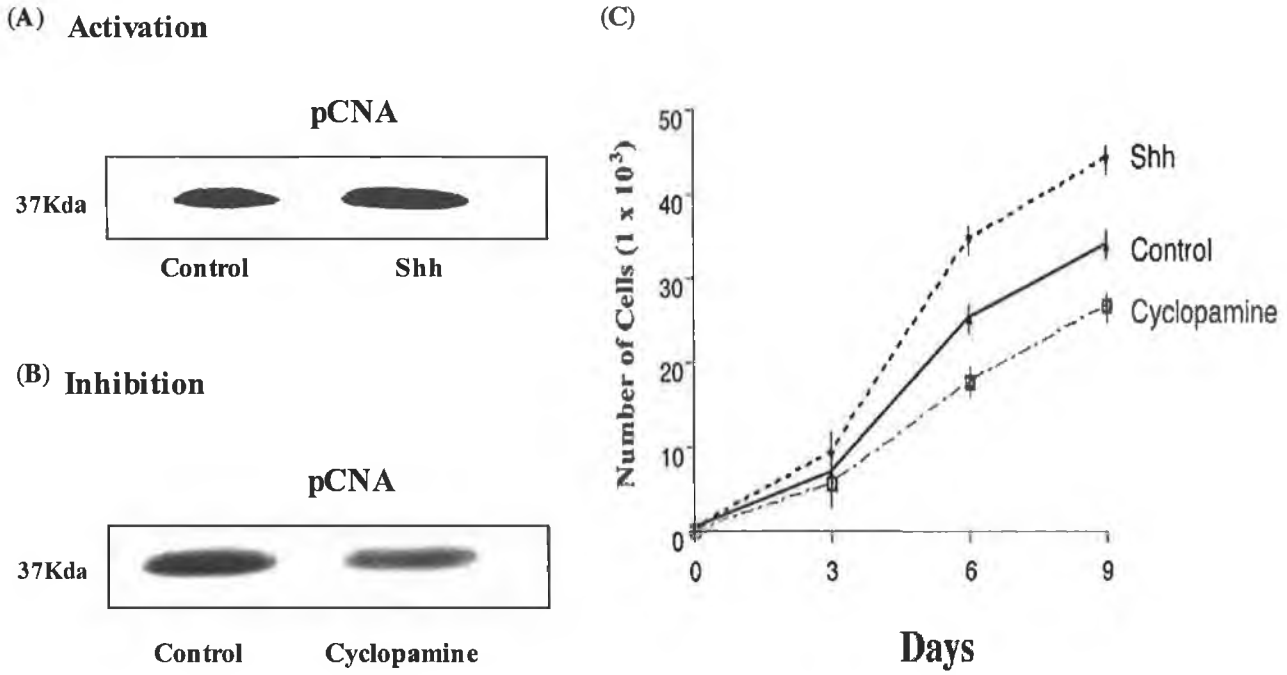


Figure 4.9 The Effect of Shh on RVSMC proliferation. A) RVSMC were treated with recombinant Shh (3.5μg) for 24 h. Serum-stimulated pCNA expression was determined after 24 h Representative blot of 3 independent experiments..B) RVSMC were treated with Cyclopamine (40μM) for 24 h and pCNA expression was determined. Representative blot of 3 independent experiments. C) RVSMCs were treated with Shh for 24 h or with Cyclopamine for 24 h. Cells were then seeded at equal densities and counted at 3 day intervals as described in section 2.1.1. The average count of 3 wells was observed and 3 independent experiments were carried out (Anova). n=3.

Figure 4.10 The Effect of Shh over-expression and Notch IC inhibition on Proliferation

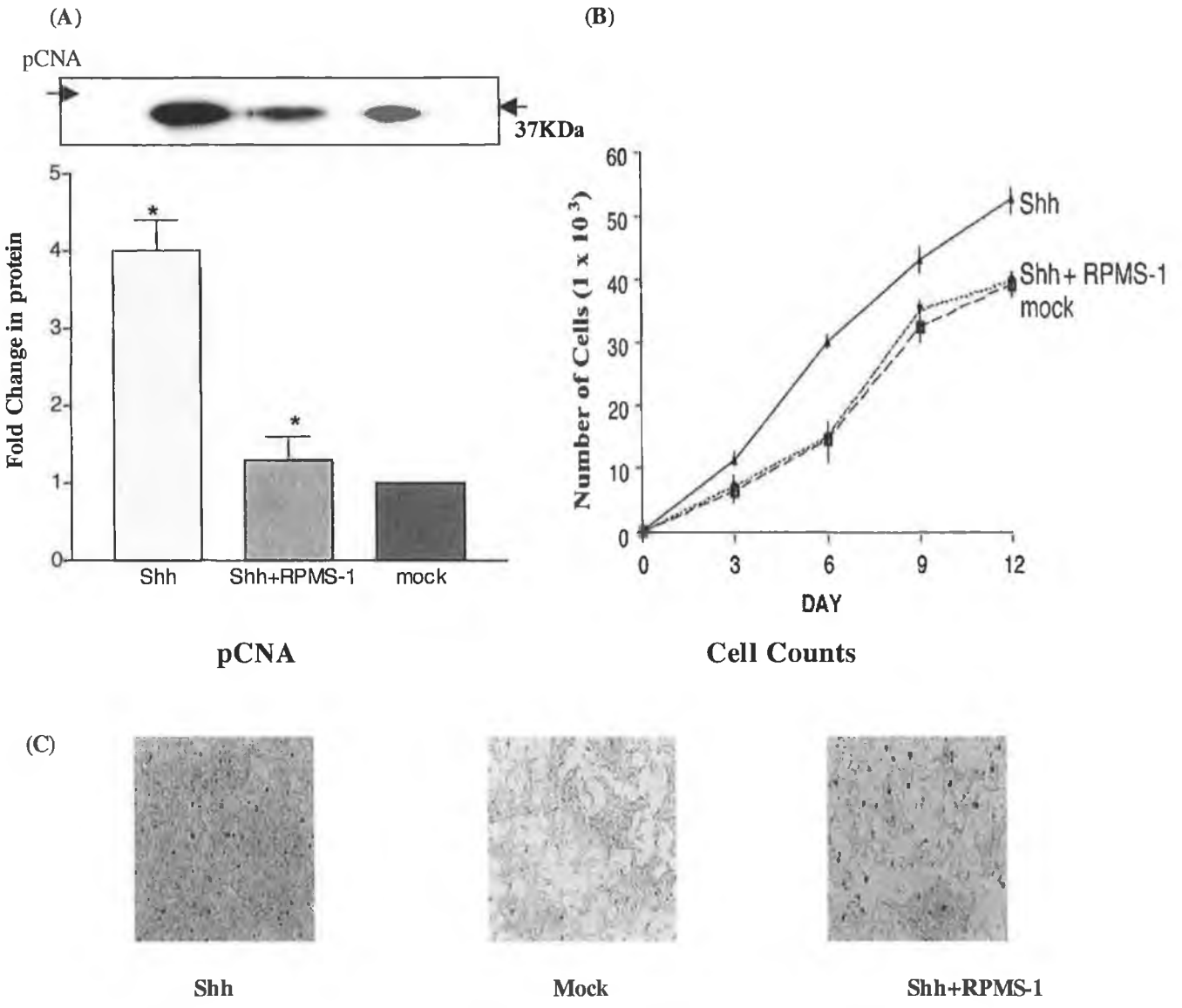


Figure 4.10 The Effect of Shh over-expression and Notch IC inhibition on Proliferation. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co- transfected with the mock vector p7pCMV, Shh plus or minus the Notch inhibitor RPMS-1. Cells were then quiesced for 48h and either analysed for serum stimulated pCNA expression at 24 h (A) or seeded at equal densities and counted at 3 day intervals over 12 days (B). C) Representative clonal proliferation assays at day 12 post transfection. Values are expressed as fold change over mock control (anova). n=3.

Figure 4.11 The Effect of Shh on RVSMC Apoptosis

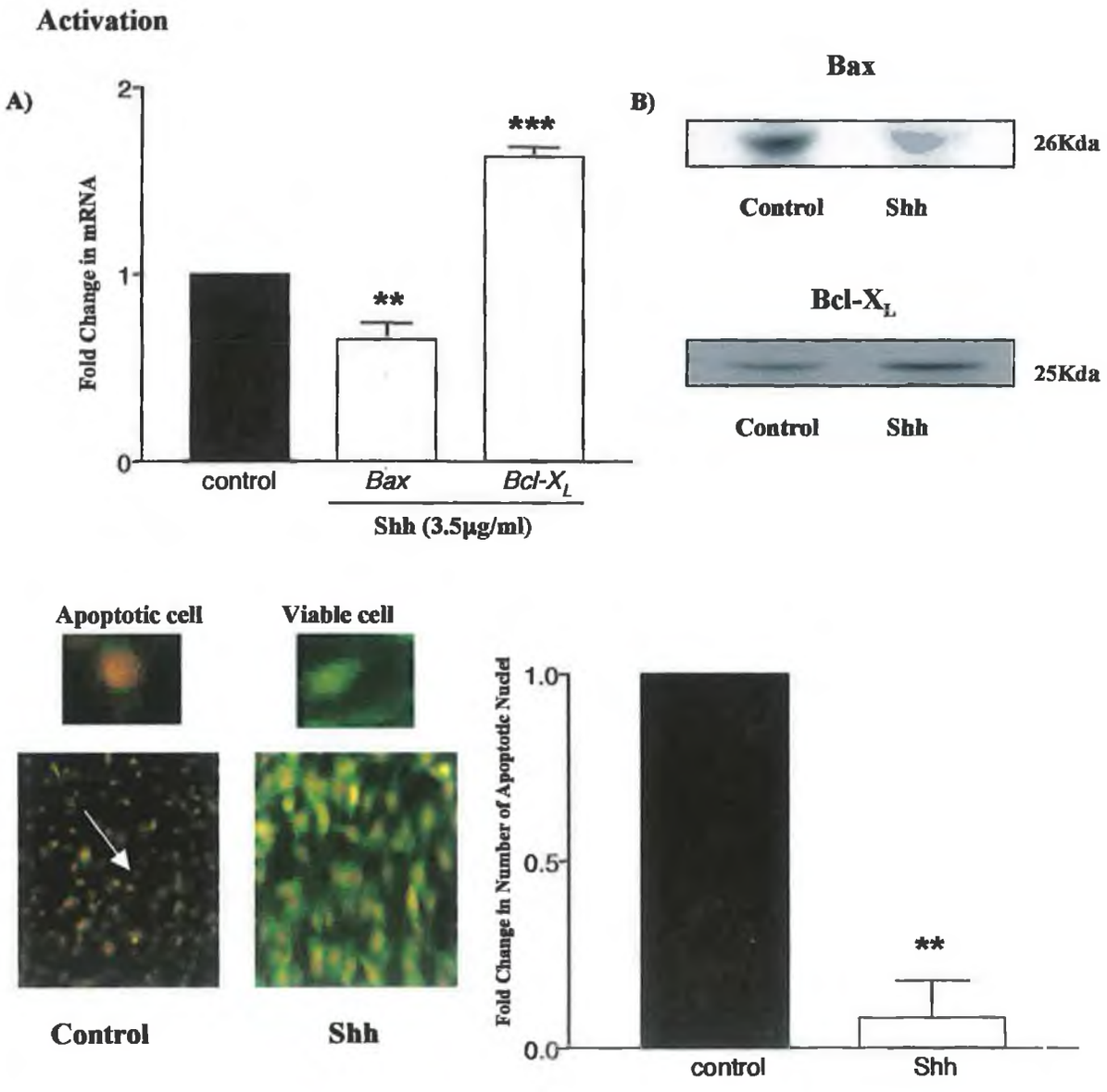


Figure 4.11 The Effect of Shh on RVSMC Apoptosis. The effect of recombinant Shh (3.5µg) for 24 h on apoptotic genes Bax and Bcl-X_L expression was determined. Samples were isolated and Bax and Bcl-X_L mRNA expression was determined by QRTPCR (A). The data was normalised to GAPDH and compared to a BSA control. Bax and Bcl-X_L Protein expression was determined by western blotting (B). Data represents a representative blot of 3 independent experiments. C) RVSMCs were treated with recombinat Shh (3.5µg) for 24 h and quiesced for 48 h. Cells were then stained with the dual stain of Acridine Orange/ Ethidium Bromide for apoptotic nuclei. Values are expressed as fold change over control .** $p<0.005$, *** $p<0.0005$ as compared to control (student's t test). n=3.

Figure 4.12 The Effect of Shh on RVSMC Apoptosis

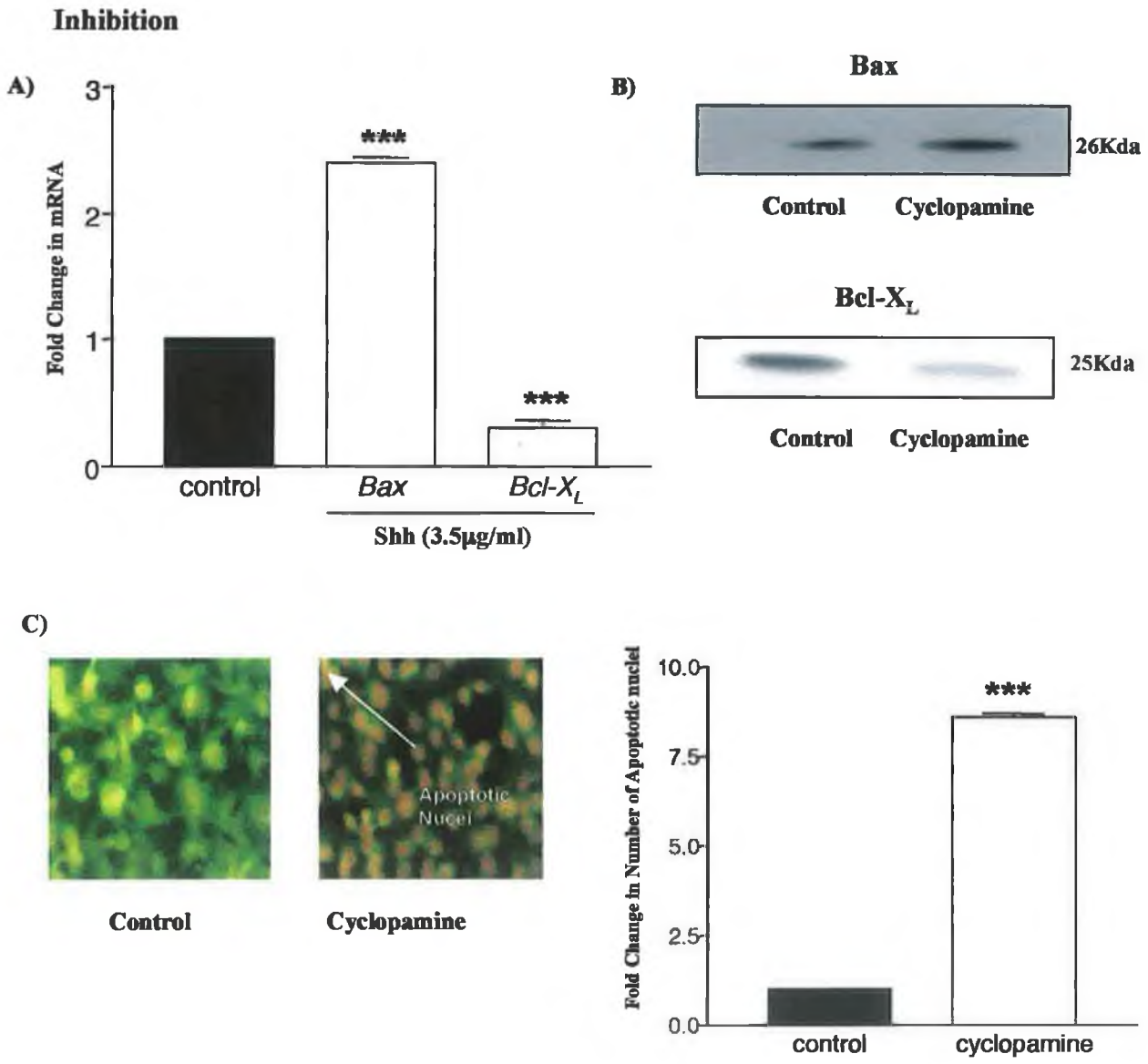


Figure 4.12 The Effect of Shh on RVSMC Apoptosis. The effect of cyclopamine (40µM) for 24 h on apoptotic genes *Bax* and *Bcl-X_L* expression was determined. Samples were isolated and *Bax* and *Bcl-X_L* mRNA expression was determined by QRTPCR (A). The data was normalised to GAPDH and compared to a BSA control. Bax and Bcl-X_L protein expression was determined by western blotting (B)..Data represents a representative blot of 3 independent experiments. C) RVSMCs were treated with recombinat Shh (3.5µg) for 24 h and quiesced for 48 h. Cells were then stained with the dual stain of Acridine Orange/Ethidium Bromide for apoptotic nuclei. Values are expressed as fold change over control , *** $p<0.0005$ as compared to control (student's t test). n=3.

Figure 4.13 The Effect of Shh Over-expression and Notch IC Inhibition on Bax expression

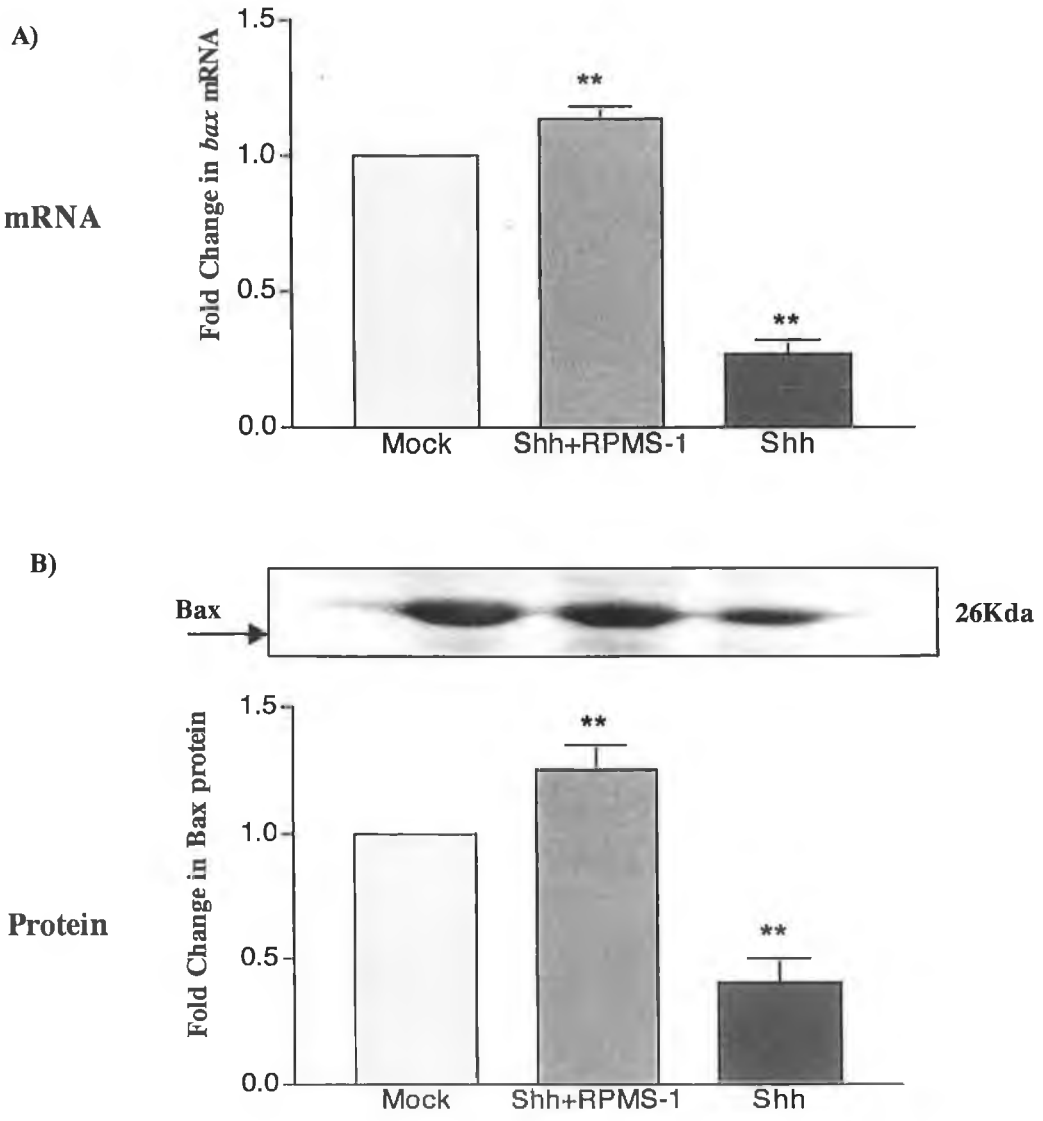


Figure 4.13 The Effect of Shh Over-expression and Notch IC Inhibition on Bax Expression. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the mock vector p7pCMV, Shh plus or minus the Notch inhibitor RPMS-1. Samples were then isolated and *Bax* mRNA levels were determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over mock control.B) RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co- transfected with the mock vector p7pCMV, Shh plus or minus the Notch inhibitor RPMS-1. Samples were then isolated and Bax protein expression was determined by western blotting. Values are expressed as fold change over control , ** $p<0.005$ as compared to control (student's t test). n=3.

Figure 4.14 The Effect of Shh over-expression and Notch IC inhibition on BcL-X_L expression

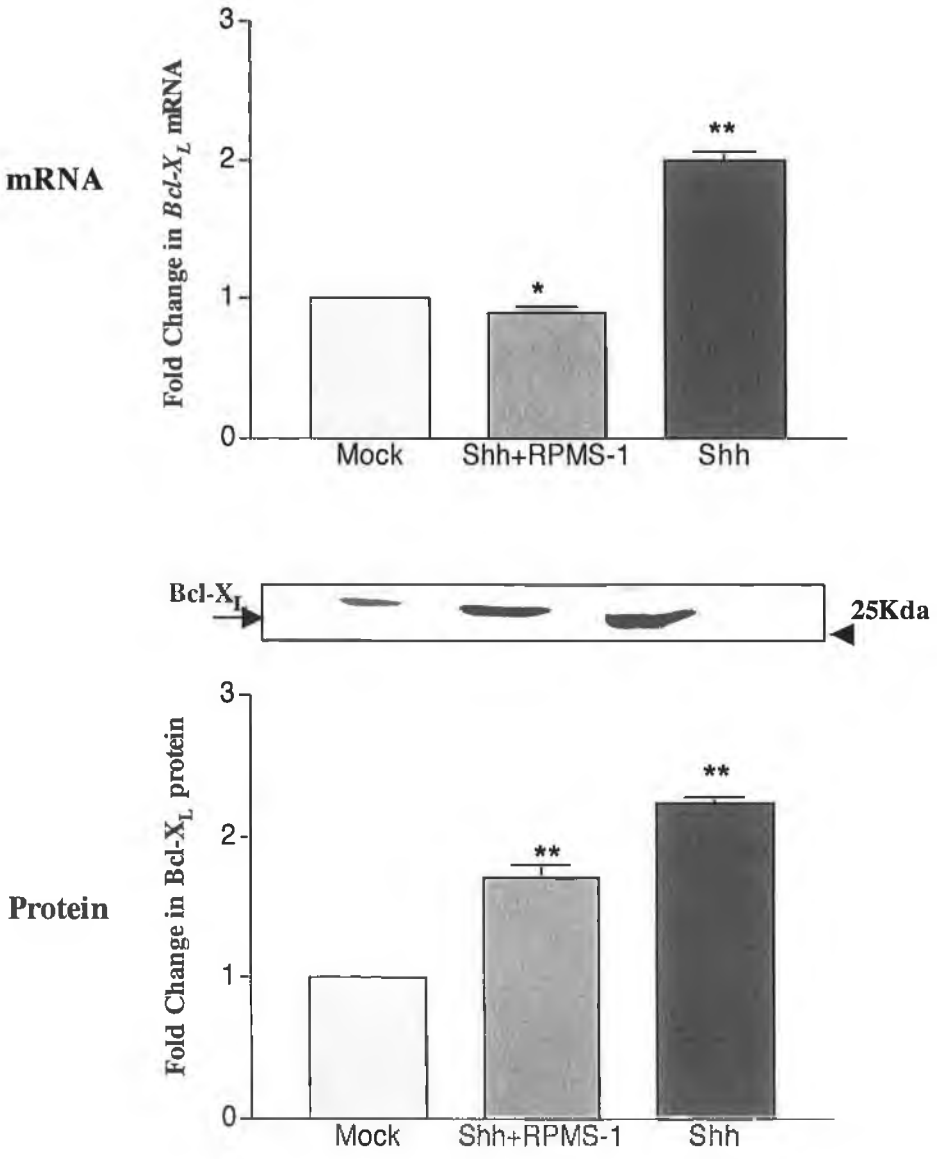


Figure 4.14 The Effect of Shh Over-expression and Notch IC Inhibition on Bcl-X_L Expression. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with themock vector p7pCMV, Shh plus or minus the Notch inhibitor RPMS-1. A) Samples were then isolated and *Bcl-X_L* mRNA levels were determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over mock control.B) RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co- transfected with the mock vector p7pCMV, Shh plus or minus the Notch inhibitor RPMS-1. Samples were then isolated and Bcl-X_L protein expression was determined by western blotting. Values are expressed as fold change over control , ** $p<0.005$, * $p<0.05$ as compared to control (student's t test). n=3.

Figure 4.15 The Effect of Shh Over-expression and Notch IC Inhibition on Apoptosis

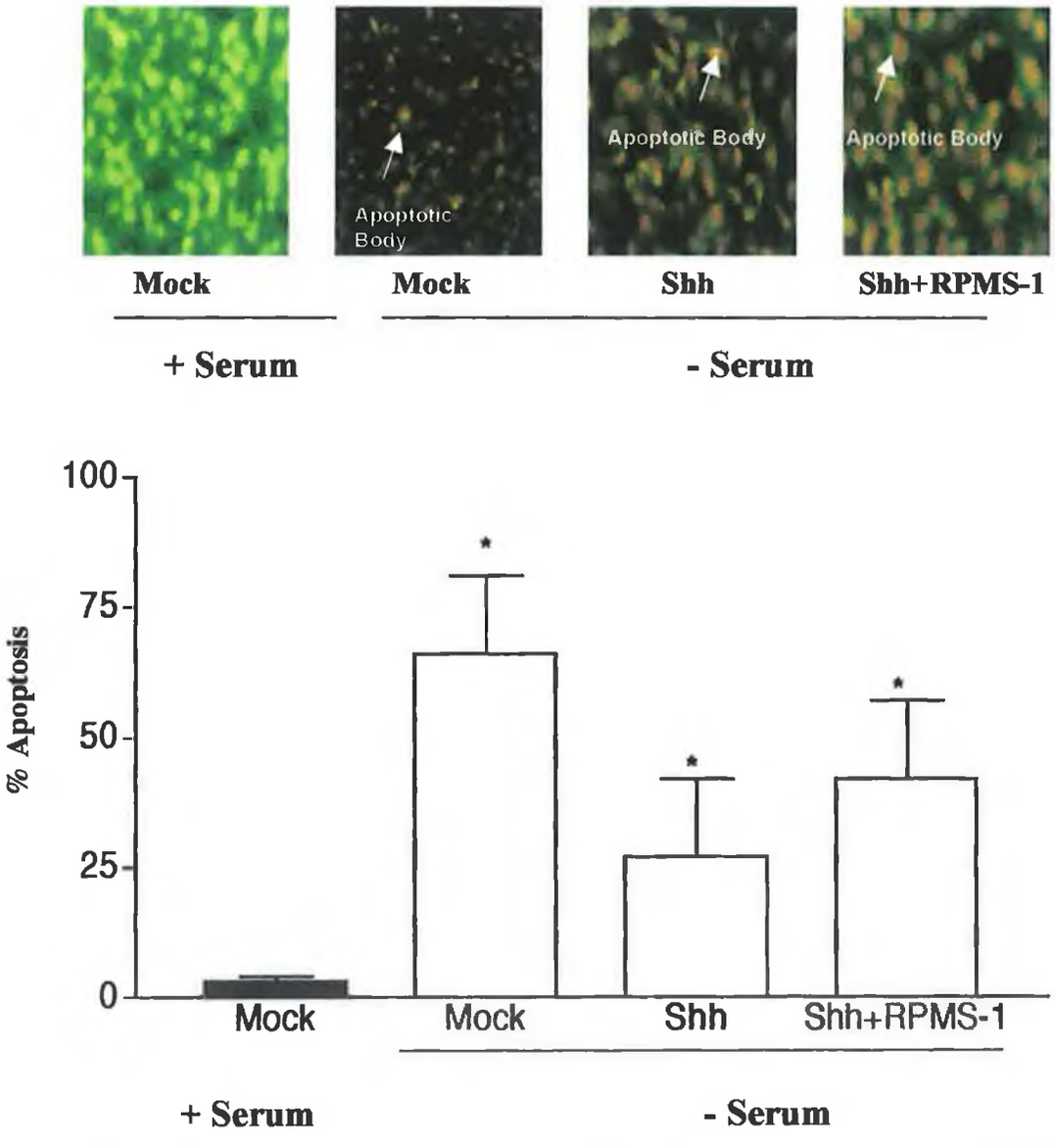


Figure 4.15 The Effect of Shh over-expression and Notch IC Inhibition on Apoptosis. RVSMCs were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, Shh or the Notch inhibitor RPMS-1. Cells were then quiesced for 48 h and then stained with the dual stain of Acridine Orange/ Ethidium Bromide for apoptotic nuclei.Pictures are representative . Values are expressed as fold change over control .* $p<0.05$, as compared to control (rank test). n=3.

Figure 4.16 The Effect of Shh on VEGF Protein Expression

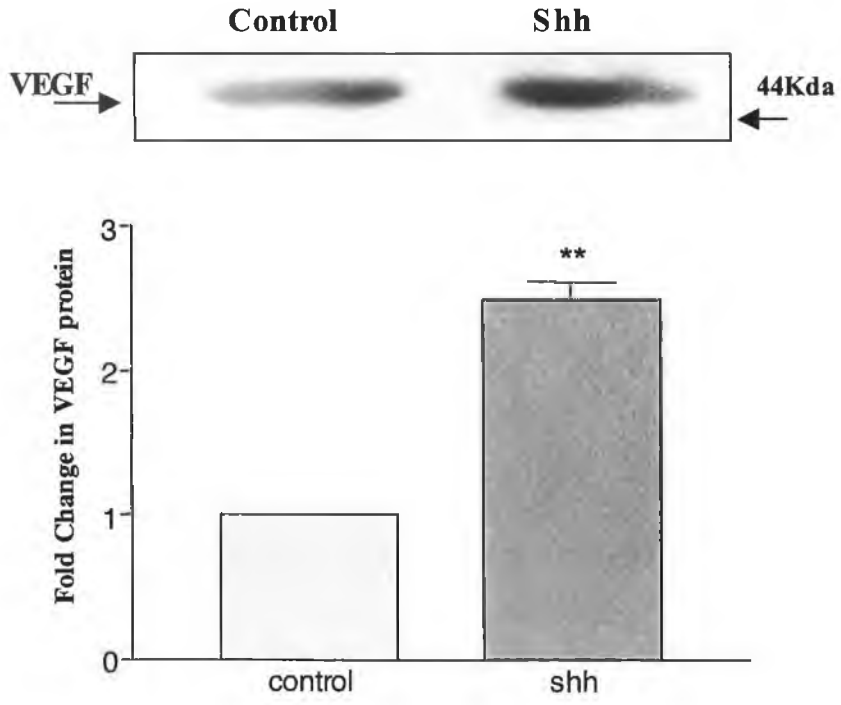


Figure 4.16 The Effect of Shh on VEGF Protein Expression. RVSMCs were treated with recombinant Shh protein (3.5μg) for 48 h and the effect on VEGF protein expression was determined. Representative blot. Values are expressed as fold change over control .* $p<0.05$, as compared to control (rank test) .n=3.

Figure 4.17 The Effect of Shh on VEGF mRNA Expression

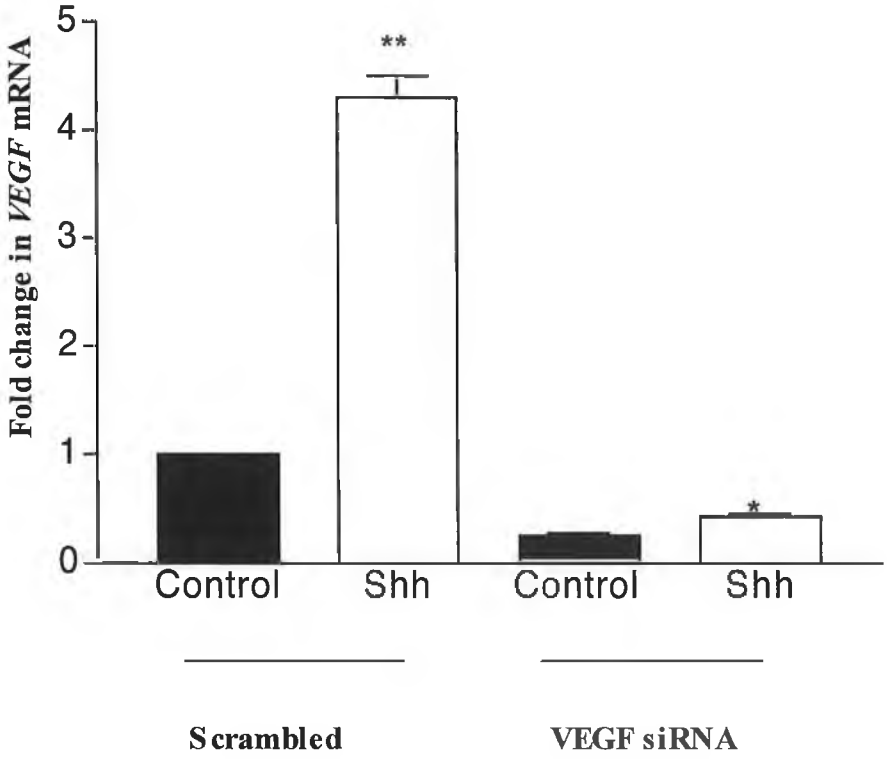


Figure 4.17 The Effect of Shh on VEGF mRNA expression. RVSMCs were transiently transfected with siRNA directed against VEGF or a scrambled control. Following overnight recovery cells were treated with recombinant Shh (3.5ug) for 48 h. Samples were isolated and *VEGF* mRNA expression was determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over control, * $p<0.05$, ** $p<0.005$ as compared to control (rank test). n=3.

Figure 4.18 The Effect of Shh Inhibition on VEGF mRNA Expression

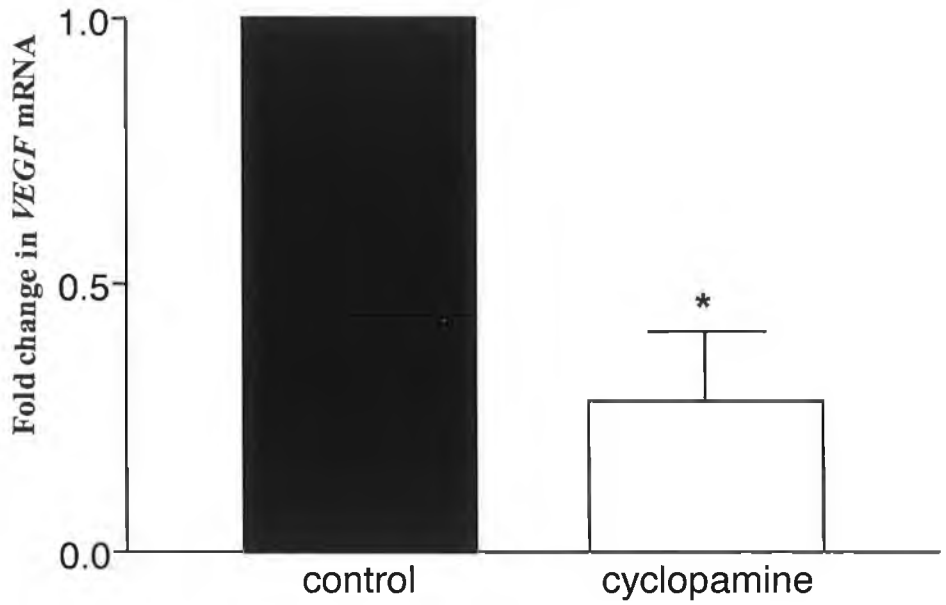


Figure 4.18 The Effect of Shh inhibition VEGF mRNA Expression. The effect of Hh inhibition with cyclopamine (40μM) for 24 h on VEGF mRNA expression was determined using QRTPR. All data was normalised to GAPDH levels and expressed as fold change over control, * $p<0.05$, as compared to control (rank test). n=3.

Figure 4.19 The Effect of VEGF on Notch Target Gene mRNA Expression

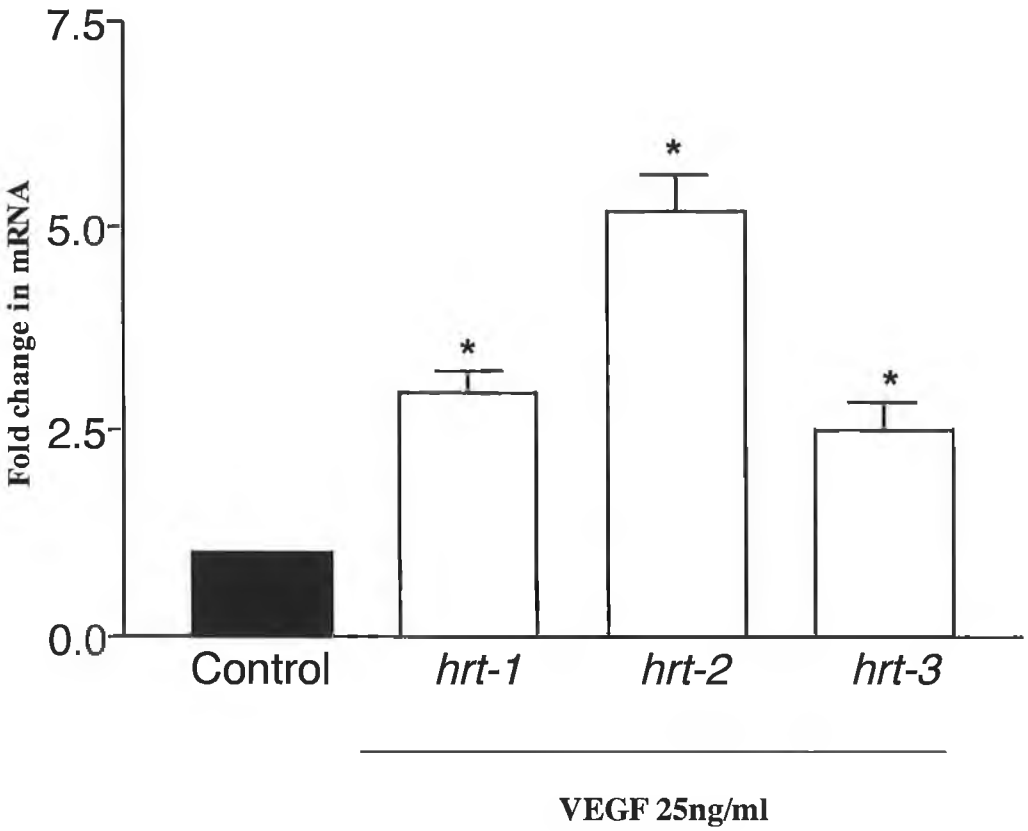


Figure 4.19 The Effect of VEGF on Notch Target gene mRNA Expression. RVSMCs were treated with recombinant VEGF (25ng) for 24 h. Samples were isolated and Notch target gene mRNA expression was determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over control, * $p<0.05$, as compared to control (rank test). n=3

Figure 4.20 The Effect of Shh and VEGF Gene Silencing on Notch Target Gene mRNA Expression

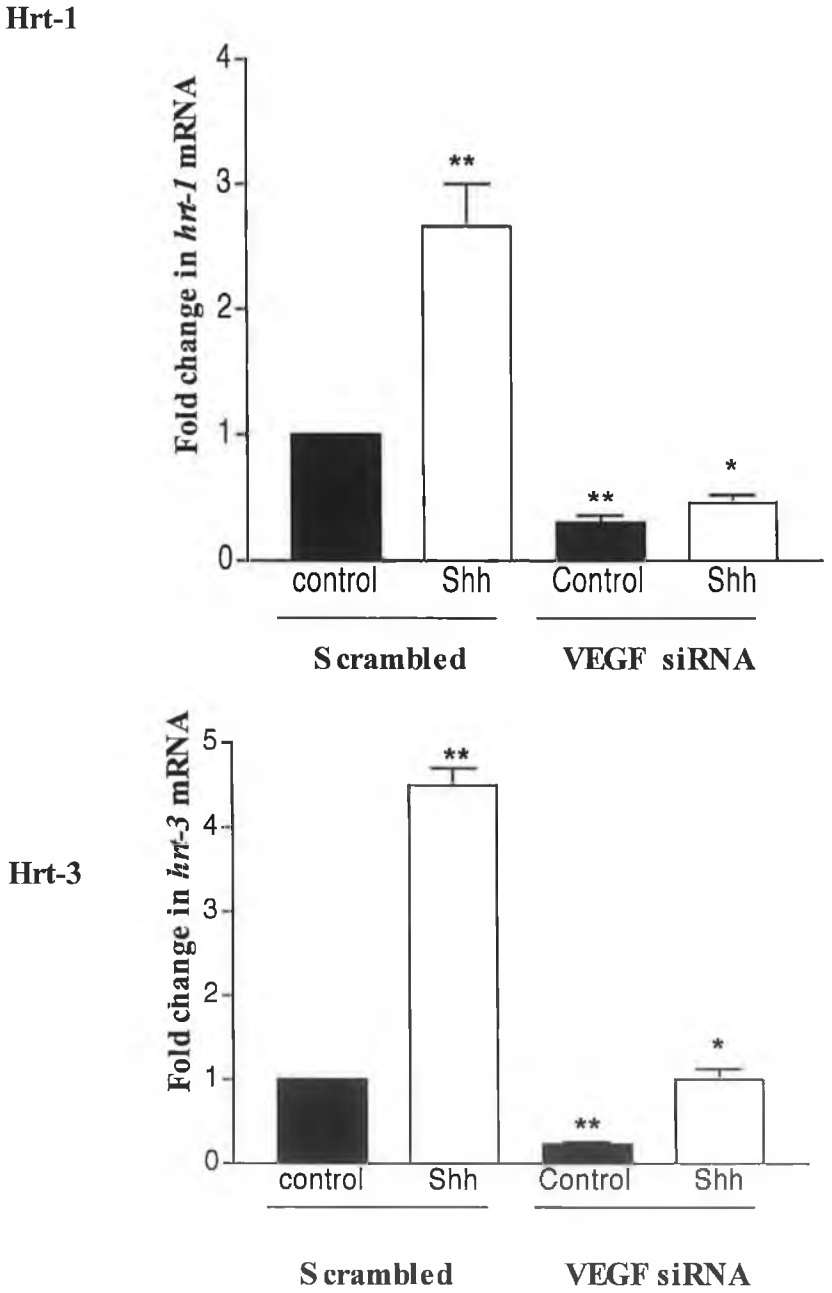


Figure 4.20 The Effect of Shh and VEGF Gene Silencing on Notch Target Gene mRNA Expression. RVSMCs were transiently transfected with siRNA directed against VEGF or a scrambled control. Following overnight recovery cells were treated with recombinant Shh (3.5ug) for 48 h. Samples were isolated and *hrt-1* and *hrt-3* mRNA expression was determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over control, ** $p<0.005$, * $p<0.05$ as compared to control (rank test). n=3.

Figure 4.21 The Effect of VEGF and Shh on Notch Target Gene mRNA expression

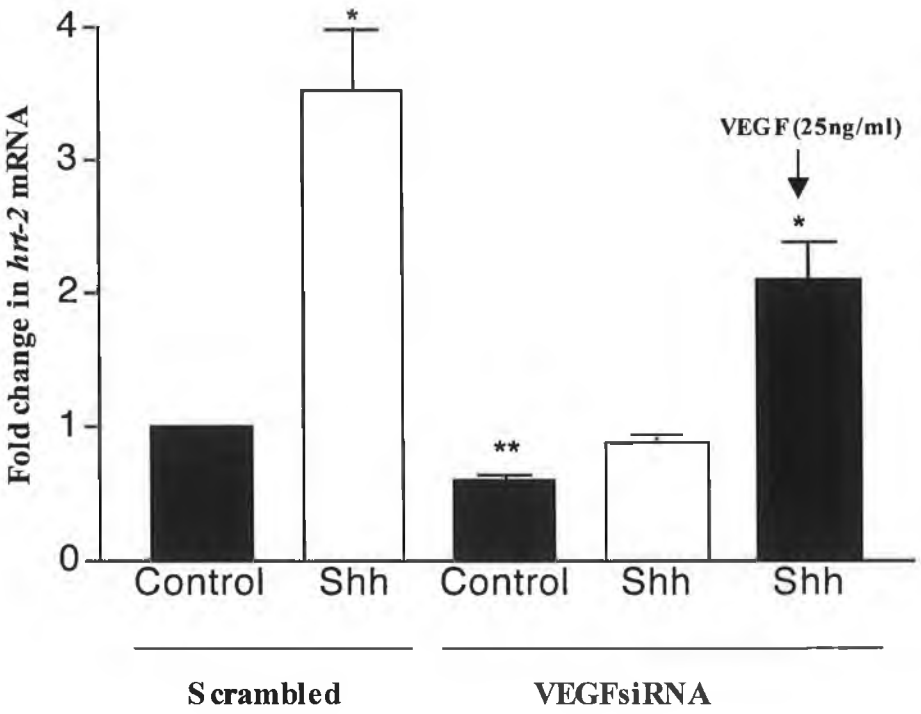


Figure 4.21 The Effect of VEGF and Shh on Notch Target Gene mRNA expression. RVSMCs were transiently transfected with siRNA directed against VEGF or a scrambled control. Following overnight recovery cells were treated with recombinant Shh (3.5ug) for 24 h. Or recombinant VEGF (25ng) for 24 h. Samples were isolated and *hrt-2* mRNA expression was determined by QRTPCR. All data was normalised to GAPDH levels and expressed as fold change over control, * $p<0.05$, ** $p<0.005$ as compared to control (rank test). n=3.

Discussion

This study has clearly established the effect of the Notch signalling pathway on VSMC growth and that it is at least in part, a CBF-1/RBP-Jκ dependent event. The functional role of CBF-1/RBP-Jκ activation of Notch target genes as a primary downstream effector system for Notch signalling in adult VSMC, has only recently been addressed (Wang *et al.,* 2002). Furthermore there has been recent evidence to suggest that the Notch pathway is involved in multiple aspects of vascular development and remodelling (Iso *et al.,* 2003), including proliferation (Wang *et al.,* 2003), apoptosis (Matsumoto *et al.,* 2002; Wang *et al.,* 2002), endothelial migration (Favre *et al.,* 2003), SMC differentiation (Shawber *et al.,* 1996) and angiogenic processes (Mailhos *et al.,* 2001). However, recent studies have also focussed on the role of a CBF-1/RBP-Jκ independent signalling in mediating the response of Notch receptor activation in several cell systems (Nofziger *et al.,* 1996; Iso *et al.,* 2003). This study describes for the first time, the regulation of endogenous signalling following inhibition of CBF-1/RBP-Jκ dependent Notch signalling or constitutive expression of functionally active Notch IC, which results in fundamental changes in VSMC growth *in vitro*. In addition, this study describes the regulation of VSMC growth by individual Notch target genes *hrt1*, *2* and *3* following selective knockdown of these genes by targeted siRNA.

There have been several studies, which have reported the expression of Notch signalling pathway components in adult vascular tissue and cells (Lindner *et al.,* 2001; Wang *et al.,* 2002). This study indicated that constitutive expression of Notch 1 and 3 IC resulted in a significant increase in the expression of both Notch 1 and 3 IC protein and Notch receptor mRNA levels concomitant with a significant increase in Notch target gene promoter activity and mRNA levels in RVSMC. In addition, by inhibiting CBF-1/RBP-Jκ-dependent signalling using both pharmacological inhibitors Brefeldin A and Monensin, in addition to molecular interventions, we reported a significant inhibition of Notch 1 and 3 IC-dependent Notch target gene promoter activity and receptor mRNA levels. This data would suggest that expression of constitutively active Notch 1 and 3 IC requires golgi trafficking mechanisms to translocate to the nucleus as Brefeldin A and Monensin, which disassemble the golgi

apparatus and causes it's collapse into the endoplasmic reticulum, both inhibited Notch IC signalling events.

The inhibition of the Notch signalling pathway clearly decreased RVSMC growth in cycling VSMC suggesting that Notch exerts a net pro-proliferative, anti-apoptotic effect in these cells. This would concur with the pro-survival role of the Notch signalling pathway, which has been established in many other cell types (Kaneta *et al.,* 2000; Mackenzie *et al.,* 2004). Studies by Wang *et al,* 2002, reported that Notch 3 IC can promote SMC proliferation by inhibiting the expression of p27KIP1, a critical cell cycle inhibitor and can promote survival through induction of c-flip, a well-established anti-apoptotic mediator. However, in contrast to this study, they reported that Notch 3 inhibits VSMC growth in a CBF-1/RBP-Jκ-independent manner, whereas we have established this anti-apoptotic effect of Notch to be a CBF-1/RBP-Jk dependent event. However, it is very likely that Notch can promote VSMC growth in both a CBF-1/RBP-Jκ-dependent and -independent manner. In fact, recent studies by Mackenzie *et al,* 2004 reported details that Notch 4 inhibits apoptosis in both a CBF-1/RBP-Jκ-independent and -dependent manner.

This study demonstrates that serum stimulation promotes endogenous Notch signalling through a CBF-1/RBP-Jκ dependent pathway and induces proliferation while in contrast, serum deprivation downregulates Notch signalling while inducing apoptosis. A similar anti-apoptotic effect of Notch 1 has been recently demonstrated in serum deprived arterial EC (Liu *et al.,* 2003). This study reported that both Notch 1 IC and Hes-1 conferred a strong resistance to serum deprivation-induced apoptosis in EC. This indicated that the Notch signalling pathway could play a significant role in regulating EC survival. In contrast to our data, Wang and co-workers, using cultured SMC reported that angiotensin II and PDGF markedly downregulated Notch 3 and Jagged –1 through ERK-dependent signalling mechanisms. Moreover, this down regulation of Jagged-1 and Notch 3 was associated with a decrease in CBF-1/RBP-Jk-mediated gene transcription and a decrease in the mRNA levels of *Hrt-1.* However, one possible explanation for this contrasting report is that Wang and colleagues utilised a clonal rat embryonic wild-type and stably transfected Notch 3 IC cell line that exhibits a de-differentiated phenotype. Moreover, stimulation of SMC growth *in vivo* following injury was associated with an increase in the expression of Notch 3 IC and Notch signalling components (Wag *et al.,* 2002). This again reinforced the

concept that stimulation of differentiated SMC growth is associated with enhanced Notch signalling (Campos *et al.,* 2002; Lindner *et al.,* 2001). Though this study clearly demonstrates that serum stimulation activates Notch signalling and subsequently promotes VSMC growth, the stimulus foe Notch IC activation following serum stimulation remains to be elucidated. There have been several serum mitogens implicated in modulating Notch receptor expression and signalling in several cell systems (Wang *et al.,* 2002; Bongarzone *et al.,* 2000). Vascular endothelial growth factor (VEGF) has been reported to induce gene expression of Notch1 and its ligand Delta-like 4 (Dll4) in human arterial endothelial cell (Liu *et al.,* 2003). In this study, 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. This study will later demonstrate that VEGF can induce the upregulation of Notch target genes *hrt-1, 2* and *3* and subsequently promote cell growth through a signalling cascade involving the Hedgehog and Notch signalling pathways.

The mechanisms on how endogenous Notch IC signalling through a CBF-1/RBP-Jk-dependent pathway promotes a pro-survival and an anti-apoptotic effect remains unclear. In an attempt to elucidate the mechanisms through which the Notch signalling pathway exerts its pro-survival, anti-apoptotic effect in VSMC, we examined the effect of the Notch signalling pathway on the Bcl-2 family of apoptotic genes. The Notch signalling pathway has been shown to regulate members of the Bcl-2 family in both vascular and non-vascular cells. Notch 1 and Notch 4 have been reported to upregulate Bcl-2 expression in T-cells and EC respectively (Deftos *et al.,* 1998; Mackenzie *et al.,* 2004). As the Bcl-2 family is central to the regulation of apoptosis in many cell types and changes in Bcl-2 family expression are often observed during the pathogenesis of vascular disease, it is likely that the Notch signalling pathway may regulate some Bcl-2 family members. Therefore, this study examined the effect of the Notch signalling pathway on the expression of both pro-apoptotic Bax and anti-apoptotic Bcl-X_L. We observed following inhibition of Notch IC, that there was a significant increase in Bax while a concurrent decrease in Bcl-X_L was reported. Furthermore, by selective knockdown of *hrt-1, 2* and *3* by siRNA, there was a significant increase in Bax expression while concurrent decrease in Bcl-X_L was observed. In addition, serum deprivation resulted in an increase in Bax and a decrease

in Bcl-X_L, further suggesting a Notch-mediated regulation of Bcl-2 family members in VSMC. Moreover, using bioinformatics this study examined whether any Notch target gene promoter sites were present on either the Bax or Bcl-X_L genes. Nakagawa *et al,* reported that the Hrt family of genes preferentially bind to an E box motif CACGTG, in addition to CAACTG, CACCTG, CACTTG and CATCTG to regulate transcription of other genes. Therefore, through sequence alignment of cloned Bax and Bcl-X_L promoters (Grillot *et al.,* 1997; Igata *et al.,* 1999), we hypothesised that the Hrt genes could bind and possibly regulate both Bax and Bcl-X_L. However, though this would require confirmation with mutational analysis, the significant changes in expression of both bax and Bcl-X_L by *Hrt* gene knockdown by siRNA, certainly adds weight to this argument.

In conclusion, this study has provided further evidence that both Notch 1 and 3 receptors acting through a CBF-1/RBP-Jκ dependent signalling pathway are an important determinant of SMC growth *in vitro*. Furthermore that Notch target genes *hrt-1, 2* and *3* are important modulators of VSMC growth by varying degrees with *hrt-1* having the most significant effect on VSMC proliferation, while *hrt-3* exerts the most significant effect on VSMC apoptosis *in vitro*. Notch receptor expression is also upregulated within the vasculature of animal models following vascular injury (Leong *et al.,* 2002; Campos *et al.,* 2002). Therefore greater knowledge of the regulation of Notch receptors and the downstream activation of Notch target genes in SMC may provide new insights into the molecular mechanisms underlying changes in vascular cell fate that underlie vascular proliferative disease.

Chapter 5

Results

The Effect of Mechanical Forces on the Notch and Hedgehog Signalling Pathways and Cell Growth *in vitro* and *in vivo*

5.1 Introduction

Biomechanical forces are known to play an important role in maintaining the normal tissue architecture of the cardiovascular system. The haemodynamic forces associated with the flow of blood play a vital role in the physiological control of vascular tone, remodelling and associated vascular pathologies. Cells of the vasculature are exposed to two principal haemadynamic forces, shear stress and cyclic strain. Shear stress is described as the dragging frictional force created due to blood flow and primarily affects EC under normal conditions. The other forces include cyclic circumferential strain, which is caused by a transmural force acting perpendicular to the vessel wall (Schwarz *et al.,* 2002). Mechanotransduction is known to play a central role in the highly co-coordinated cellular response of the vasculature to changes in haemadynamic stimulation. Transduction of biomechanical stimuli leads to the activation of cellular signalling mechanisms that can alter cell fate. It is these alterations in cell fate in response to haemadynamic stimulation that are fundamental to the pathogenesis of vascular disease (Osol *et al.,* 1995). Strain-induced changes in SMC growth participates in the local vascular response to hypertension (Koller *et al.,* 2002), late lumen loss and restonosis after vascular interventions in addition to plaque vulnerability during atherosclerosis (Schwarz *et al.*, 2002, Brooks *et al.,* 2005).

VSMC, which constitute the major component of the blood vessel wall, absorb most of the pressure–induced cyclic strain. Blood vessels are continuously exposed to mechanical forces, which is essential to develop and maintain a differentiated and functional VSMC phenotype (Birukov *et al,*.1998). The pathogenic role of VSMC growth is increasingly recognized in cardiovascular disease. Since changes in VSMC growth are also apparent during vascular morphogenesis and modelling of the embryonic vasculature (Brookes *et al.,* 2004) (Majesky *et al.,* 2003), the control of these cell fate decisions in adult cells may share similar signalling pathways. This study aimed to investigate the effect of cyclic strain on both the Notch and Hh signalling pathways and in addition, determine the effect of mechanical force on VSMC growth. Furthermore, we aimed to elucidate whether the cyclic strain-induced changes in VSMC growth are regulated by the Notch signaling pathway. A Flexercell

Tension Plus™ strain unit was used in this study to regulate pressure to flexible bottomed pronectin-coated Bioflex plates, thus allowing VSMC to be exposed to defined levels of cyclic strain. VSMCs were exposed to 10% cyclic strain which is accepted as normal physiological conditions *in vivo*. Cardiovascular disease states, such as hypertension, can increase the level of cyclic strain on VSMC up to 30% (Li and Xu, 2000). Biomechanical signals induce a highly restricted transcriptional response in SMC that include genes that can modify vascular structure (Xu *et al.*, 2000). As components of the Notch and Hh signaling pathways are co-ordinately regulated in vascular tissue (Wang *et al.*, 2002), (Pola *et al.*, 2001), the present study examined the specific role of cyclic strain on endogenous Notch and Hh signaling components in VSMC and their contributory role in controlling the growth response of these cells following strain.

To further address the role of mechanical forces associated with blood flow on the regulation of Notch and Hh signalling and subsequently on vascular cell fate we determined the effect of altered biomechanical forces in an *in vivo* environment. We examined the relative expression of Notch and Hh signalling components in addition to the growth response *in vivo* using two models of vascular injury. The models of altered haemadynamic forces and subsequent vascular remodelling analyzed in this study were the rat carotid artery and portal vein ligated models. The carotid artery ligation provides a model of bilateral carotid remodelling in a single animal. Hence, the simultaneous reduction in blood flow in the left (ligated) carotid artery and the subsequent increase in blood flow in the right carotid artery allows us to examine the effects of altered haemadynamics on expression of Notch, Hh and other relevant genes of interest in a single animal. This partial ligation model used in this study and by Korshunov and Berk (2003) shows that the partial ligation of the left carotid artery results in a dramatic decrease of 90% in blood flow in the left carotid artery, with the maintenance of an intact endothelium with no thrombosis. Furthermore, a concomitant increase of 70% in blood flow was observed in the right carotid artery. This particular model exhibits vessel enlargement or outward remodelling in both the left and right carotid arteries, which is maximal at 7 days post-ligation. Carotid ligation resulted in greater remodelling in the right carotid artery, with an increased lumen and only a subtle increase in medial or adventitial layers. In contrast, the left

carotid artery displayed dramatic increases in both medial and adventitial layers.

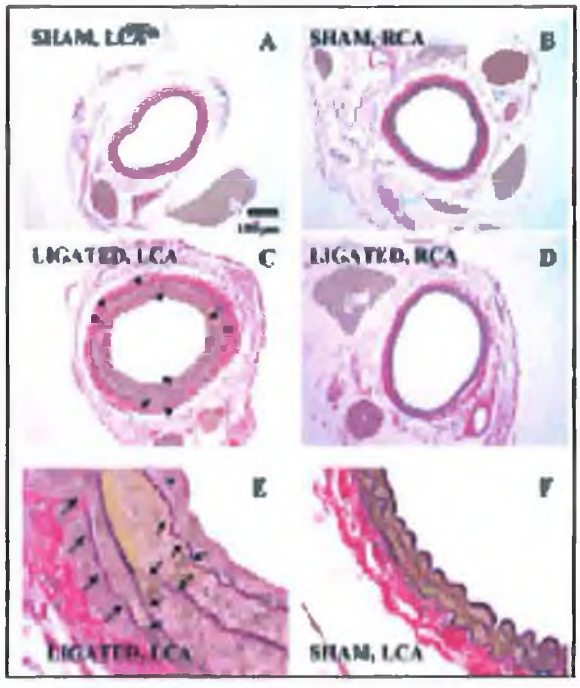


Figure 1: Carotid remodelling. Verhoeff Van Giesson staining of sham, right carotid artery (RCA) and left carotid artery (LCA) 14 days post-ligation. Sham (A, B, F); Ligated (C, D, E). Elastic fibres, black; nuclei, brown; collagen, red; other tissue structure, yellow. Partial ligation resulted in significant intimal formation (in mouse model only) in the LCA, with small breaks (arrows) and thinning in the internal and external elastic laminae. In contrast, the elastic laminae and the media of shams remained intact (Korshunov and Berk 2003).

In addition to the carotid ligation model we also utilized portal vein ligation (PVL) as a model of altered heamadynamic forces and compared this to sham-operated control. Portal vein ligation induces pre-hepatic portal hypertension, which is characterized by increased cardiac output and increased plasma volume (Yokoyama *et al.*, 2001). As a result of this increased cardiac output and plasma volume, blood pressure and therefore biomechanical forces are increased in ligated animals as compared to sham-operated controls. Previous studies by Yokoyama (2002), observed increases in portal

pressure from 5.41±0.6 mm Hg in sham-operated animals as compared to 13.2±0.9 mm Hg in portal vein ligated rats

The aim of this chapter was to investigate the effect of altered biomechanical forces both in vitro and in vivo on Notch and Hh signalling pathway expression and the subsequent effect on VSMC growth.

5.2 Results

5.2.1 Notch 3 Receptor Promotes SMC Proliferation and Inhibits Apoptosis Through Activation of Notch Target Genes in a CBF-1/RBP-Jk-dependent Manner.

The co-expression of RPMS-1 to inhibit CBF-1/RBP-Jκ-dependent signalling resulted in a significant increase of 31±9.0% in the number of apoptotic nuclei when compared to mock controls (**Fig. 5.1 A**). Over expression of constitutively active Notch 3 IC also resulted in a significant reduction in the *bax/bcl-xL* ratio such that steady state *bax* mRNA levels decreased by 48±3.0% while *bcl-X_L* mRNA levels increased by (62±12.0%) when compared to mock controls (**Fig. 5.1 B** and **C**). Moreover, inhibition of endogenous CBF-1/RBP-Jκ-dependent signaling following expression of RPMS-1 resulted in an increase of 42±3.0% in the pro-apoptotic *bax* mRNA levels while concomitantly inhibiting anti-apoptotic *bcl -$_XL$* mRNA levels by 38±2.0% (**Fig. 5.1 B** and **C**). Furthermore, the changes in the *bax/bcl-xL* ratio following over expression of constitutively active Notch 3 IC were reversed following co-expression with RPMS-1 (**Fig. 5.1 B** and **C**). In addition, selective knockdown of a Notch target gene, *hrt-2* with siRNA resulted in a significant increase of 410±87.0% in *bax* mRNA levels while significantly decreasing *bcl-$_XL$* mRNA by 45±13.2% when compared to the scrambled siRNA control (**Fig. 5.2**).

5.2.2 Cyclic Strain Induces Changes in SMC Growth *In Vitro*.

Cyclic strain (24h, 10%) significantly decreased the expression of pCNA, a marker for cell proliferation by 40±4.0% after 24 h (**Fig. 5.3 B**) while concomitantly decreasing the proliferation rate of SMC for up to 10 days post strain by 33±2.5% (**Fig. 5.3 A**). In parallel cultures, serum-deprivation of SMC for 48 h, a known ubiquitous pro-apoptotic stimulus for SMC, caused a marked fold increase of 1.9±.05 in SMC Bax protein expression while concurrently inhibiting Bcl-$_X$L expression by 72±6.0% (**Fig. 5.4 B**). Cyclic strain also increased the Bax/Bcl-$_X$L ratio by increasing the expression of Bax by a fold of 1.24±.03 and decreasing the expression of Bcl-X_L, respectively by 61±8.0%, when compared to unstrained cells (**Fig. 5.4 A**). The change

in the Bax/Bcl-xL ratio was further confirmed by examining *Bax* and *Bcl-X_L* mRNA levels following strain. Strain significantly increased the levels of proapoptotic *bax* mRNA by 31.0±8.0% and decreased the levels of *Bcl-X_L* mRNA by 29.0±17.0% when compared to unstrained cells (**Fig. 5.4 C**).

5.2.3 Cyclic Strain Inhibits Notch Signaling in SMC *In Vitro*

Cyclic strain (10%, 24 h) caused a significant decrease in Notch signalling pathway components. Notch 1 and Notch 3 protein expression (both Notch IC and the full-length receptor (Notch EC)) was significantly decreased by 17.0±2.0% and 25.0±3.0% respectively (**Fig. 5.5 A**) concomitant with a significant decrease in the expression of Notch ligand, jagged-1 protein expression by 30.0±6.0% (**Fig. 5.5 C**). In addition, Hrt-1 and Hrt-3 protein expression (**Fig. 5.6 A**) was significantly decreased by 12.0±1.0% and 11.0±2.0% respectively following strain. Cyclic strain also significantly decreased *Notch 1* and *Notch 3* receptor steady state mRNA levels by 57.0±11.0% and 50.0±2.9% respectively with respect to static control (**Fig. 5.5 B**). Similarly, a significant decrease of 35.0±4.0% in *jagged-1* mRNA levels was also observed due to cyclic strain (**Fig. 5.5 D**), concomitant with a significant decrease in *hrt-1, hrt-2* and *hrt-3 by* 39.0±1.0%, 47.0±17.0% and 42.0±12.0% respectively and a significant decrease of 42.0±12.0% and *hes-5* 57.0±11.0% in *hes-1* and *hes-5* mRNA levels by when compared to unstrained controls (**Fig. 5.6 B**). The effects of cyclic strain on the Notch signalling pathway were both force- and time-dependent. VSMC were subjected to 0, 5, 10 and 15% cyclic strain and assayed for Notch receptor and Notch target gene mRNA expression (**Fig. 5.10**). Both *notch 3* and *hes-5* exhibited a significant force-dependent decrease in mRNA expression. The percentage decrease in *notch 3* mRNA levels was 26.0±1.0, 50.0±3.0 and 64.0±0.8 at 5%, 10%, and 15% cyclic strain, as compared to the static control (**Fig. 5.7 A**). *Hes-5* mRNA expression was similarly decreased with increasing levels of cyclic strain, with a decrease of 38.0±13.0% at 5% cyclic strain, 57.0±11.0% at 10% cyclic strain, and 61.0±14.0% at 15% cyclic strain, as compared to static control (**Fig. 5.7 B**). *Notch 3* and *hes-5* mRNA levels were temporally reduced following exposure to strain for up to 24 h when compared to the unstrained controls (**Fig. 5.8 A** and **B**). VSMC exposed to 10% cyclic strain over a period of 24 h resulted in a significant temporal decrease in

Notch 3 mRNA expression of 23.0±7.0%, 58.0±6.0% and 64.0±3.0% at 4 h, 8 h, and 24 h post-cyclic strain respectively, as compared to static control (0 h timepoint). *Hes-5* mRNA expression showed a similar temporal decrease of (27.0±4.0%), (55.0±6.0%) and (25.0±3.0%) at 4 h, 8 h, and 24 h post-cyclic strain respectively, as compared to static control (0 h timepoint). For subsequent studies, regulation of Notch signaling was examined following exposure to 10% cyclic strain for 24 h. There was also a significant temporal decrease in baseline transactivation CBF-1/RBP-Jκ-dependent-promoter activity in cells exposed to strain when compared to unstrained controls at all times examined (6, 10 and 24 h) with strain maximally decreasing CBF-1/RBF-Jκ transactivation by 82.36±1.96% after 6 h (**Fig. 5.9 A**). Cyclic strain also significantly decreased Hrt-1 activity at all times examined post strain, with maximal inhibition occurring after 8 h with a significant decrease of 65.8±0.5% (**Fig. 5.9 B**).

5.2.3 Cyclic Strain Inhibits Notch Signaling in a Gi-MAPK-dependent Manner.

Since cyclic strain regulates SMC fate through various mechanosensitive pathways (Lehoux *et al.,* 2003; Xu *et al* 2000), the role of inhibitory Gi-proteins and MAPK in mediating the strain-induced response was examined. Pre-treatment of cells with the Gi-protein inhibitor, pertussis toxin (100ng/ml for 24 h) significantly inhibited baseline levels of Notch 3 IC protein expression by 40.0±10% (**Fig. 5.10 A**) and *Notch 3* mRNA 74.0±11% in unstrained cells (**Fig. 5.10 B**). Moreover, the strain-induced decrease in Notch 3 IC expression and *Notch 3* mRNA levels was attenuated following pertussis toxin treatment (**Fig. 5.10 A** and **B**). Furthermore, *hrt-2* mRNA baseline levels were significantly inhibited following pre-treatment of cells with the Gi-protein inhibitor, pertussix tosin (100ng/ml for 24 h). In addition, the strain-induced decrease in *hrt-2* mRNA was also attenuated following pre-treatment of cells with the Gi-protein inhibitor, pertussix tosin (100ng/ml for 24 h) (**Fig. 5.11A**). The role of inhibitory Gi-proteins in mediating the strain response on proliferation was examined. Pre-treatment of cells with the Gi-protein inhibitor, pertussis toxin (100ng/ml for 24 h) significantly inhibited baseline levels of pCNA, a marker for proliferation by 26.0±9.0%. Furthermore the strain-induced decrease in pCNA expression was significantly blocked following pertussis toxin treatment (**Fig. 5.11B**). In addition to the role of inhibitory Gi-protein, the MAPK pathway involvement in

mediating the strain-induced response was investigated. Following pre-treatment of cells with the MAPK inhibitor, PD98059, there was a significant inhibition in baseline levels of Notch 3 IC protein expression of 51.0±7.0% while concurrently inhibiting the strain-induced decrease in Notch IC expression (**Fig. 5.12**).

5.2.4 Effect of Notch Signaling on Cyclic Strain-induced Changes in SMC Proliferation and Apoptosis *In Vitro*

The recovery from strain-induced decreases in Notch signalling was confirmed by demonstrating that over expression of Notch 3 IC recovered the strain-induced decrease in *Notch 3* and *hrt-2* mRNA to levels that were comparable with unstrained cells (**Fig. 5 .13 A** and **B**). Hence we investigated whether over expression of Notch 3 IC could recover the strain-induced changes in SMC proliferation and apoptosis. In parallel cultures, cyclic strain decreased pCNA expression in mock controls by 54.0±5.0%, an effect that was significantly attenuated following over expression of Notch 3 IC (**Fig. 5.14**). Moreover, the cyclic strain-induced increase in the percent of apoptotic nuclei of 22.0±10% in mock controls was significantly attenuated following over expression of Notch 3 IC (**Fig. 5.15**). The effect of Notch 3 IC over expression on cyclic strain-induced changes in Bcl-2 family mRNA levels were also investigated. Cyclic strain significantly decreased *bcl-X_L* mRNA levels by 48.0±10% when compared to mock controls, an effect that was significantly attenuated following over expression of Notch 3 IC (**Fig. 5.16A**). In contrast, the cyclic strain-induced increase in *bax* mRNA levels of 1.52±.21 compared to mock controls was further enhanced to a significant increase of 2.1±.08 following over expression of constitutively active Notch 3 IC (**Fig. 15.16 B**). In parallel studies, inhibition of Notch IC by co-expression with RPMS-1 further enhanced the strain-induced increase in *bax* mRNA by 1.95±.05 while concomitantly potentiating the strain-induced decrease in *Bcl-X_L* mRNA levels by 0.15±.05 when compared to mock controls (**Fig. 5.17A** and **B**). The inhibition of Notch IC also further enhanced the strain-induced decrease in pCNA expression from a 32.0±8.0% to a 48.0±8.0% decrease as compared to mock controls (**Fig 5.17C**).

5.2.5 Cyclic Strain Inhibits Hedgehog Signaling in SMC *In Vitro*

Cyclic strain (10%, 24 h) caused a significant decrease in Hedgehog signalling pathway components. Hedgehog ligands, *Shh, Ihh* and *Dhh* mRNA expression was significantly decreased 44.0±2.8%, 46.0±7.9% and 35.0±5.4% respectively following strain while Hh target gene mRNA expression of *Ptc1* and *gli-2* also was significantly decreased by 29.0±1.0% and 29.0±1.0% (**Fig. 5.18**). Moreover Shh and Ptc1 protein expression was significantly decreased 62.0±5.0% and 23.0±5.0% respectively following cyclic strain (10%, 24 h) (**Fig. 5.19A** and **B**). For subsequent studies, regulation of Hh signaling was examined following exposure to 10% cyclic strain for 24 h. There was a significant temporal decrease in baseline transactivation of Gli-promoter activity in cells exposed to strain when compared to unstrained controls. This temporal decrease was maximum at 24 h with a 71.0±9.0% decrease in Gli-promoter activity (**Fig. 5.20**).

5.2.6 Effect of Notch Signaling on Cyclic Strain-induced Changes in Hedgehog Signalling in SMC *In Vitro*

The recovery from strain-induced decreases in Hh signaling was confirmed by demonstrating that over expression of Notch 3 IC recovered the strain-induced decrease in *gli-2* and *Ptc-1* mRNA to levels that were comparable with unstrained cells. Cyclic strain-induced decreases in Ptc1 mRNA expression compared to unstrained controls was reduced from 45.0±5.0% to 5.0±5.0% while concurrently cyclic strain-induced decreases in *gli-2* mRNA expression was reduced from 30.0±15.0% to a 0.1±.20 fold increase respectively (**Fig. 5.21A** and **B**).

To determine whether the effect of altered mechanical forces extrapolates to an *in-vivo* situation, we investigated Notch and Hh signalling pathway expression and subsequent levels of growth. Two *in-vivo* models of vascular injury and remodeling were used where animal models were subjected to either carotid artery or portal vein ligation.

The Effect of Carotid Ligation on Notch and Hh Signaling *In-Vivo*

Notch and Hh component expression levels were examined in the carotid arteries of sham-operated animals (data on level of expression in right and left carotid of sham animals pooled) and compared to that of the right and left carotid arteries of ligated animals. Blood flow was significantly altered in both the right and left carotid arteries of ligated animals where flow rates remained constant in sham-operated animals (**Fig. 5.23**). Over the time period examined of 0, 3 and 28 days, flow rates in the right carotid were significantly increased to 165±13% and 175±8.0% at day 3 and 28 respectively over sham-operated control. Concomitantly, blood flow rates in the left carotid decreased at day 3 and 28 to 5.0±0.8% and 3.0±0.4% respectively over sham-operated controls post- ligation.

Post-Carotid Ligation Day 3

With blood flow and subsequent mechanical forces altered in both the right and left carotid arteries of ligated animals, Notch and Hh component expression levels were examined. There was an increase of 1.19±0.04 in Notch 1 IC protein expression in the right carotid artery compared to control and a significant increase of 1.21±0.02 in the left carotid artery of the ligated animal (**Fig. 5.24**). Furthermore, Notch 3 IC protein expression was significantly increased in both the right and left carotid arteries of ligated animals to 1.87±0.26 and 1.77±0.23 over sham operated control respectively (**Fig. 5.25**). Notch target gene protein expression was also increased as compared to sham- operated controls (**Fig. 5.26**). HRT-1 protein expression was increased to 2.07±0.1 and 2.0±0.2 over sham-operated control in the right and left arteries of ligated animals respectively. HRT-2 expression was increased by 3.18±0.32 and 3.0±0.32 fold in the right and left carotid arteries respectively. Likewise, fold increases of 2.63±0.27 and 2.58±0.23 in HRT-3 expression were evident in the right and left carotid arteries respectively. A similar pattern was evident for the up-regulation of Hes-1 and Hes-5 protein expression. Significant fold increases of 1.86±0.08 and 1.87±0.21, and 2.4±0.02 and 1.99±0.02 evident in the right and left carotids of ligated animals for Hes-1 and Hes-5 respectively. In addition, the level of Hes-5 protein expression was significantly decreased in the left ligated artery when

compared to the contralateral right carotid. Hh component expression was also significantly increased as a result of carotid ligation. Shh protein expression was increased to 3.38±0.36 and 3.37±0.02 over sham –operated control in the right and left arteries of ligated animals respectively (**Fig. 5.27A**). Similarly, Ihh expression was increased by 2.19±0.12 and 2.25±0.14 fold in the right and left carotid arteries respectively (**Fig. 5.27B**). Likewise, Hh target gene Ptc-1 expression was increased to 1.61±0.04 and 1.58±0.07 fold in the right and left carotid arteries respectively (**Fig. 5.27C**). In parallel studies we investigated this effect of altered mechanical forces *in-vivo* using the rat carotid artery ligation model on proliferation. Using pCNA as a marker for proliferation, there was a subtle but significant increase in expression in the right and left carotid arteries of the ligated animal over the sham operated control of 1.28±0.02 and 1.24±0.05 respectively (**Fig. 5.28**).

Post-Carotid Ligation Day 28

The expression of Notch receptors and target genes were subsequently analyzed in animals at 28 days post-carotid ligation. Again, protein expression was analyzed in the right and left carotid arteries of ligated animals, and expressed as fold change over sham-operated control level.

Notch 1 IC protein levels were decreased in both the right and left carotid arteries of ligated animals (**Fig. 5.29**). Notch 1 IC expression was decreased by 37.0±15.0% and 11.0±4.0% in the right and left carotid arteries respectively, as compared to sham-operated control. A similar expression pattern was evident for Notch 3 IC protein expression, with significant decreases in both the right 42.0±7.0% and left 18.0±3.0% carotid arteries as compared to sham-operated controls (**Fig. 5.30**). In addition, Notch 3 IC protein expression was significantly decreased in the right carotid artery of the ligated animal, as compared to the left carotid artery. Subsequent analysis of Notch target gene protein expression levels revealed a similar pattern of expression to that of the Notch receptors (**Fig. 5.31**). Analysis of the right carotid artery of ligated animals revealed a significant decrease of 40.0±7.0% in HRT-1 protein expression, with respect to sham-operated animals (**Fig. 5.31A**). This decrease in HRT-1 expression was also evident in the left ligated carotid artery 15.0±6.0%. As with Notch IC

receptor expression, HRT-1 expression was significantly decreased in the right carotid artery of the ligated animal, as compared to the left carotid artery.

Similarly, HRT-2 expression was significantly decreased in the right carotid of the ligated animal by 37.0±11.0% of as compared to sham-operated control, with a less pronounced decrease of 16.0±6.0% evident in the contralateral left carotid artery (**Fig. 5.31B**). Analysis of HRT-3 protein expression also revealed a significant percentage decrease in expression in both the right 49.0±4.0% and left 19.0±5.0% carotid arteries of ligated animals, as compared to sham-operated controls (**Fig. 5.31C**). In addition, the level of HRT-3 protein expression between the right and left carotid arteries was significantly different, with a substantial decrease in the right carotid over the contralateral left carotid artery. The expression pattern of the Hes family of target genes followed a similar pattern to that of the related HRT family. Hes-1 protein expression was significantly decreased in the right carotid artery 43.0±6.0% of ligated animals as compared to sham-operated controls (**Fig. 5.31D**). The contralateral left carotid artery exhibited a slight fold decrease in Hes-1 protein expression over control 9.0±5.0%, this level of protein expression was significantly different to that evident in the right carotid artery. Similarly, Hes-5 protein expression was significantly decreased in the right artery 51.0±13.0% as compared to sham-operated control, and a less pronounced fold decrease of 25.0±28.0% was evident in the contralateral left carotid artery (**Fig. 5.31E**). The expression of Hh signaling components were subsequently analyzed in animals at 28 days post-carotid ligation. Again, protein expression was analyzed in the right and left carotid arteries of ligated animals, and expressed as fold change over sham-operated control level. Analysis of Shh protein expression revealed a significant percentage decrease in expression in both the right 57.0±3.0% and left 36.0±11.0% carotid arteries of ligated animals, as compared to sham-operated controls (**Fig. 5.32A**). In addtion, the level of Shh protein expression between the right and left carotid arteries was significantly different, being substantially decreased in the right carotid over the contralateral left carotid artery. A similar pattern of expression was evident for both Ihh and Hh target gene Ptc-1. Ihh protein expression decreased by 61.0±3.0% and 44.0±1.5% in the right and left carotid arteries respectively as compared to sham-operated controls while Ptc-1 expression decreased by 28.0±9.0% in the right while decreasing by 12.0±9.0% in the left. In both cases, expression between the left and right carotid arteries was

significantly different, being substantially decreased in the right carotid over the contralateral left carotid artery. In parallel studies we investigated this effect of altered mechanical forces *in-vivo* using the rat carotid artery ligation model at day 28 on proliferation. Using pCNA as a marker for proliferation, there was no significant difference in expression in the right carotid arteries of the ligated animal over the sham-operated control. However there was a subtle but significant fold increase of 1.24±.09 in pCNA expression in the left carotid artery as compared to sham-operated controls (**Fig. 5.33**). In addition, the level of pCNA expression between the right and left carotid arteries was significantly different, being decreased in the right carotid over the contralateral left carotid artery.

Portal Vein Ligation

In addition to the carotid ligation model, a portal vein ligation model was also used to analyze the effect of altered mechanical forces on the Notch signalling pathway *in vivo.* Rat portal veins were ligated, and subsequently isolated from ligated and sham-operated models at timepoints over a period of 2-15 days post-ligation. Splanchnic blood flow and splenic pressure (an index of portal venous pressure) was measured at each timepoint post-ligation in both sham-operated and ligated animals. We measured blood flow using a Transonic flow probe, and splenic pressure was measured using a Doppler probe, with measurements adjusted according to the weight of the animal. Two days following PVL splanchnic blood flow was significantly decreased in the ligated versus sham-operated animal, as a result of the obstruction to portal inflow caused by ligation (**Fig. 5.34A**). Splanchnic blood flow returned to sham-operated control levels 4 days post-ligation, but was significantly increased, as compared to sham-operated control, in the ligated animal by 44.0±17.0%, 44.0±13.0% and 92.0±6.0% at 6, 9, and 15 days post-ligation respectively. Analysis of splenic pressure, and hence portal venous pressure, revealed significant increases in pressure in the ligated animals at all timepoints post-ligation (**Fig. 5.34B**). Splenic pressure was increased by 76.2±17.6% and 79.1±19.1%, as compared to sham-operated control, at 2 and 4 days post ligation respectively. Similarly, increases of

34.1±14.4%, 62.8±18.9% and 49.2±14.1% in splenic pressure were observed in the ligated animals at 6, 9 and 15 days post ligation respectively.

We then analyzed expression levels of Notch receptors, ligand and target genes in the ligated models, and compared to that of the sham-operated animals at each timepoint (arbitrarily assigned a value of 1). The data presented represents the mean of two vessels pooled for each timepoint. We found that the Notch signalling pathway generally appears to exhibit a triphasic pattern of expression in response to portal vein ligation over the time period studied. There was generally a decrease initially in expression of components of the signaling pathway at 2 days post-ligation. Expression of Notch signaling pathway components were then increased over sham-operated control levels at 4 days post-ligation, followed by an attenuation of this increase, maintained over 6 - 15 days post-ligation. Notch 1 IC receptor expression remained unchanged in the ligated animal 2 days post-ligation, as compared to the sham-operated control, arbitrarily assigned a value of 1 (**Fig. 5.35A**). However, at 4 days post ligation there was a considerable fold increase in Notch 1 IC protein with the level of Notch 1 IC protein increased in the ligated animal by 60% over sham-operated control. Notch 1 IC protein levels examined at 6, 9 and 15 days post-ligation decreased by 58%, 50% and 75%, as compared to sham-operated control for each timepoint. It is interesting to note that Notch 1 IC expression changes in both sham-operated and ligated animals following surgery, which is represented in (**Fig. 5.35B**). Notch 1 IC expression in the sham-operated animal decreases from days 2-6 post-surgery, and subsequently increases from days 6-15 post-surgery.

A similar expression pattern was evident for Notch 3 IC protein (**Fig. 5.36A**). An initial decrease in Notch 3 IC protein expression was observed at 2 days post-ligation by 11% as compared to sham-operated control, this expression was subsequently increased at 4 days post-ligation, to 55% over sham-operated control. However, this increase in Notch 3 IC protein expression was attenuated at 6, 9, and 15 days post-ligation by 61%, 43% and 58% as compared to sham-operated controls. As with Notch 1 IC, Notch 3 IC expression is altered in sham-operated animals following surgery (**Fig. 5.36B**). Notch 3 IC protein expression decreases from 2-6 days post-surgery, subsequently increases at day 9 and decreases at day 15 post-surgery. Therefore, a tri-phasic pattern of Notch 3 IC expression is exhibited in sham-operated animals post-surgery. Similarly, analysis of Jagged protein expression revealed an

initial decrease of 12% with respect to sham-operated control 2 days post-ligation, and a subsequent increase in Jagged expression levels 4 days post-ligation, to 50% over sham-operated control samples (**Fig. 5.37A**). As was evident with the pattern of Notch receptor expression, this increase in Jagged expression was attenuated at 6 and 9 days post-ligation to expression levels of 14% and 11% lower than sham-operated control. Jagged expression levels remained similar to those of sham-operated control in the ligated animal 15 days post ligation. Jagged expression is also altered in the sham-operated animals following surgery (**Fig. 5.37B**). Jagged expression decreases post-surgery from day 2 - day 4, subsequently increases from day 4 - day 9, and decreases at day 15, thus exhibiting a similar tri-phasic expression pattern to Notch 3 IC.

In addition, we analyzed the expression levels of Notch target genes, which revealed a similar pattern of expression to that of the Notch receptors. Analysis of HRT-1 expression revealed a considerable decrease of 40% in expression 2 days post-ligation, as compared to sham-operated control (**Fig. 5.38A**). A subsequent increase in HRT-1 protein expression, to 30% over sham-operated control, was observed 4 days post-ligation. This increase was attenuated to expression levels of 50%, 40% and 60% decreased versus sham-operated control at 6, 9, and 15 days post-ligation respectively. The pattern of HRT-1 expression alteration in sham-operated control animals following surgery is illustrated in (**Fig. 5.38B**). HRT-1 expression in sham-operated animals is decreased from days 2 - 4 post-surgery, subsequently increased at 6 days post-surgery, and remains unchanged thereafter.

Similar to the pattern of HRT-1 expression, a 37% decrease in HRT-2 protein expression was observed in the ligated vessel 2 days post-ligation, as compared to sham-operated control (**Fig. 5.39A**). HRT-2 protein expression analysis at 4 days post-ligation revealed a 60% increase in expression over sham-operated control, which was attenuated at 6, 9 and 15 days post-ligation to expression levels of 53%, 8% and 14% decreased versus sham-operated control. The changes in HRT-2 expression following surgery in the sham-operated animal are illustrated in (**Fig. 5.39B**), as with HRT-1, a minimal change in HRT-2 expression is evident following surgery, as compared to the change in expression described following PVL. In addition, HRT-3 protein expression levels decreased slightly by 10% as compared to control levels 2 days post-ligation, however, an increase of 46% over control levels

was observed 4 days post-ligation (**Fig. 5.40A**). Again, this increase was considerably attenuated at day 6 and day 15 post-ligation, with expression levels being decreased by 47% and 33% respectively, as compared to sham-operated control levels. This attenuation was also evident 9 days post-ligation but to a lesser degree, with HRT-3 expression levels being 20% increased over sham-operated control. HRT-3 expression levels decrease in the sham-operated animals from day 2 - day 4 following surgery, and subsequently increase gradually from day 4 - day 15 after surgery (**Fig. 5.40B**). Unlike the Notch receptors, or the HRT target genes studied, the Hes target genes exhibited an increase in protein expression of 33% and 70% over sham-operated control 2 days post-ligation respectively (**Fig. 5.41A** and **5.42A**). The subsequent expression pattern of these target genes, however, mirrors that of the other components of the Notch signaling pathway studied. Protein expression of HES-1 was increased 4 days post-ligation by 54% fold over sham-operated control (**Fig. 5.41A**). An attenuation of this increase was observed at 6, 9 and 15 days post ligation respectively. Hes-1 expression levels were decreased by 42% and 37% compared to sham-operated control at 6 and 15 days post-ligation respectively. Hes-1 expression was increased by 10% over sham-operated control at 9 days post-ligation, but is decreased as compared to Hes-1 expression levels at 4 days post-ligation. Similarly, Hes-5 protein expression levels were found to be increased by 35% over control 4 days post-ligation, which is an attenuation of the 70% increase in Hes-5 expression evident 2 days post-ligation (**Fig. 5.42A**).

This increase was further attenuated at 6, 9 and 15 days post-ligation expression levels decreased by 67%, 46% and 65% respectively, as compared to sham-operated control. As with other components of the Notch signaling pathway, both Hes-1 and Hes-5 expression levels are altered following surgery. Hes-1 expression gradually increases from day 2 - day 15 post-surgery (**Fig. 5.41B**), whereas Hes-5 expression increases from day 2 - day 6 post-surgery, subsequently decreases at day 9, and increases at day 15 post-surgery to expression levels similar to those seen at day 6 post-surgery (**Fig. 5.42B**).

In addition to protein expression of the Notch signaling pathway post portal vein ligation, we also analyzed mRNA expression at days 2 and 15 as compared to sham-operated controls. Between 5-7 vessels were pooled and at both days 2 and 15 a significant decrease in Notch signaling component expression was evident. At day 2,

Notch 1 mRNA expression was decreased by 59.7±.05% as compared to sham-operated control (**Fig. 5.43**). Furthermore Notch target gene mRNA expression was also significantly decreased at day 2. *Hrt-1, hrt-2, hrt-3* and *hes-5* mRNA expression was decrease by 24.0±.06%, 44.0±.11%, 54.0±.09% and 53.0±.04% respectively as compared to s ham-operated controls (**Fig. 5.43**). In addition, the effect of portal vein ligation on Hh signaling component mRNA expression was also determined at day 2 post ligation. As with the Notch signaling pathway, Hh signaling component expression was significantly decreased as compared to sham–operated controls. Shh and *smo* expression was decreased by 64.8±.04% and 37.0±.02% respectively (**Fig. 5.43**). Similarly at day 15 a decrease in Notch signaling component expression was observed. Notch 1 mRNA expression was decreased by 44.0±.12% as compared to sham-operated controls (**Fig. 5.44**). Similarly *hrt-1, hrt-2 and hrt-3* exhibited decreases in expression of 71.0±.01%, 77.0±.04% and 60.0±.05% respectively (**Fig. 5.44**). This data clearly shows altered expression of Notch and Hh signaling as a result of increased mechanical forces as a result of portal vein ligation. In addition, we addressed the effect of portal vein ligation on apoptosis by analyzing *bax* and *bcl-*X_L mRNA expression at days 2 and 15 post ligation. A significant fold increase in *bax* expression of 3.84±.08 and 3.23±.15 was observed at day 2 (**Fig. 5.43**) and 15 (**Fig. 5.44**) respectively as compared to sham-operated control. Moreover, *bcl-*X_L expression was significantly decreased by 83.0±.02% in ligated animals at day15 as compared to sham-operated controls (**Fig. 5.44**). In parallel studies the effect of portal vein ligation on VEGF expression was determined. At day 15 post portal vein ligation a significant decrease of 73.0±.08% was observed as compared to sham-operated controls (**Fig. 5.44**).

Figure 5.1 The Effect of Notch IC Inhibition on Apoptosis

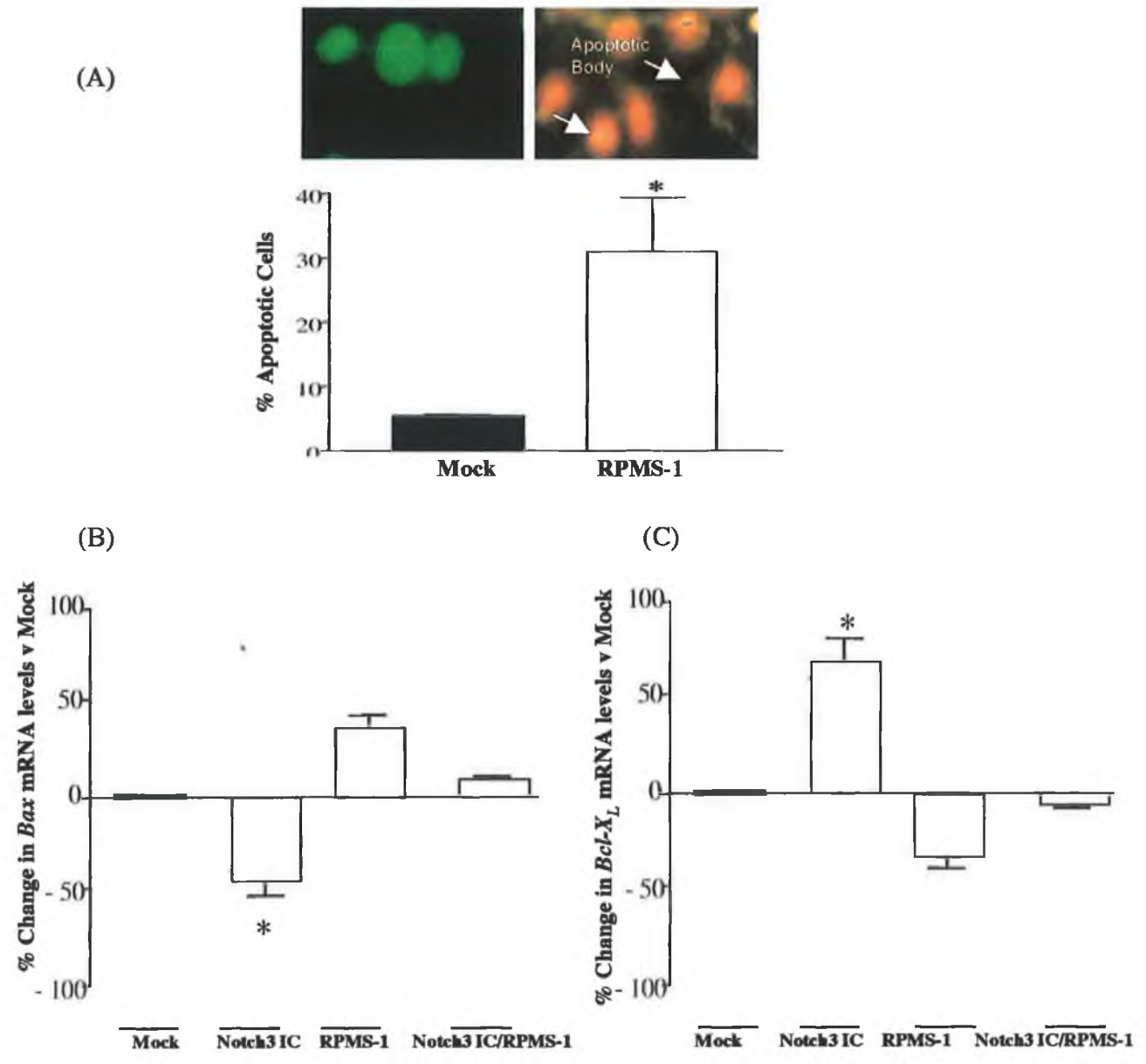


Figure 5.1 The Effect of Notch IC Inhibition on RVSMC Apoptosis. (A) RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or the Notch inhibitor RPMS-I. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μ g/ml, 48 h). Cells were stained with the Acridine orange/Ethidium Bromide dual stain and viewed under a fluorescent microscope. A) Representative images. Apoptotic cells, white arrow. Graph shows cumulative data of percentage increase in the number of apoptotic cells due to Notch inhibition. (B) RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC plus or minus the Notch inhibitor RPMS-I. RNA was isolated and assayed for *Bax* (B) and *Bcl-x_L* (C) expression as indicated using quantitative real time PCR analysis. Expression was normalized to GAPDH levels, and expressed as % increase over control (= the value obtained with mock transfected cells arbitrarily assigned a value of 1) n=3, * $p<0.05$ as compared to mock transfected control (student's t test).

Figure 5.2 The Effect of Notch IC Inhibition on Apoptosis

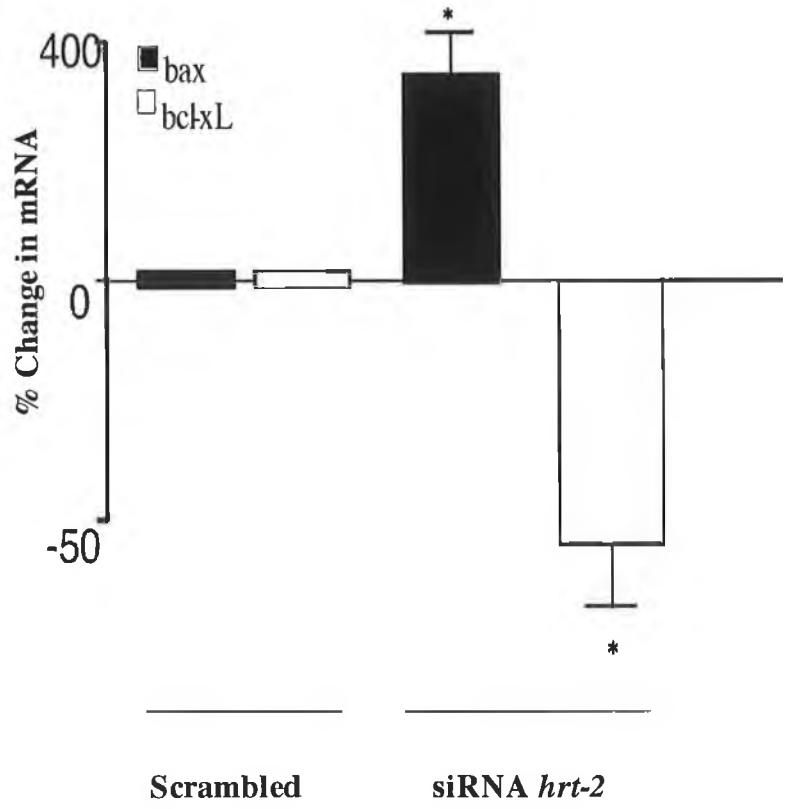


Figure 5.2 The Effect of Notch IC inhibition on RVSMC Apoptosis. RVSMCs were transiently transfected with siRNA directed against *hrt-2* or a scrambled control. Following overnight recovery RNA was isolated and assayed for *Bax* and *Bcl-x_L* expression as indicated using quantitative real time QRTPCR analysis. Expression was normalized to GAPDH levels, and expressed as % increase over control (= the value obtained with mock transfected cells arbitrarily assigned a value of 1) n=3, * $p<0.05$ as compared to mock transfected control (student's t test).

Figure 5.3 Cyclic Strain Induces Changes in SMC Growth *in vitro*.

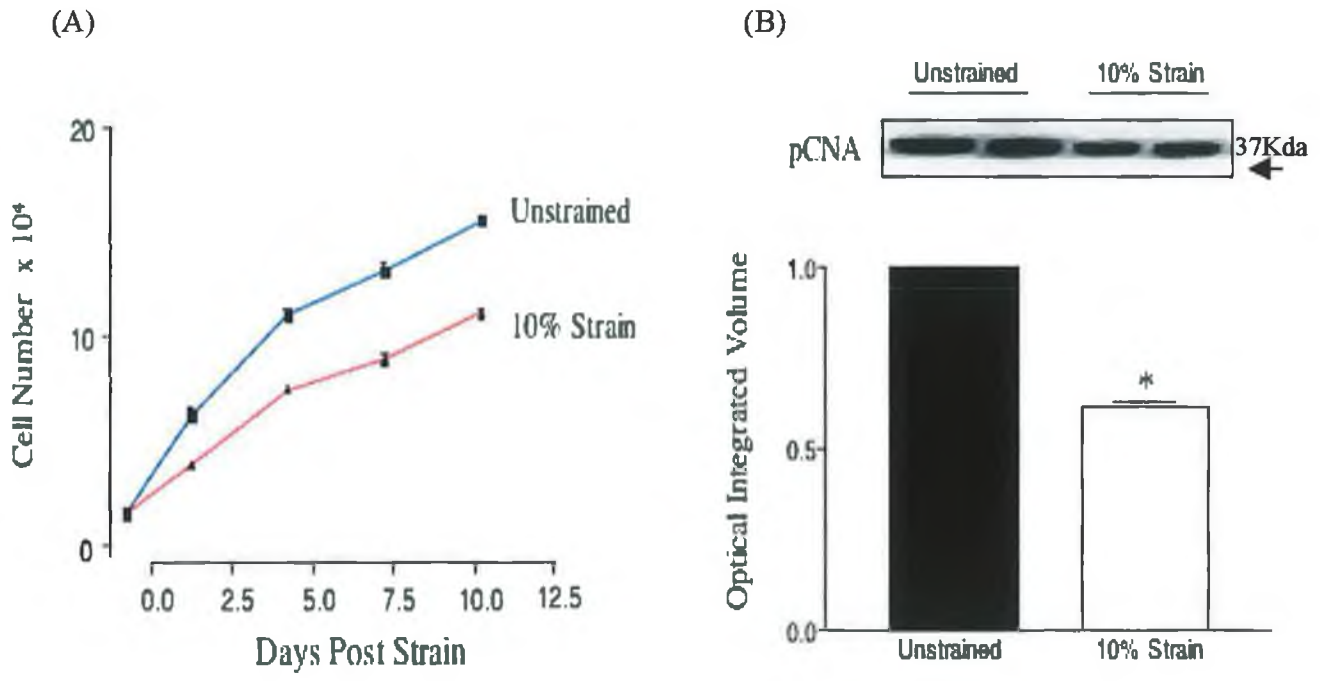


Figure 5.3 Cyclic Strain Induces Changes in SMC Growth in vitro. (A) RVSMC were seeded onto Flexercell™ plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to cyclic strain (10%, 24 h). Cells were then counted at 2 day intervals using a haemacytometer. The average of 3-wells was observed. (B) RVSMC were seeded onto Flexercell™ plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to cyclic strain (10%, 24 h). Protein was then isolated and pCNA expression was determined by western blot analysis. Values are expressed as fold change over static (arbitrarily assigned a value of 1), n=3. * $p<0.05$ as compared to static control (student's t test).

Figure 5.4 Cyclic Strain Induces Changes in SMC Growth *in vitro.*

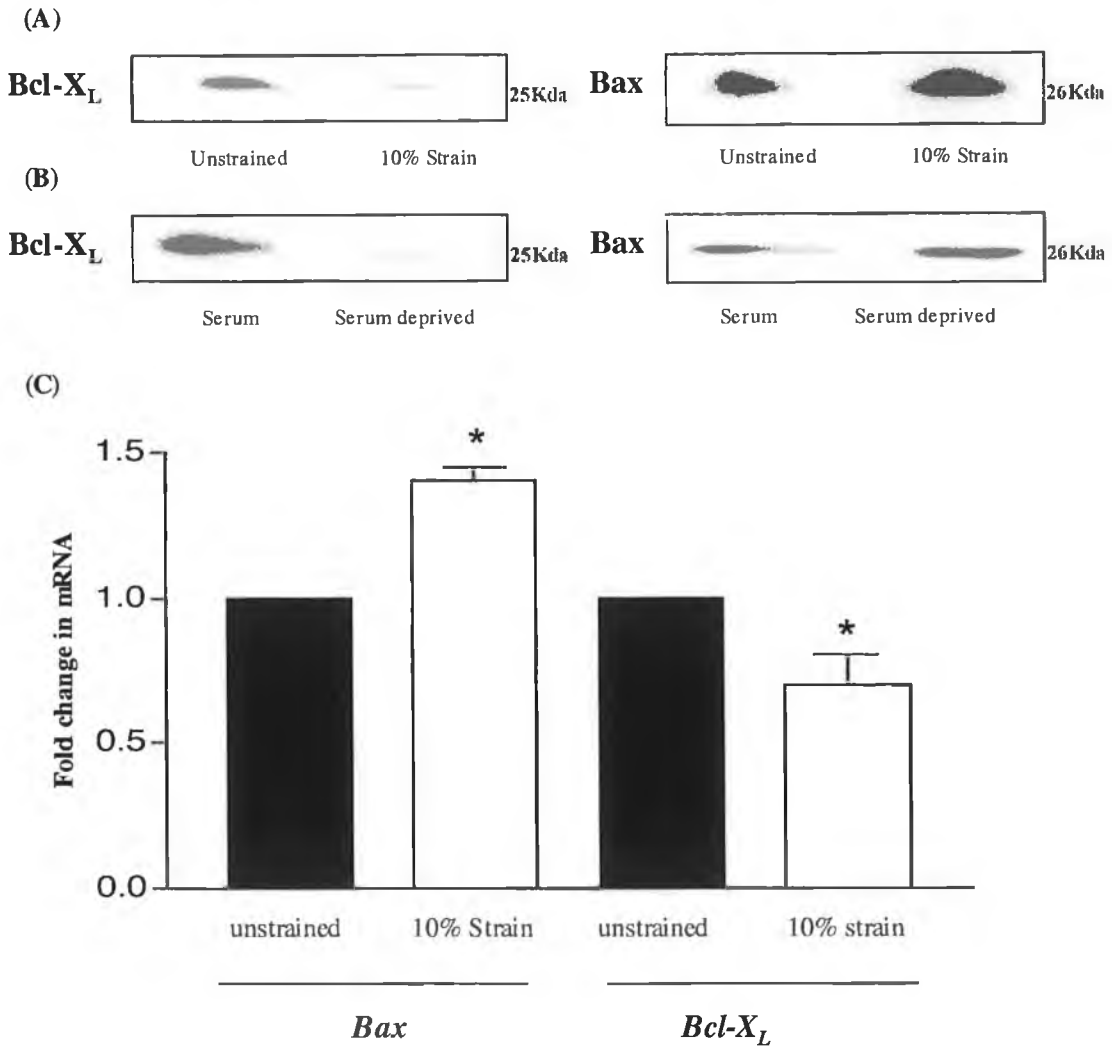


Figure 5.4 Cyclic Strain Induces Changes in SMC Growth *In Vitro*. **A**) RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to cyclic strain (10%, 24 h). Protein was then isolated and Bax and Bcl-X_L expression was determined by western blot analysis .(B) RVSMC were quiesced for 48hrs, protein was then isolated and Bax and Bcl-X_L expression was determined by western blot analysis and compared to serum containing controls. (**C**) RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to cyclic strain (10%, 24 h). RNA was then isolated and Bax and Bcl-X_L expression was determined by using quantitative real time PCR analysis. Expression was normalized to GAPDH levels, and expressed as fold increase over control (= the value obtained with unstrained cells arbitrarily assigned a value of 1) n=3, * $p<0.05$ as compared to unstrained control (student's t test).

Figure 5.5 Cyclic Strain Inhibits Notch Signalling in SMC *in vitro*

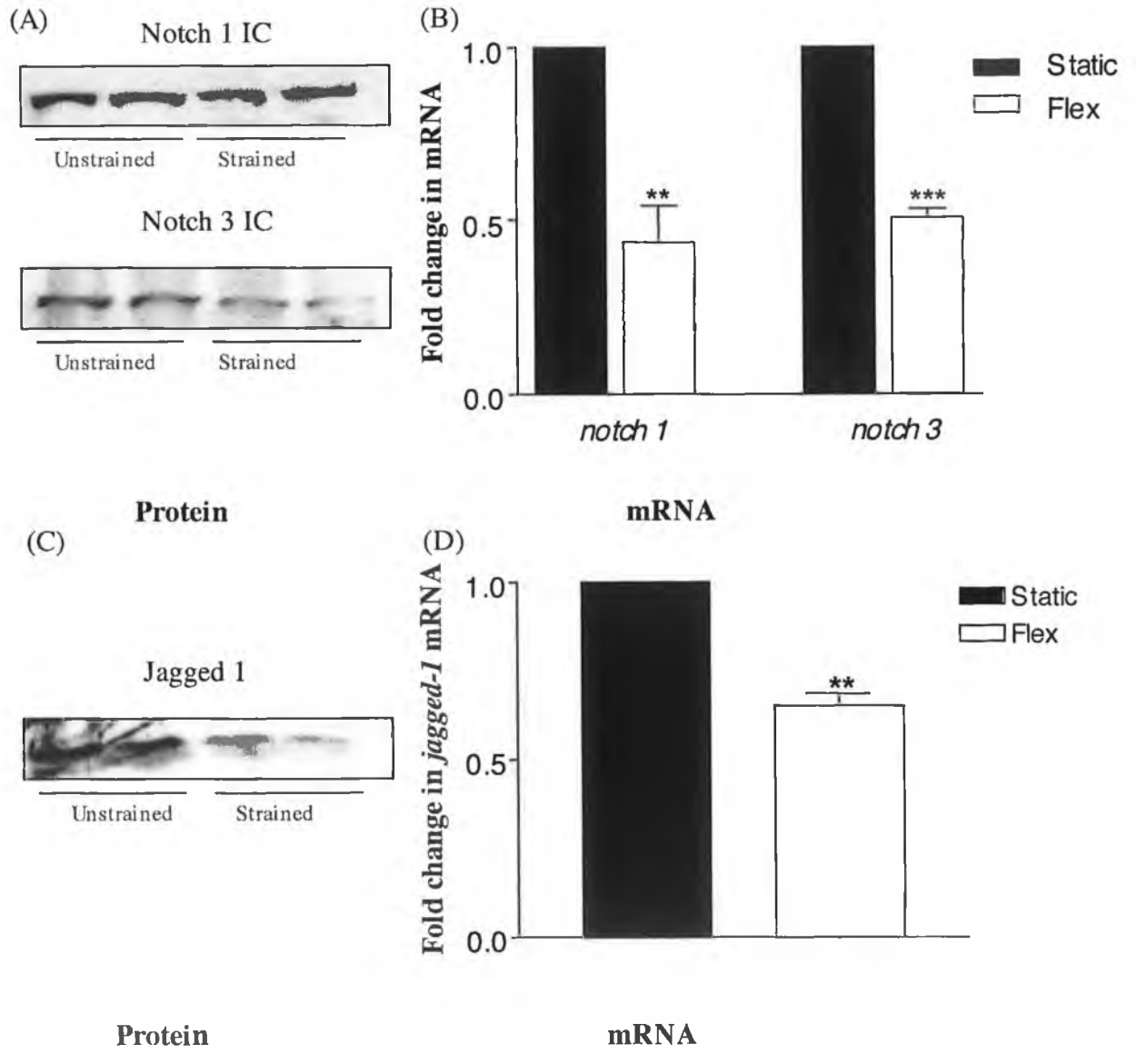


Figure 5.5 Cyclic Strain Inhibits Notch Signaling in SMC *in vitro*. **A**) RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to cyclic strain (10%, 24 h). Protein was then isolated and Notch 1 IC and Notch 3 IC (A) and Jagged 1 (C) expression was determined by western blot analysis. In parallel cultures RNA was extracted and Notch 1 and Notch 3 (B) and Jagged 1 (D) expression was determined by using QRTPCR analysis. Expression was normalized to GAPDH levels, and expressed as fold increase over control (= the value obtained with unstrained cells arbitrarily assigned a value of 1) n=5, ** $p<0.005$, *** $p<0.0005$ as compared to unstrained control (student's t test).

Figure 5.6 Cyclic Strain Inhibits Notch Signaling in SMC *in vitro*

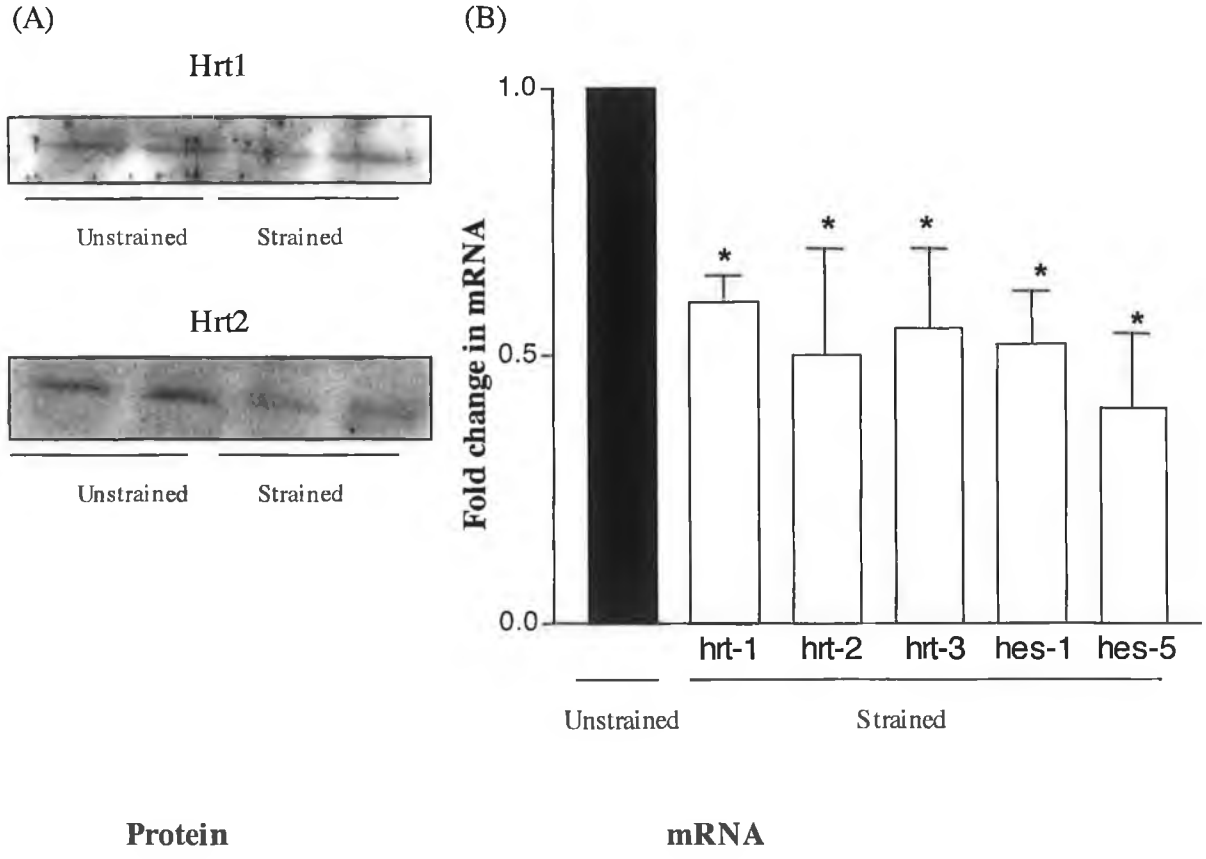


Figure 5.6 Cyclic Strain Inhibits Notch Signaling in SMC *in vitro*. (**A**) RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to cyclic strain (10%, 24 h). Protein was then isolated and Hrt-1 and Hrt-2 expression was determined by western blot analysis. (B) In parallel cultures RNA was extracted and *hrt-1,hrt-2,hrt-3 hes-1* and *hes-5* expression was determined by using quantitative real time PCR analysis. Expression was normalized to GAPDH levels, and expressed as fold increase over control (= the value obtained with unstrained cells arbitrarily assigned a value of 1) n=5, * $p<0.05$ as compared to unstrained control (student's t test).

Figure 5.7 Force-dependent Effect of Cyclic Strain-induced Decrease in Notch Signalling Pathway mRNA Expression in RVSMC

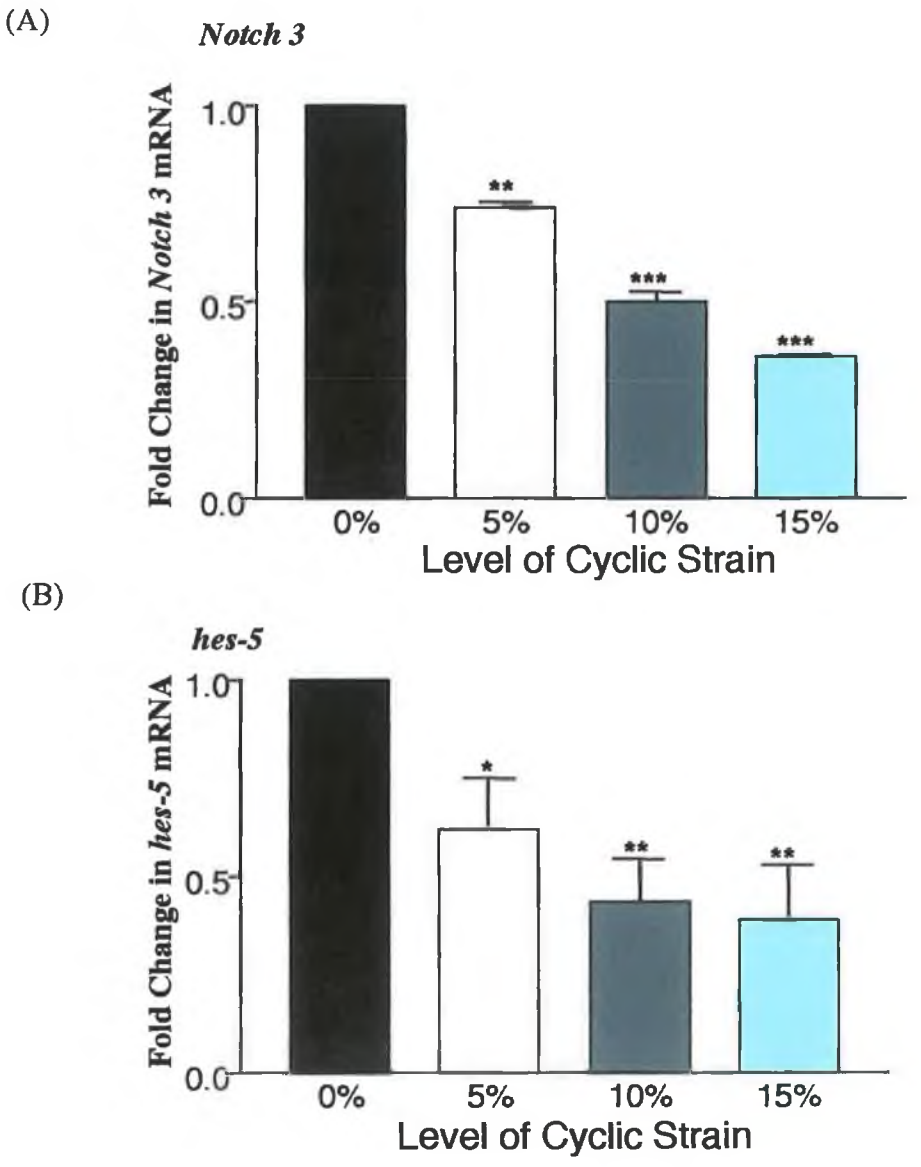
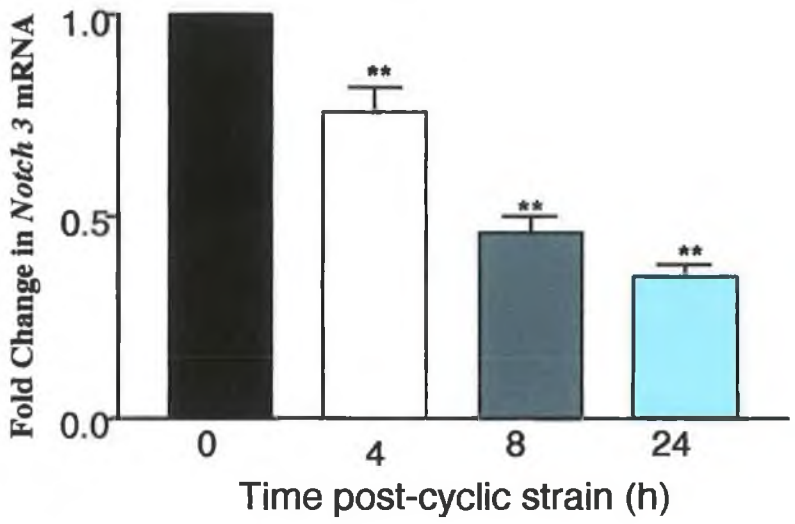


Fig 5.7 Force-dependent Effect of Cyclic Strain-induced decrease in Notch Signalling Pathway mRNA Expression in RVSMC. RVSMC were seeded onto Flexercell™ plates at 1 x 10^5 cells/well. Following a 24 h growth period, cells were subjected to differing levels of cyclic strain, 0%, 5%, 10% or 15% for 24 h as indicated. Samples were isolated at 24 h post-strain and subsequently assayed using quantitative PCR for notch 3 (A), and hes-5 (B) mRNA expression. All values were normalized to GAPDH levels, and expressed as a fold change over static (0%) control (= the value obtained with static RVSMC mRNA levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate. * $p<0.05$, ** $p<0.005$,*** $p<0.0005$ as compared to unstrained control (student's t test). n=3.

Figure 5.8 Time-dependent Effect of Cyclic Strain-induced Decrease in Notch Signalling Pathway mRNA Expression in RVSMC

(A) ***Notch 3***



(B) ***hes-5***

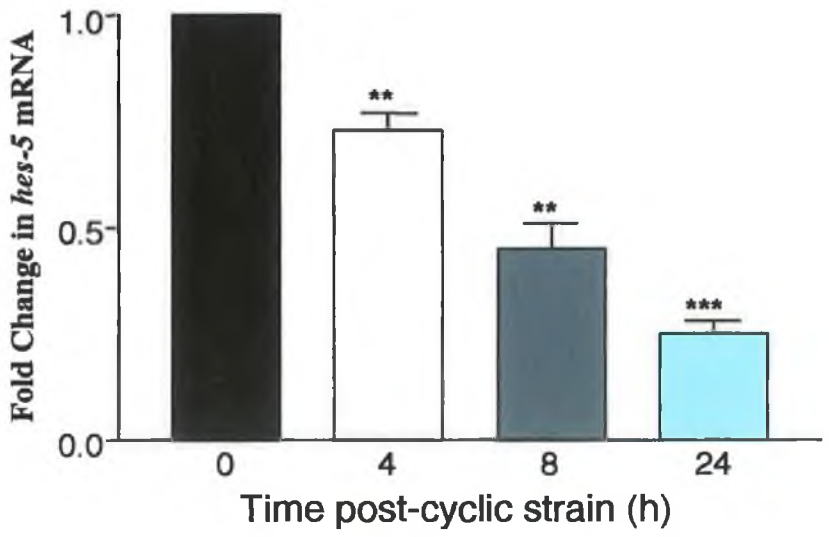


Fig 5.8 Time-dependent Effect of Cyclic Strain-induced Decrease in Notch Signalling Pathway mRNA Expression in RVSMC. RVSMC were seeded onto Flexercell™ plates at 1 x 10^5 cells/well. Following a 48 h growth period, cells were subjected to cyclic strain over a period of 24 h, as indicated. Samples were isolated at 0 h, 4 h, 8 h, and 24 h post-strain and subsequently assayed using quantitative PCR for Notch 3 (A) and hes-5 (B) mRNA expression. All values were normalized to GAPDH levels, and expressed as a fold change over static (0%) control (= the value obtained with static RVSMC mRNA levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate. ** $p<0.005$, *** $p<0.0005$ as compared to static control (student's t test).

Figure 5.9 Cyclic Strain Inhibits Notch Signaling in SMC *in vitro*

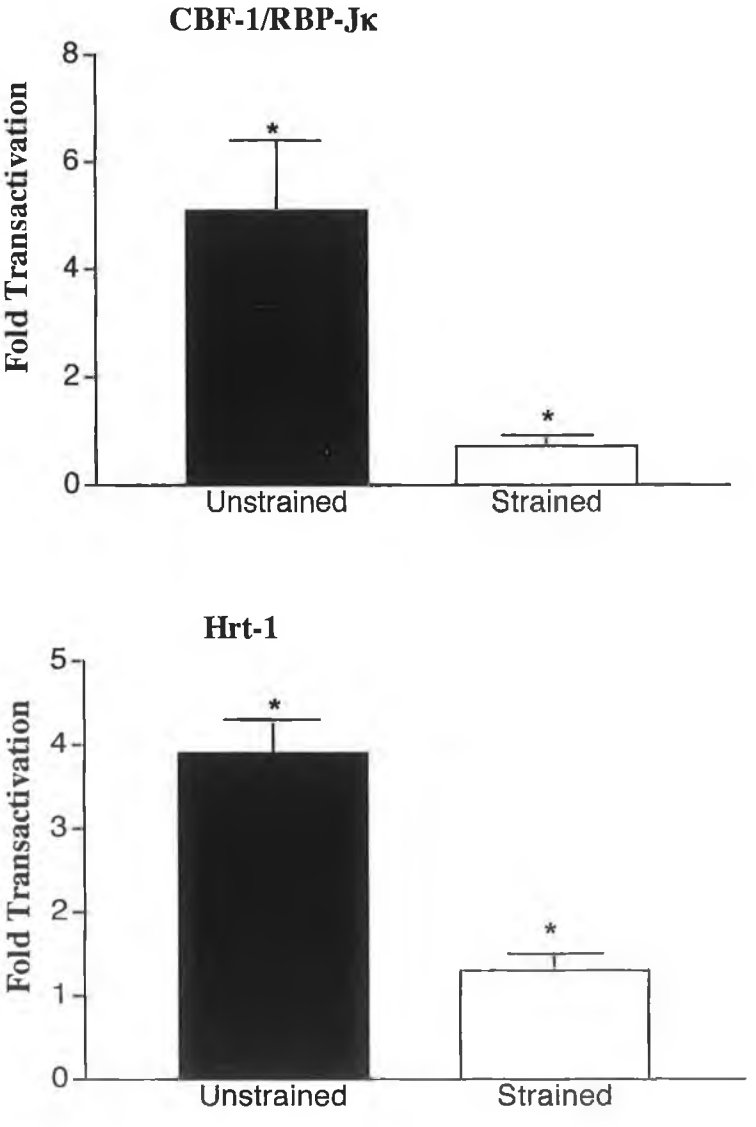


Figure 5.9 Cyclic Strain Inhibits Notch Signaling in SMC *in vitro*. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. RVSMC were transiently transfected with the CBF-1 or the hrt1 luciferase-tagged reporter plasmid. Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated. Samples were isolated at 24 h post-strain and assayed for luciferase activity. Luciferase assays were normalized to β-galactosidase activities and protein levels, n=4, and expressed as fold increase over control (= the value obtained with unstrained cells arbitrarily assigned a value of 1). *$p<0.05$ as compared to unstrained control n=3 (student's t test).

Figure 5.10 Cyclic Strain Inhibits Notch Signalling in a Gi-dependent Manner

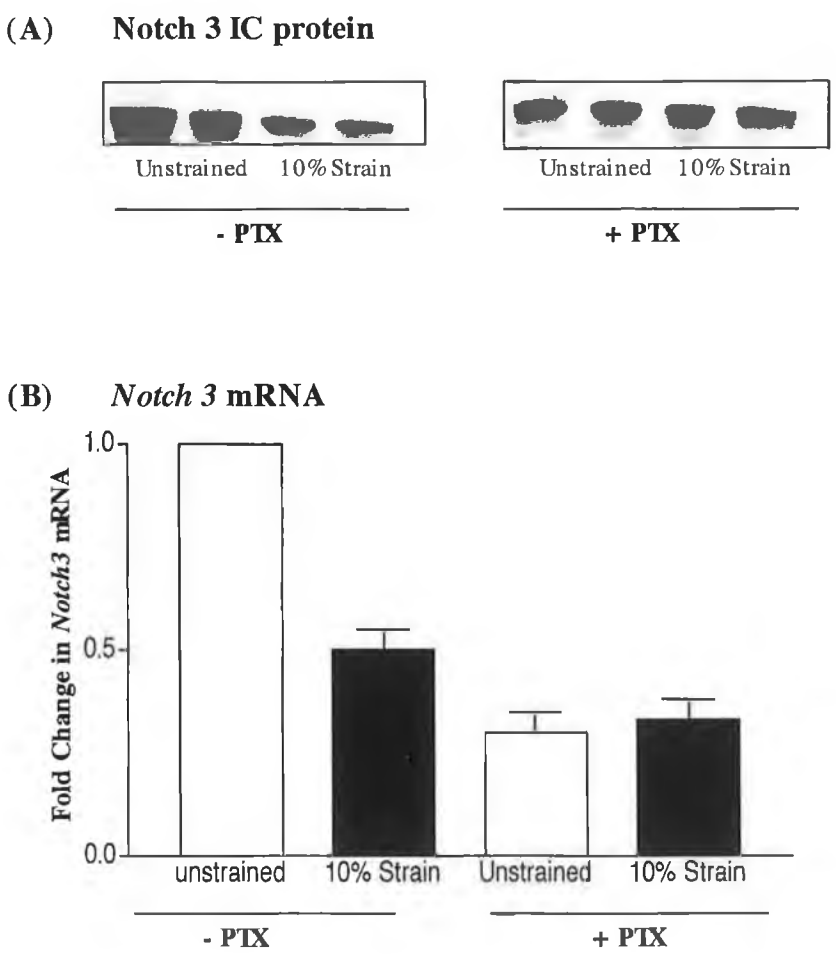


Figure 5.10 Cyclic Strain Inhibits Notch Signalling in a Gi-dependent Manner. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. Following a 48 h growth period, cells were treated with PTX(100ng/ml) and subjected to cyclic strain over a period of 24 h, as indicated. Samples were isolated post-flex and subsequently assayed using western blotting and quantitative PCR for Notch 3 protein(A) and mRNA(B) expression. (B) All values were normalized to GAPDH levels, and expressed as a fold change over static (0%) (-PTX) control (= the value obtained with static (-PTX) RVSMC mRNA levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate as compared to static control. n=3.

Figure 5.11 Cyclic Strain Inhibits Notch Signalling in a Gi-dependent Manner

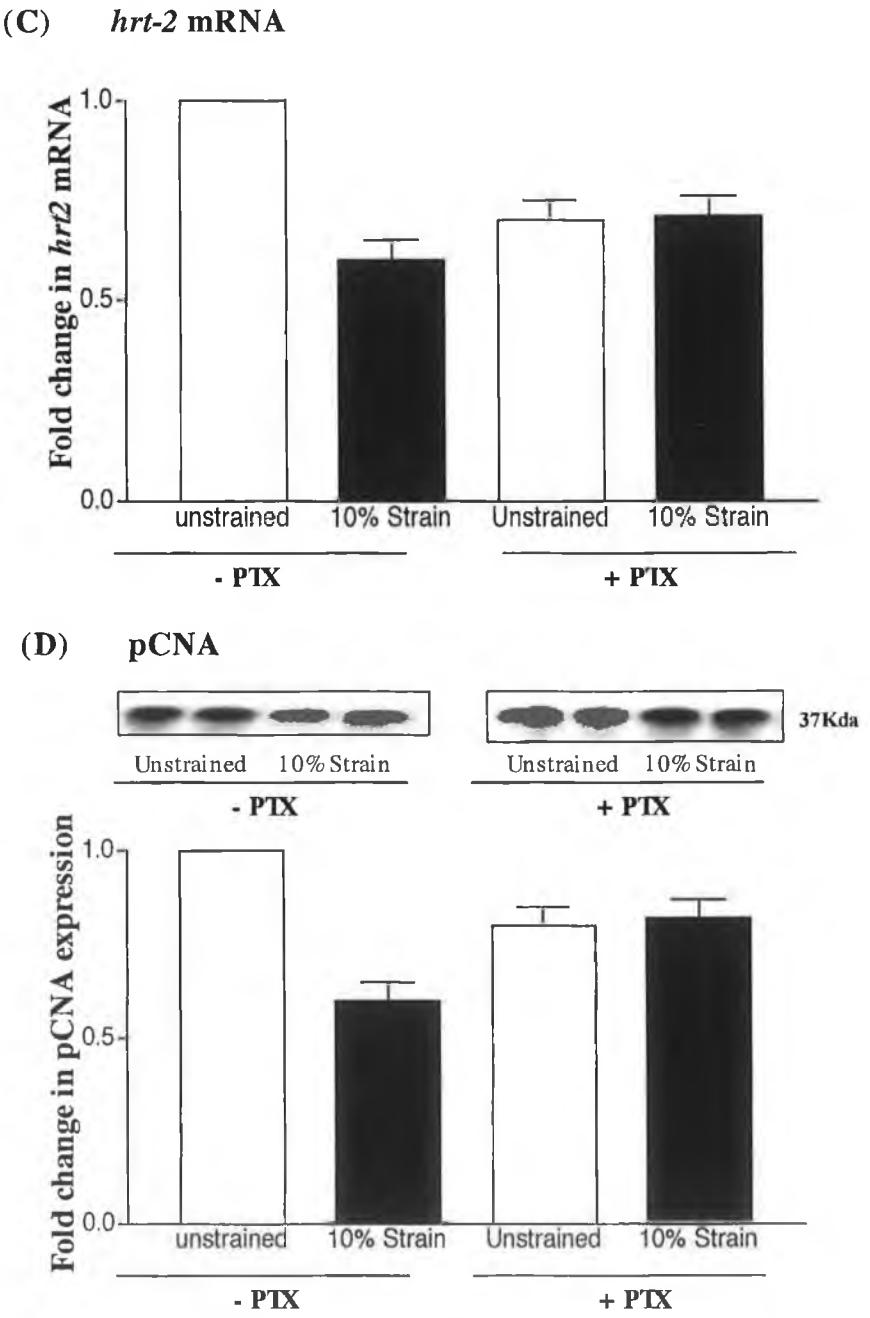


Figure 5.11 Cyclic Strain Inhibits Notch Signalling in a Gi-dependent Manner. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. Following a 48 h growth period, cells were treated with PTX (100ng/ml) and subjected to cyclic strain over a period of 24 h, as indicated. Samples were isolated post-flex and subsequently assayed using western blotting and quantitative PCR for hrt-2 mRNA expression (A) and pCNA protein expression (B). (A) All values were normalized to GAPDH levels, and expressed as a fold change over static (0%) (-PTX) control (= the value obtained with static (-PTX) RVSMC mRNA levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate. as compared to static control. n=3 (student's t test).

Figure 5.12 Cyclic Strain Inhibits Notch Signalling in a MAPK- dependent Manner

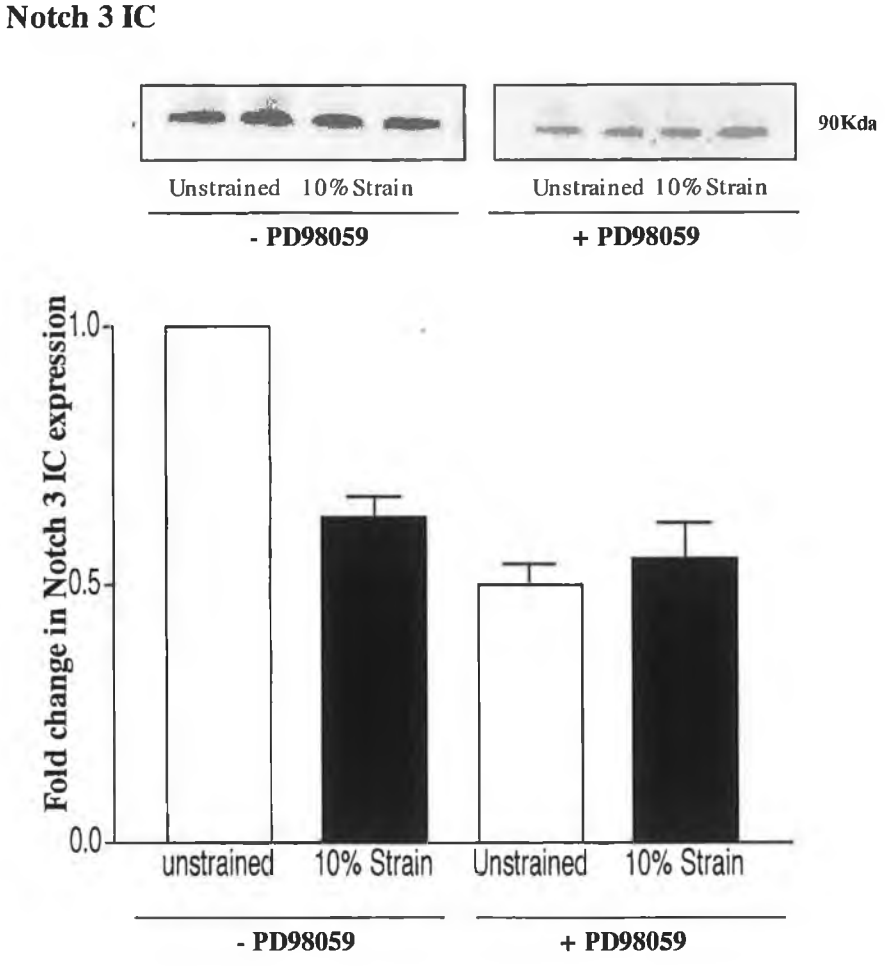


Figure 5.12 Cyclic Strain Inhibits Notch Signalling in a MAPK- dependent Manner. RVSMC were seeded onto Flexercell™ plates at 1 x 10^5 cells/well. Following a 48 h growth period, cells were treated with PD98059 (100ng/ml) and subjected to cyclic strain over a period of 24 h, as indicated. Samples were isolated post-flex and subsequently assayed using western blotting and quantitative PCR for Notch3 protein expression(A) and mRNA expression (B). (B) All values were normalized to GAPDH levels, and expressed as a fold change over static (0%) (-PD) control (= the value obtained with static (-PD) RVSMC mRNA levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate. as compared to static control.n=3

Figure 5.13 The Effect of Notch3 IC over expression in Strained RVSMC

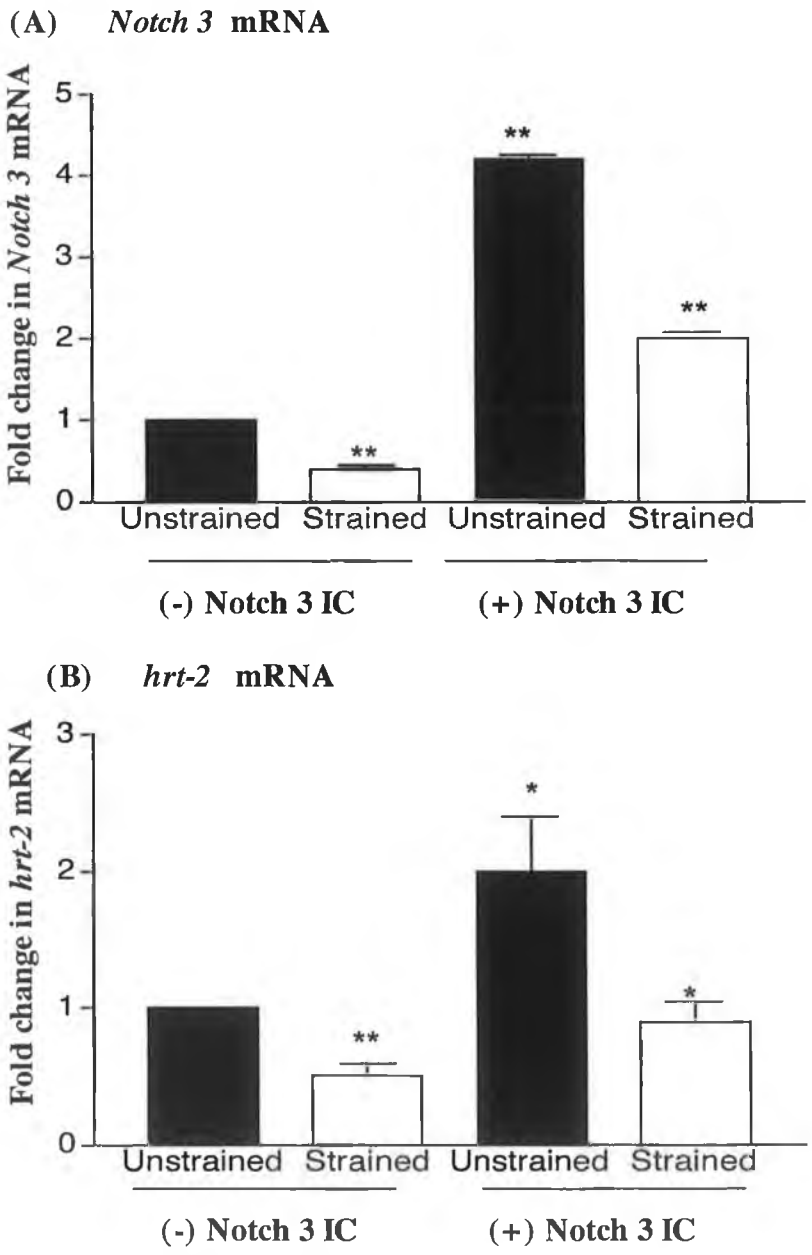


Figure 5.13 The Effect of Notch3 IC over expression in Strained RVSMC. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later. and subsequently exposed to cyclic strain (10%, 24 h). *Notch 3* mRNA levels (A) and *hrt-2* mRNA (B) were measured using quantitative RT-PCR, and expressed as fold increase over control (= the levels of target gene present in static mock transfected cells) n=3, $^{**}p<0.005$, $^{*}p<0.05$ as compared to static control (student's t test).

Figure 5.14 The Effect of Notch3 IC over expression in Strained RVSMC on Proliferation

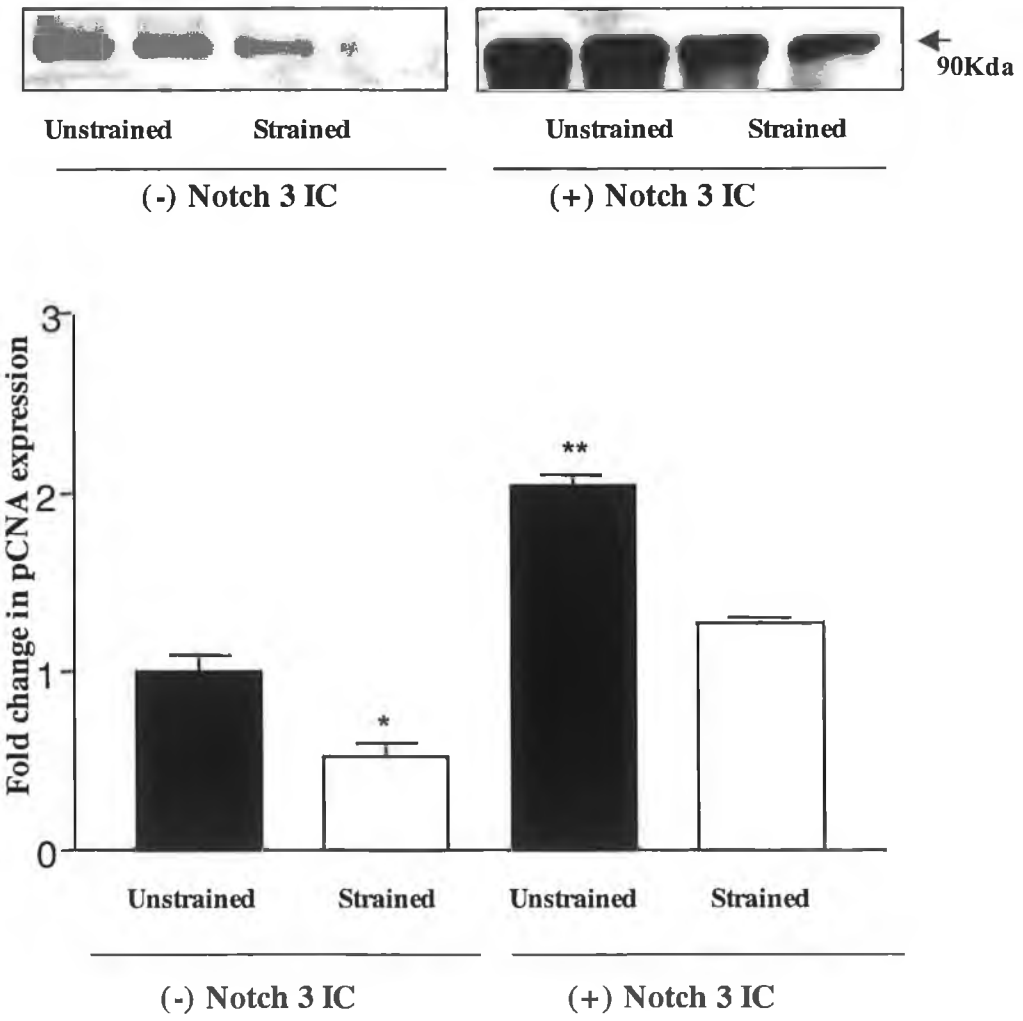


Figure 5.14 The Effect of Notch3 IC over expression in Strained RVSMC on Proliferation. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later and subsequently exposed to cyclic strain (10%, 24 h). Western blot analysis was carried out and pCNA protein expression was measured, and expressed as fold increase over control (= the levels of target gene present in static mock transfected cells) n=3, $^{**}p<0.005$, $^{*}p<0.05$ as compared to static control (student's t test). n=3.

Figure 5.15 The Effect of Notch IC over Expression on Apoptosis

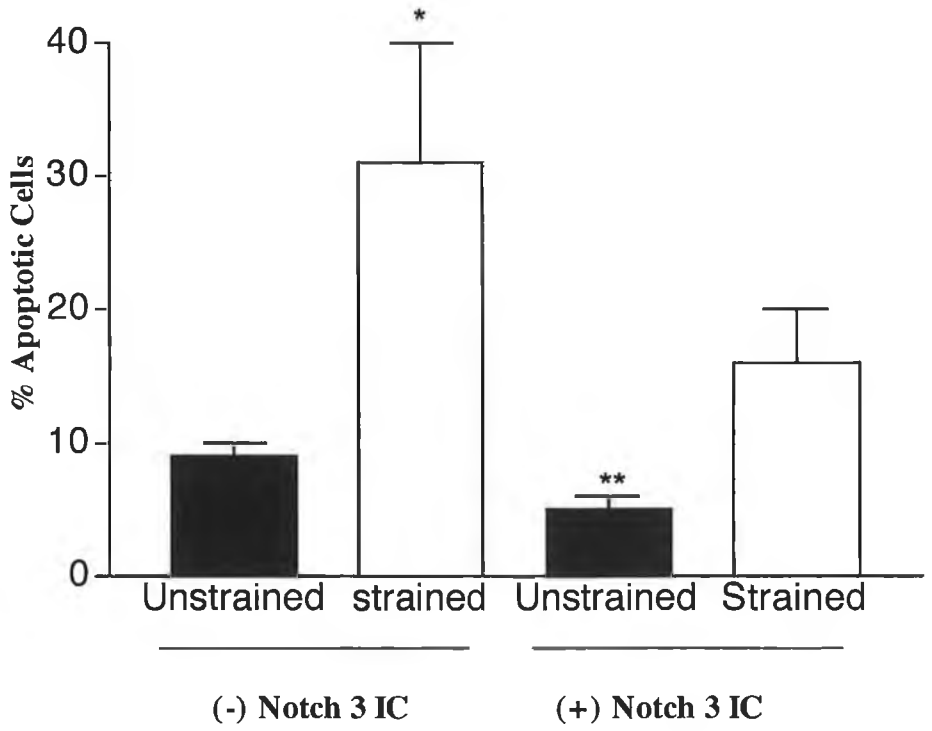


Figure 5.15 The Effect of Notch3 IC over expression in Strained RVSMC on Apoptosis. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later and subsequently exposed to cyclic strain (10%, 24 h). Cells were stained with AO/EtBr dual stain and viewed under a fluorescent microscope. Apoptotic cells were then counted and expressed as % change in the number of apoptotic cells as compared to control (= the levels of target gene present in static mock transfected cells) n=3, $^{**}p<0.005$, $^{*}p<0.05$ as compared to static control (student's t test).

Figure 5.16 The Effect of Notch IC over Expression on Apoptosis

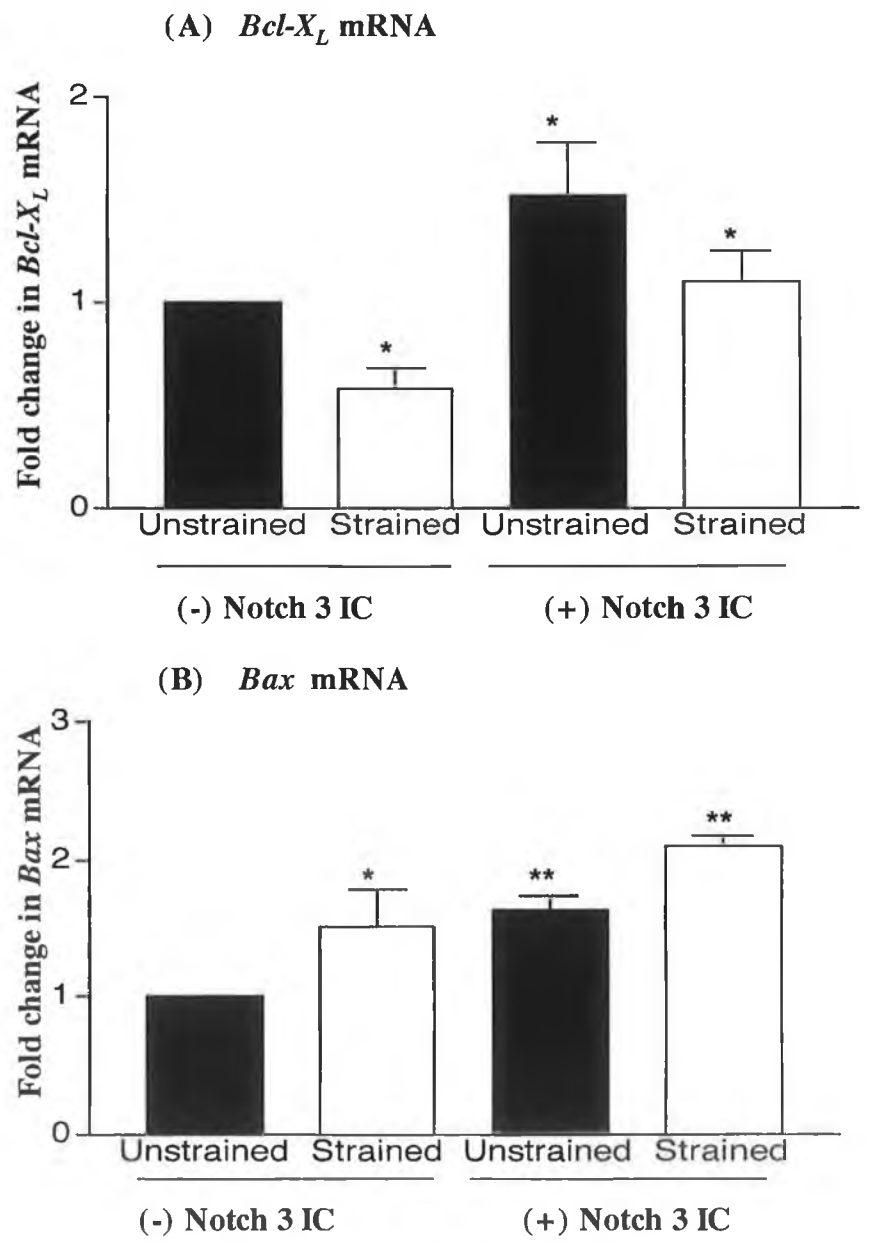


Figure 5.16 The Effect of Notch3 IC over Expression in Strained RVSMC on Apoptosis. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later and subsequently exposed to cyclic strain (10%, 24 h). Bcl-X_L mRNA levels (A) and Bax mRNA (B) were measured using quantitative RT-PCR, and expressed as fold increase over control (= the levels of target gene present in static mock transfected cells) n=3, $^{**}p<0.005$, $^{*}p<0.05$ as compared to static control (student's t test)

Figure 5.17 The Effect of Notch IC Inhibition on Strained RVSMCs

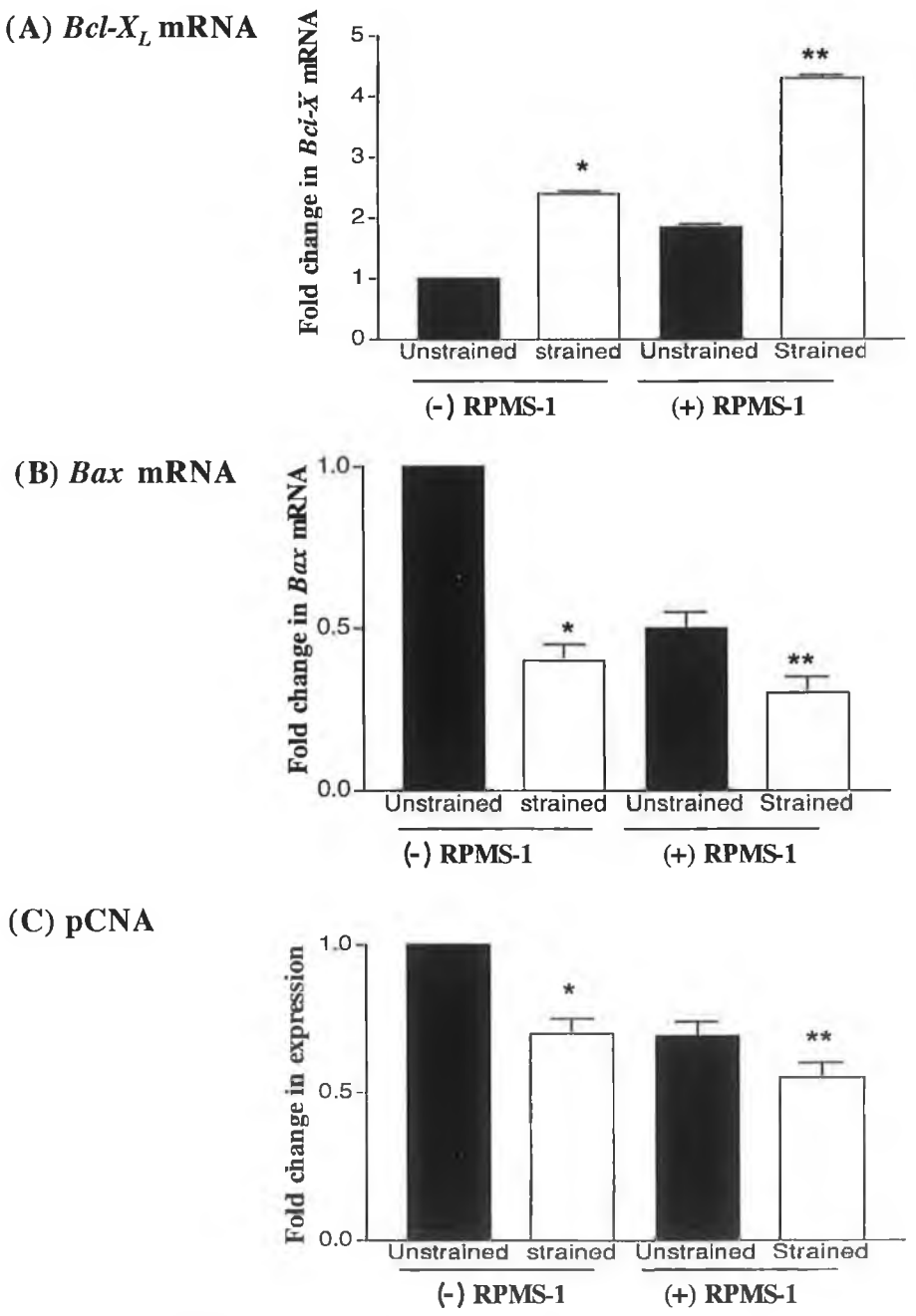


Figure 5.17 The Effect of Notch IC Inhibition on Strained RVSMC. RVSMC were seeded onto Flexercell™ plates at 1 x 10^5 cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later and subsequently exposed to cyclic strain (10%, 24 h). *Bcl-X_L* mRNA levels (A), *Bax* mRNA (B) and pCNA (c) was measured and expressed as fold increase over control (= the levels of target gene present in static mock transfected cells) n=3, $^{**}p<0.005$, $^{*}p<0.05$ as compared to static control (student's t test).

Figure 5.18 Cyclic Strain Inhibits Hedgehog Signalling in SMC *in vitro*

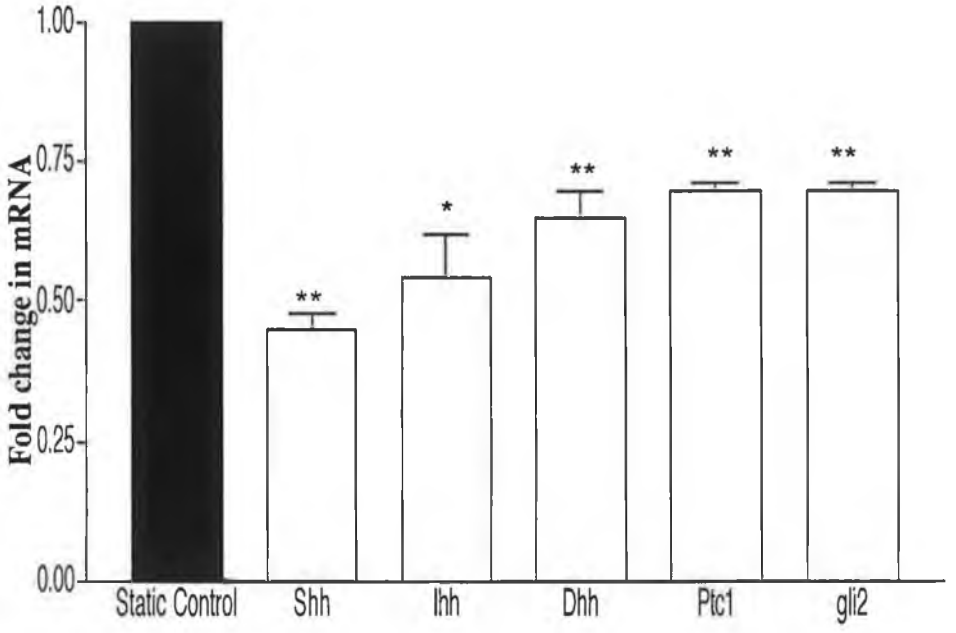


Figure 5.18 Cyclic Strain Inhibits Hedgehog Signalling in SMC in vitro. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. RVSMC were transiently transfected with the CBF-1 or the hrt1 luciferase-tagged reporter plasmid. Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated. Samples were isolated at 24 h post-strain and subsequently assayed using quantitative PCR for Hh signalling pathway component mRNA expression. All values were normalized to GAPDH levels, and expressed as a fold change over static (0%) control (= the value obtained with static RVSMC mRNA levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate. * $p<0.05$, ** $p<0.005$ as compared to static control (student's t test).n=3.

Figure 5.19 Cyclic Strain Inhibits Hedgehog Signalling in SMC *in vitro*

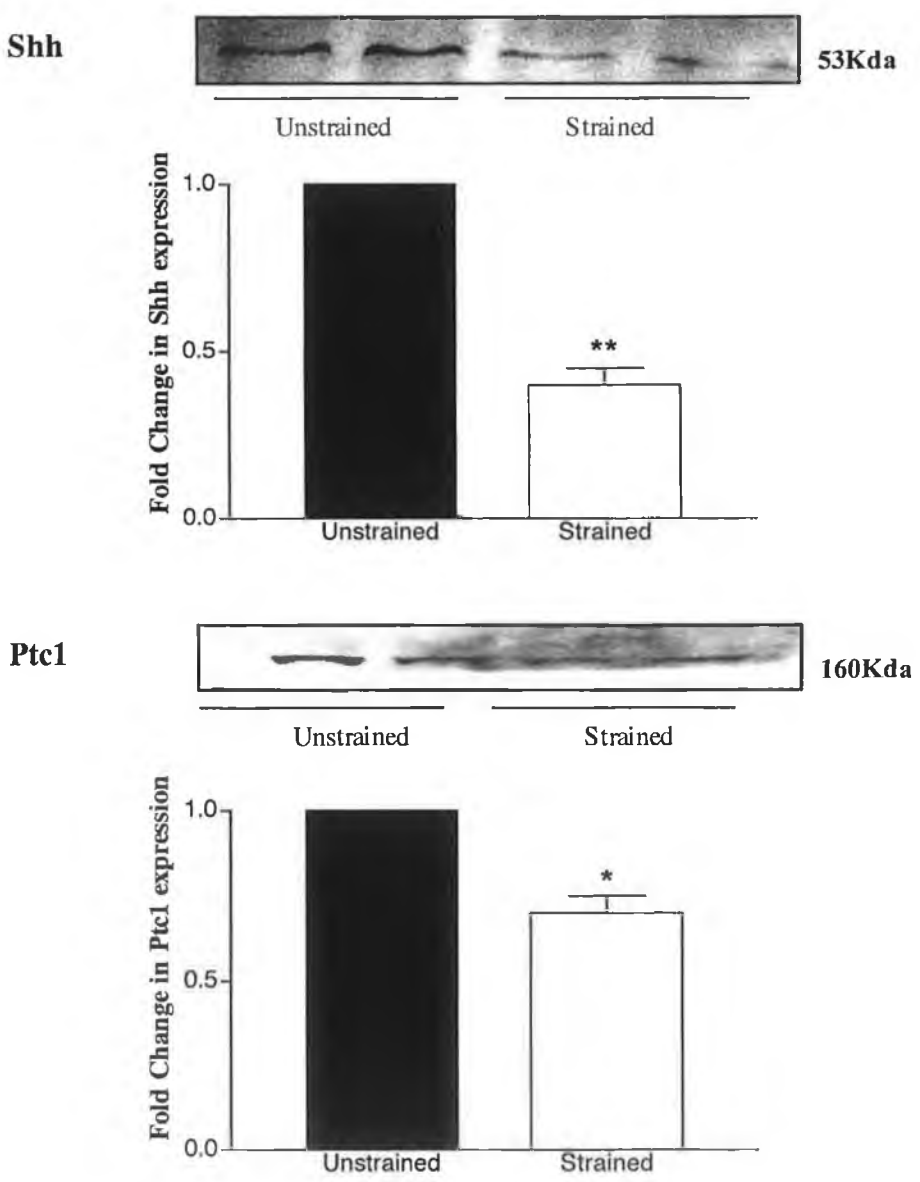


Figure 5.19 Cyclic Strain Inhibits Hedgehog Signalling in SMC *in vitro*. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. RVSMC were transiently transfected with the CBF-1 or the hrt1 luciferase-tagged reporter plasmid. Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated. Samples were isolated at 24 h post-strain and subsequently assayed using western blotting forShh (A) and Ptc-1 (B) protein expression. All values were expressed as a fold change over static (0%) control (= the value obtained with static RVSMC protein levels, arbitrarily assigned a value of 1), representative experiment, mean of 6 wells, experiment performed in triplicate. * $p<0.05$, ** $p<0.005$ as compared to static control (student's t test). n=3.

Figure 5.20 Cyclic Strain Inhibits Hedgehog Signalling in SMC *in vitro*

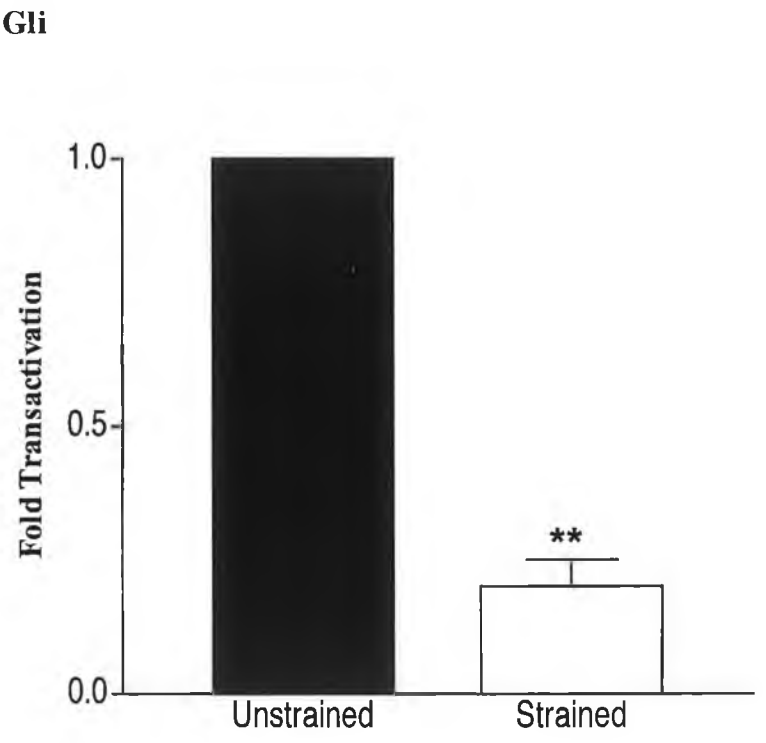


Figure 5.20 Cyclic Strain Inhibits Hedgehog Signaling in SMC in vitro. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well. RVSMC were transiently transfected with the Gli-luciferase-tagged reporter plasmid. Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated. Samples were isolated at 24 h post-strain and assayed for luciferase activity. Luciferase assays were normalized to β-galactosidase activities and protein levels, n=4, and expressed as fold increase over control (= the value obtained with unstrained cells arbitrarily assigned a value of 1). **$p<0.005$ as compared to unstrained control. n=3 (student's t test).

Figure 5.21 The Effect of Notch 3 IC over-Expression on Hedgehog Target Gene mRNA Expresssion in Strained RVSMC

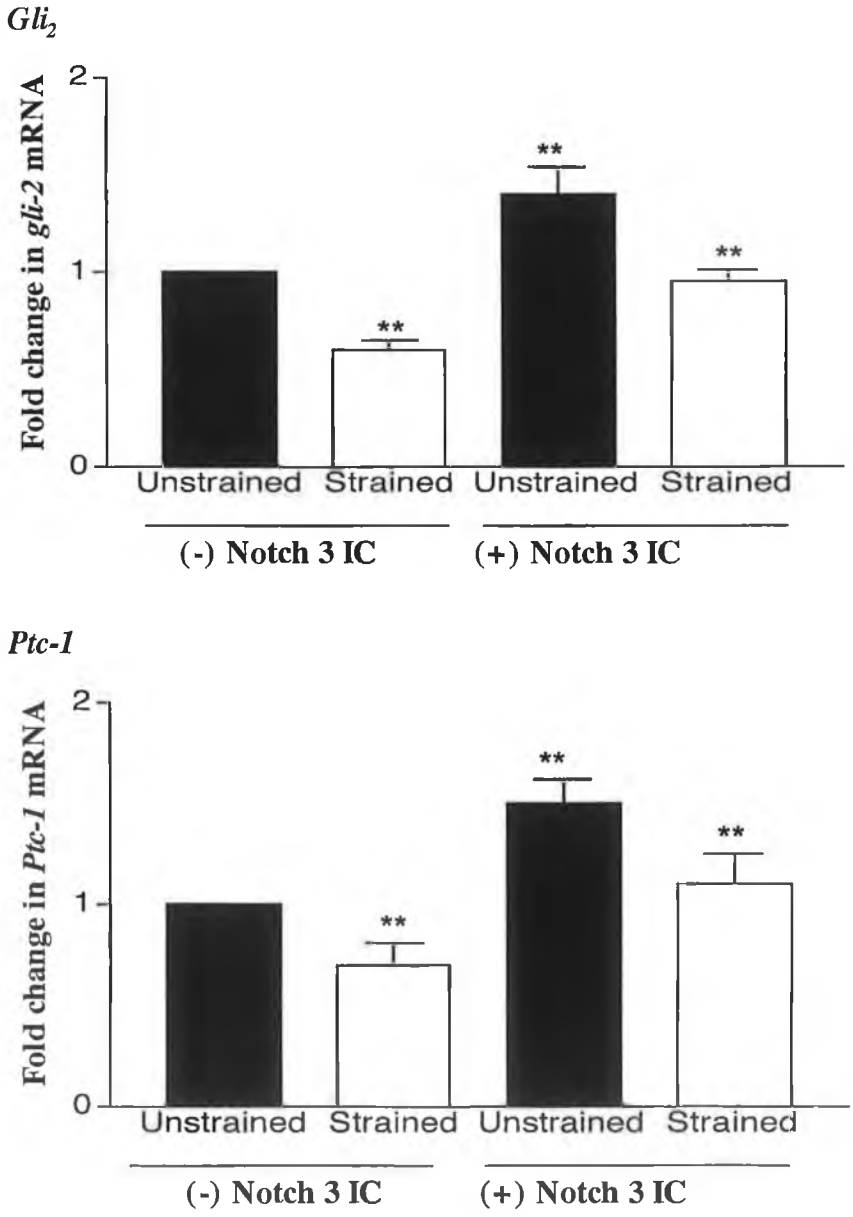
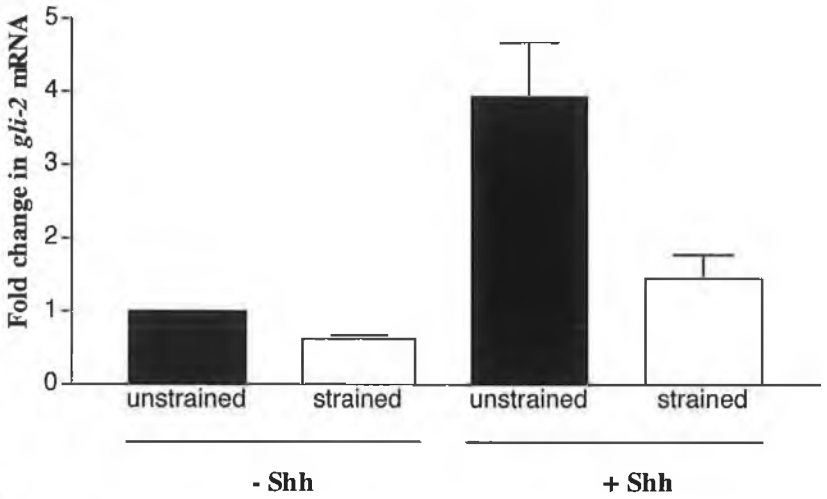


Figure 5.21 The Effect of Notch 3 IC over-Expression on Hedgehog target gene mRNA Expresssion in Strained RVSMC. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later and subsequently exposed to cyclic strain (10%, 24 h). gli-2 mRNA levels (A) and ptc-1 mRNA (B) were measured using quantitative RT-PCR, and expressed as fold increase over control (= the levels of target gene present in static mock transfected cells) n=3, $^{**}p<0.005$ as compared to static control (student's t test)

Figure 5.22 The Effect of Shh on Hedgehog and Notch Target Gene mRNA Expresssion in Strained RVSMC.

Gli$_2$



Hrt-3

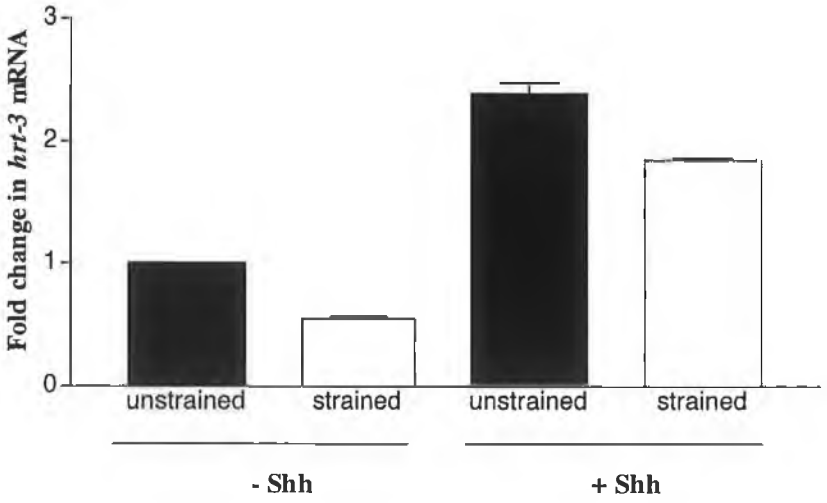


Figure 5.22 The Effect of Shh on Hedgehog and Notch Target Gene mRNA Expression in Strained RVSMC. RVSMC were seeded onto Flexercell^{TM} plates at 1 x 10^5 cells/well, and were treated with recombinant Shh(3.0ug, 24 h). Following overnight recovery, cells were subsequently exposed to cyclic strain (10%, 24 h). *gli-2* mRNA levels (A) and *hrt-3* mRNA (B) were measured using quantitative RT-PCR, and expressed as fold increase over control (= the levels of target gene present in static mock transfected cells) n=3.

Fig 5.23 Blood Flow in Young Rats Following Carotid Ligation

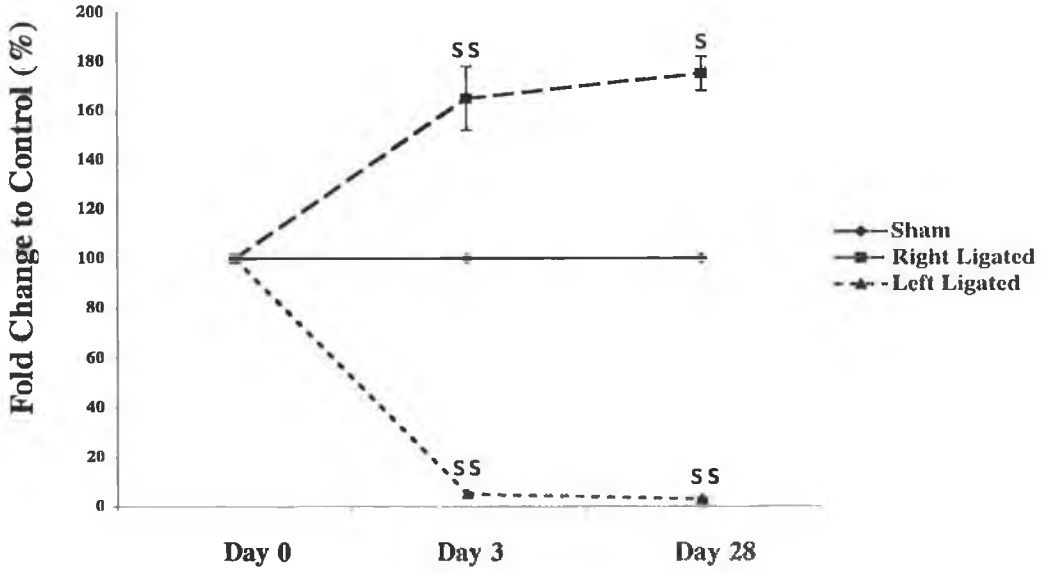


Fig 5.23 Graphs Showing Blood Flow Rates in Young Rats Post-Carotid Ligation Compared to Sham Control Animals. The left carotid artery of young rats was ligated, and the blood flow rates measured in the left and right carotid arteries of both ligated and sham-operated animals using a Transonic flowprobe (g/ml/min) at 0, 3, and 28 days post-ligation. Results are expressed as percentage increase in flow rate over sham operated animals, arbitrarily assigned a value of 100%. ^{S} $p<0.05$, ^{SS} $p<0.005$ as compared to sham-operated control (student's t test).n=3.

Fig 5.24 Changes in Notch 1 Receptor Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation

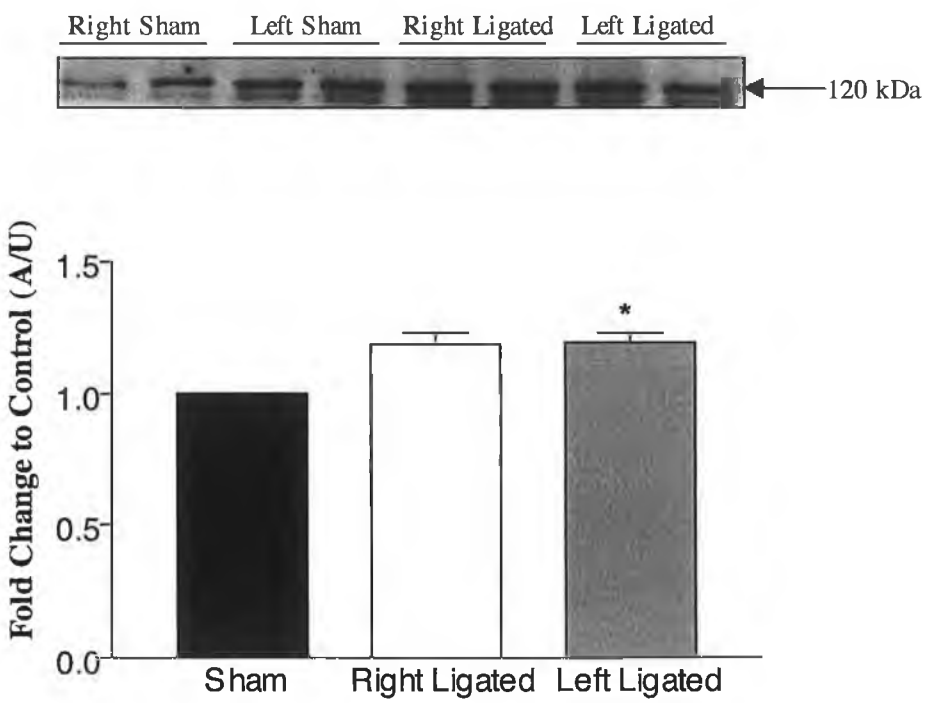


Fig 5.24 Changes in Notch 1 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation. Protein extracted from these vessels was assayed for Notch 1 using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation. * $p<0.05$ as compared to sham-operated control (student's t test).

Fig 5.25 Changes in Notch 3 Receptor Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation

Notch 3

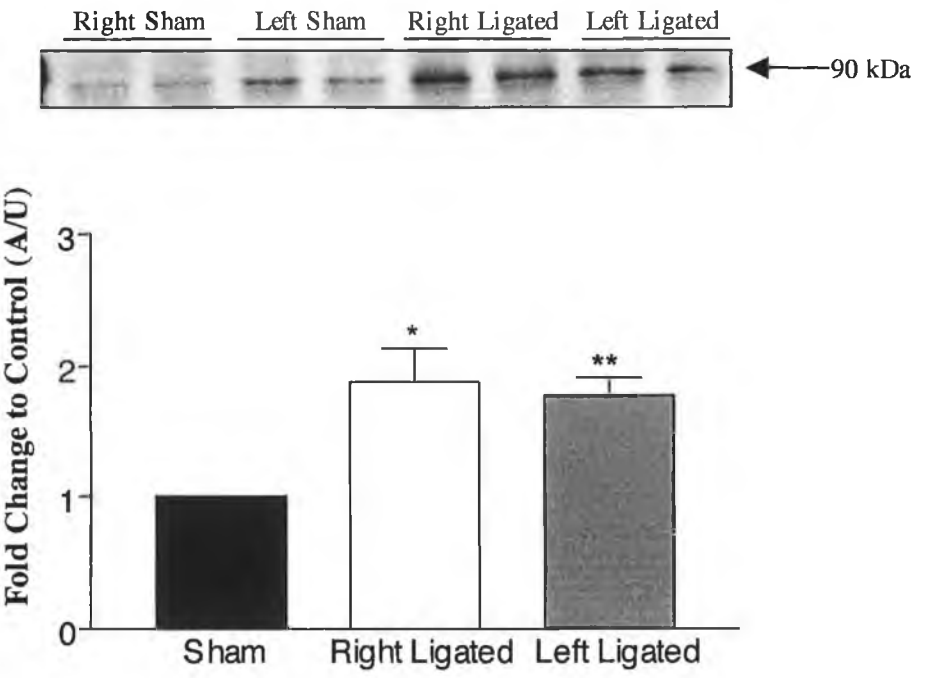
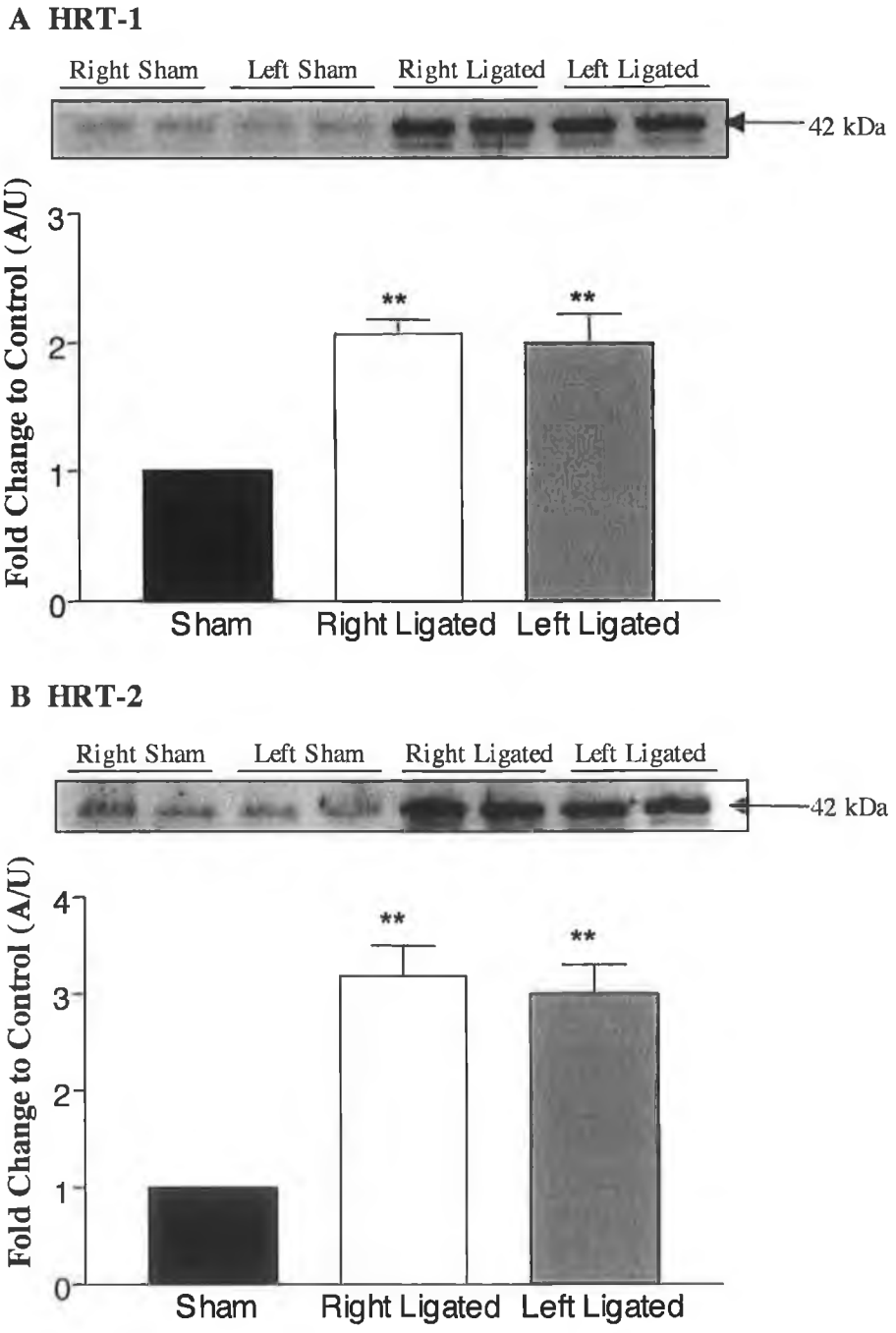
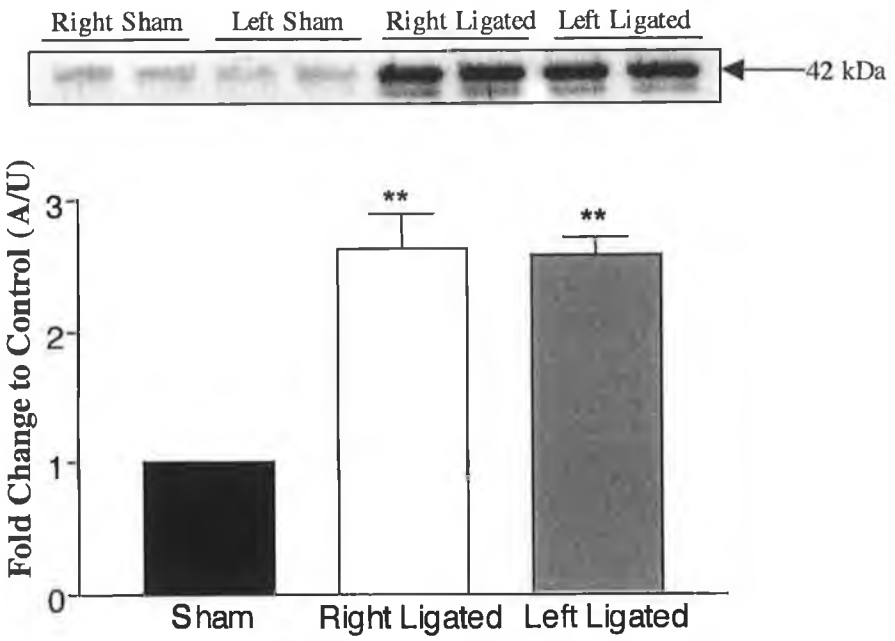


Fig 5.25 Changes in Notch 3 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats was ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation. Protein extracted from these vessels was assayed for Notch 3 using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation. $^{*}p<0.05$, $^{**}p<0.005$ as compared to sham-operated control (student's t test).

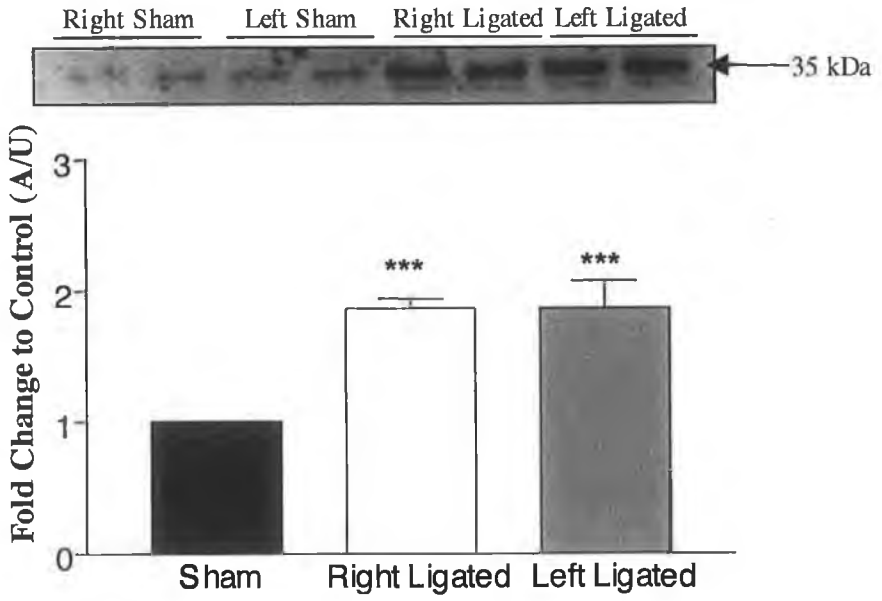
Fig 5.26 Changes in Notch Target Gene Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation



C HRT-3


Right Sham
Left Sham
Right Ligated
Left Ligated
42 kDa
Fold Change to Control (A/U)
0
1
2
3
**
**
Sham
Right Ligated
Left Ligated

D Hes-1


Right Sham
Left Sham
Right Ligated
Left Ligated
35 kDa
Fold Change to Control (A/U)
0
1
2
3
***
***
Sham
Right Ligated
Left Ligated

E Hes-5

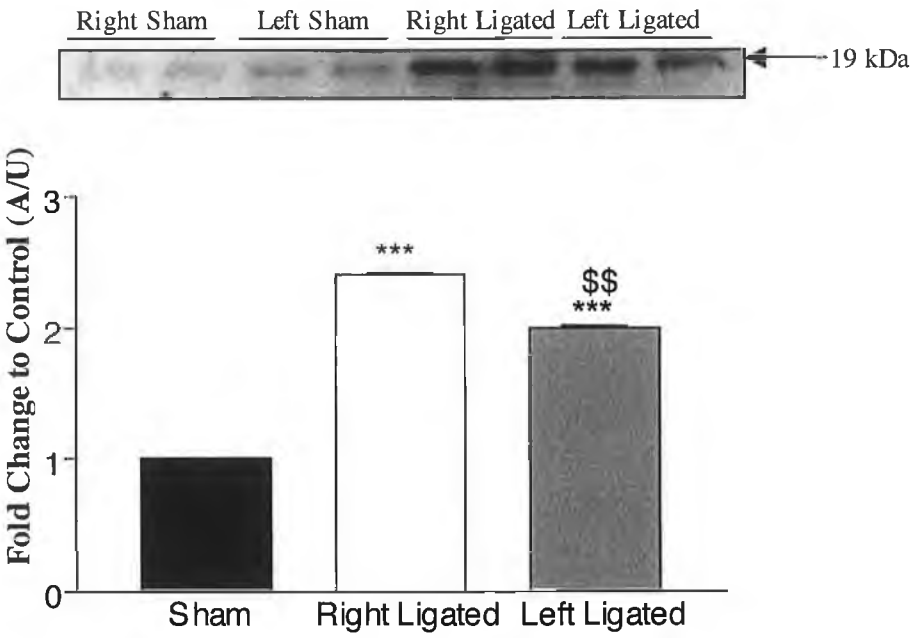
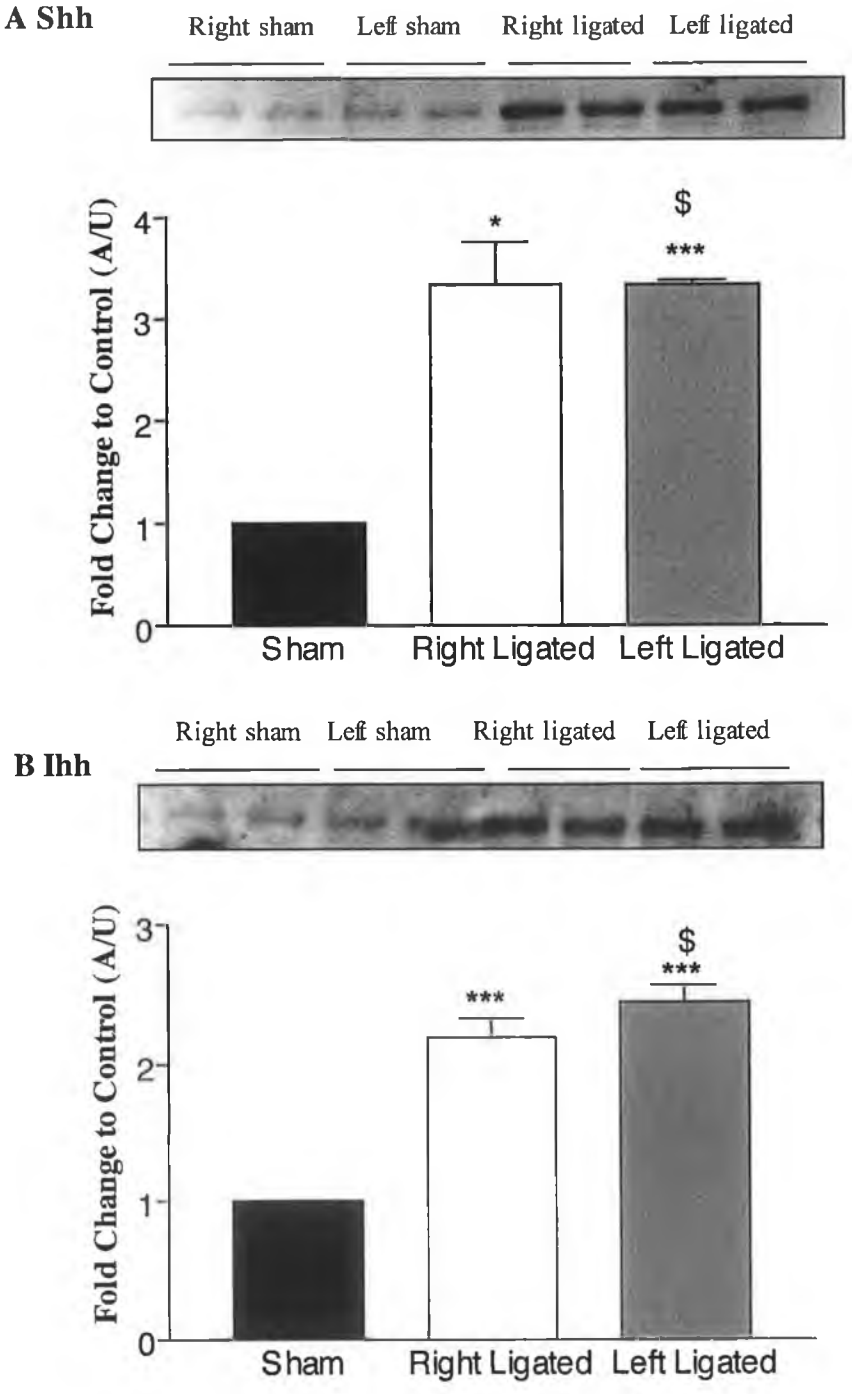


Fig 5.26 Changes in Notch Target Gene expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation. Protein extracted from these vessels was assayed for HRT-1 (A), HRT-2 (B), HRT-3 (C), Hes-1 (D) and Hes-5 (E) using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation. ** $p<0.005$, *** $p<0.0005$ as compared to sham-operated control, $ $$ $p<0.005$ as compared to right artery of ligated animal (student's t test).

Fig 5.27 Changes in Hedgehog Signaling Gene Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation



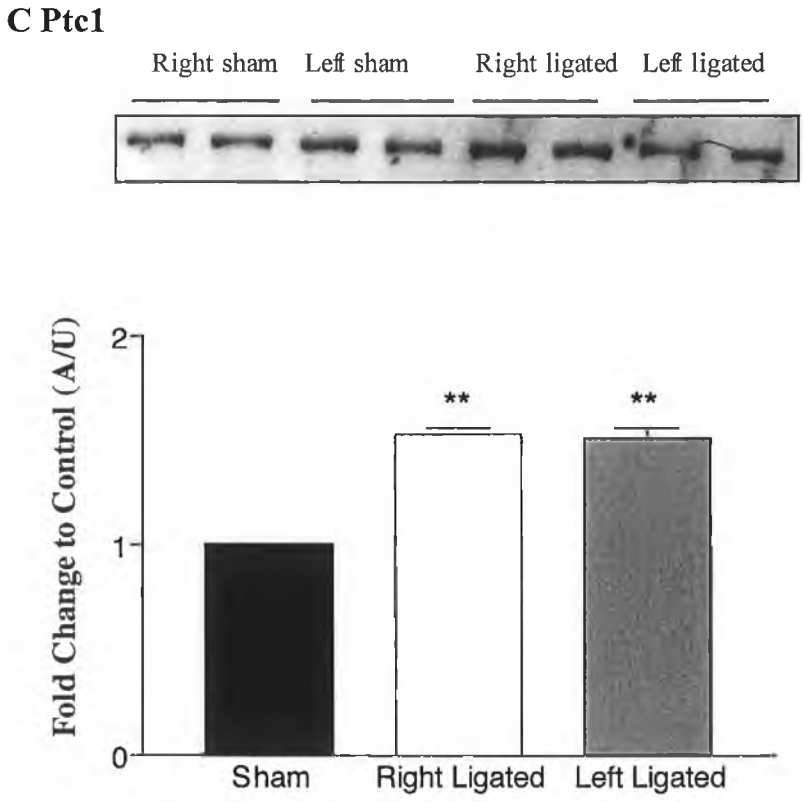


Fig 5.27 Changes in Hedgehog Gene expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation. Protein extracted from these vessels was assayed for Shh- (A), Ihh-(B) and Ptc1 (E) using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation *$p<0.05$, ** $p<0.005$, *** $p<0.0005$ as compared to sham-operated control, $^{\$}$ $p<0.05$ as compared to right artery of ligated animal (student's t test).

Fig 5.28 Changes in pCNA Expression in Ligated versus Sham Operated Animals 3 Days Post-Carotid Ligation

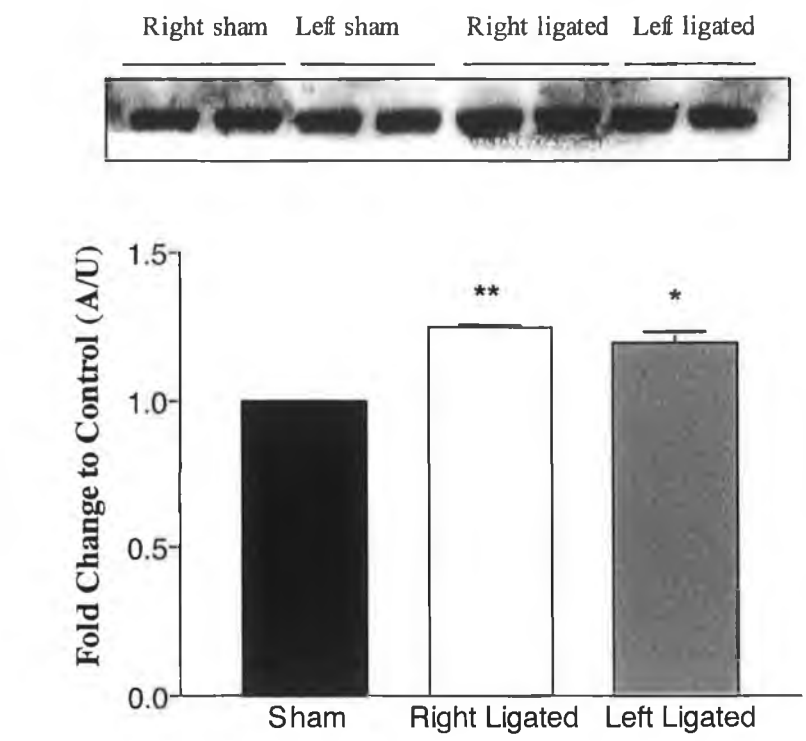


Fig 5.28 Changes in pCNA expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation. Protein extracted from these vessels was assayed for pCNA expression using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation. ** $p<0.005$, * $p<0.05$ as compared to sham-operated control (student's t test).

Fig 5.29 Changes in Notch 1 Receptor Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation

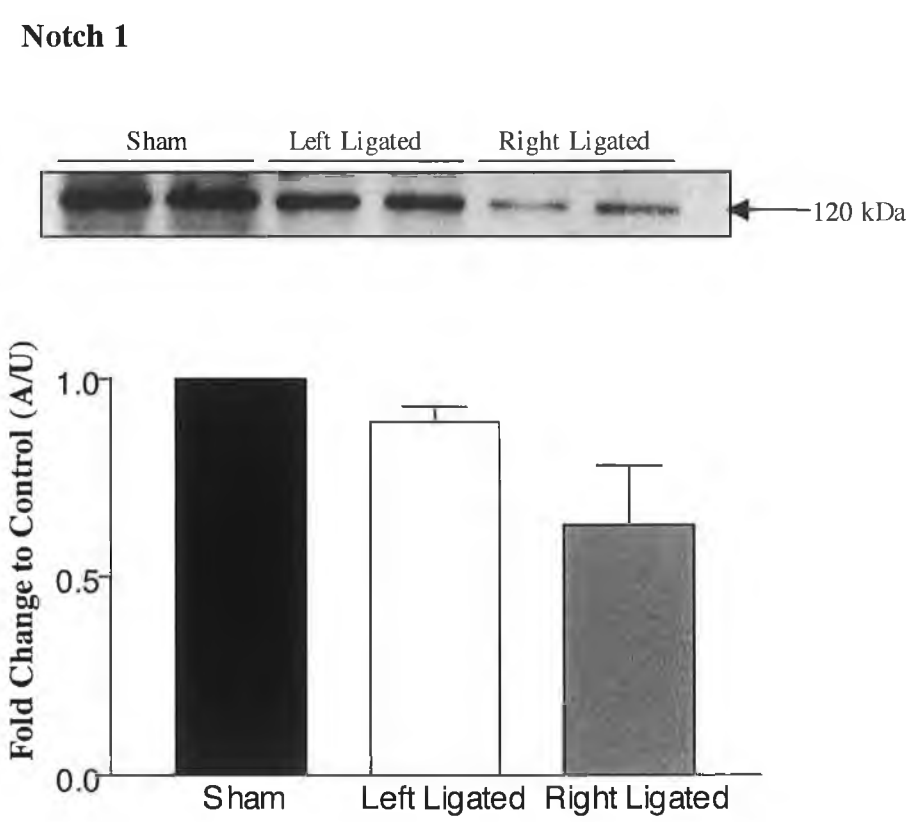


Fig 5.29 Changes in Notch 1 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 28 days post ligation. Protein extracted from these vessels was assayed for Notch 1 using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation.

Fig 5.30 Changes in Notch 3 Receptor Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation

Notch 3

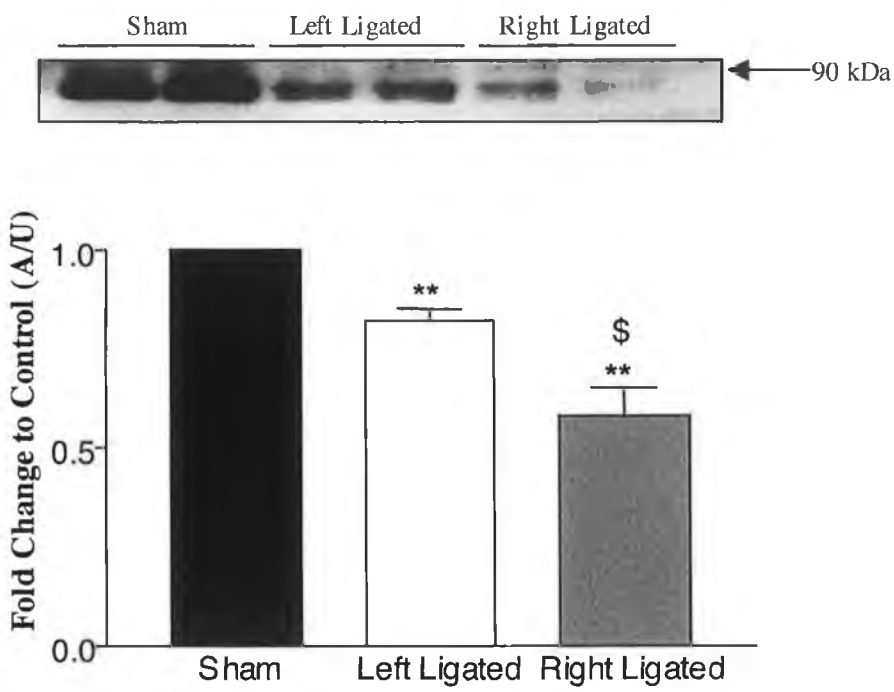
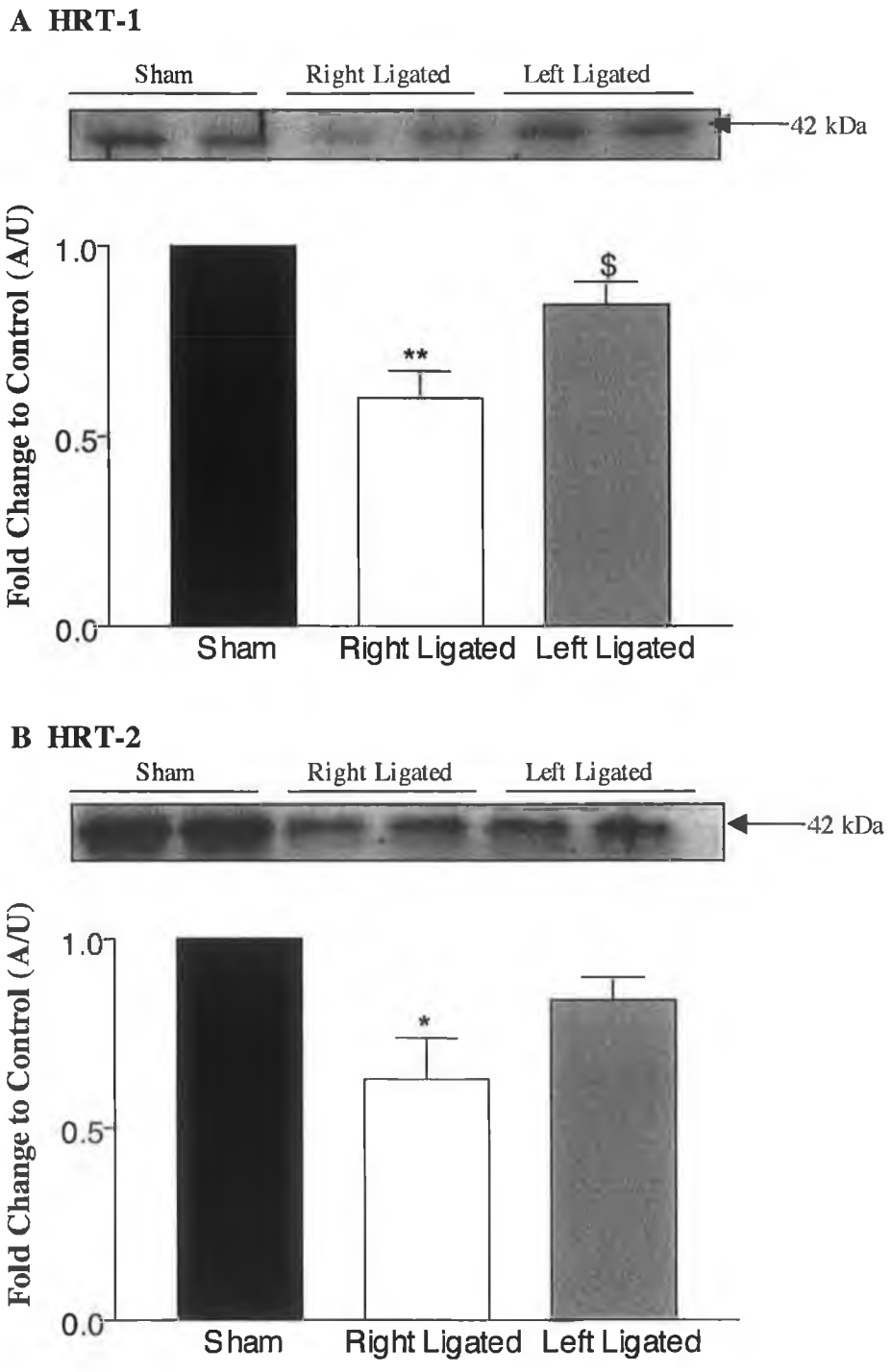
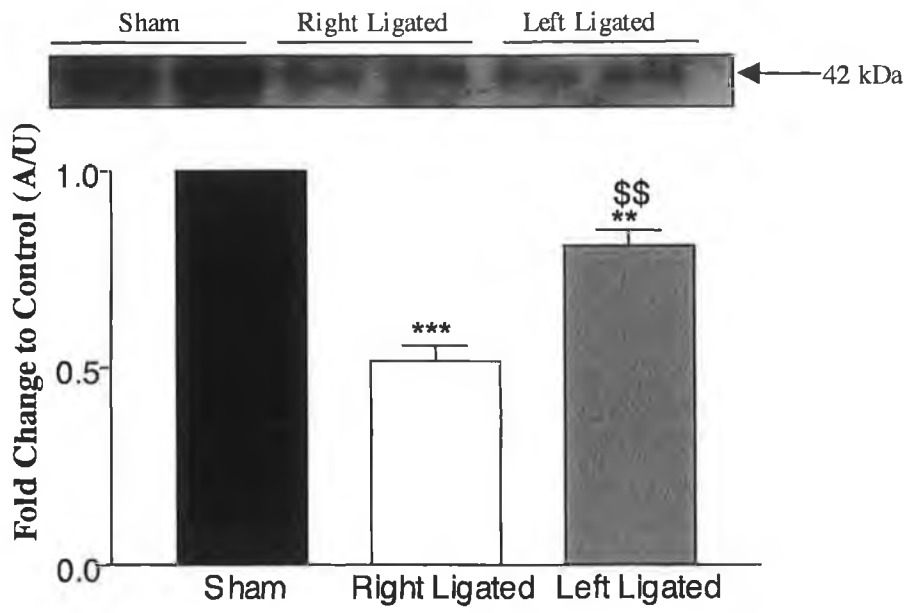


Fig 5.30 Changes in Notch 3 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 28 days post ligation. Protein extracted from these vessels was assayed for Notch 3 using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation.** $p<0.005$ as compared to sham-operated control, $ $p<0.05$ as compared to left ligated artery (student's t test).

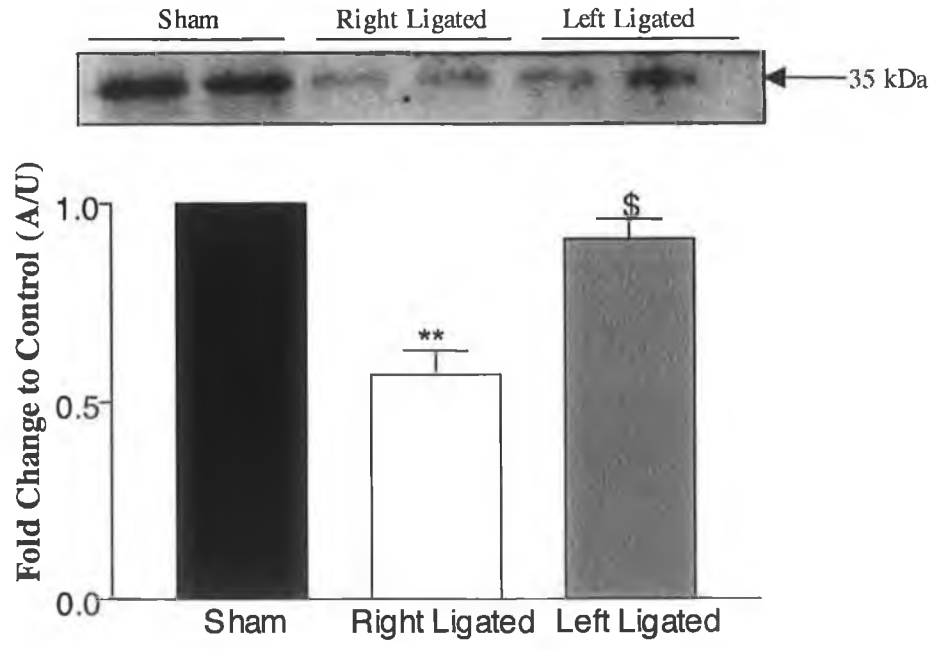
Fig 5.31 Changes in Notch Target Gene Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation



C HRT-3


Sham
Right Ligated
Left Ligated
42 kDa
Fold Change to Control (A/U)
1.0
0.5
0.0
***
$$
**
Sham
Right Ligated
Left Ligated

D Hes-1


Sham
Right Ligated
Left Ligated
35 kDa
Fold Change to Control (A/U)
1.0
0.5
0.0
**
$
Sham
Right Ligated
Left Ligated

E Hes-5

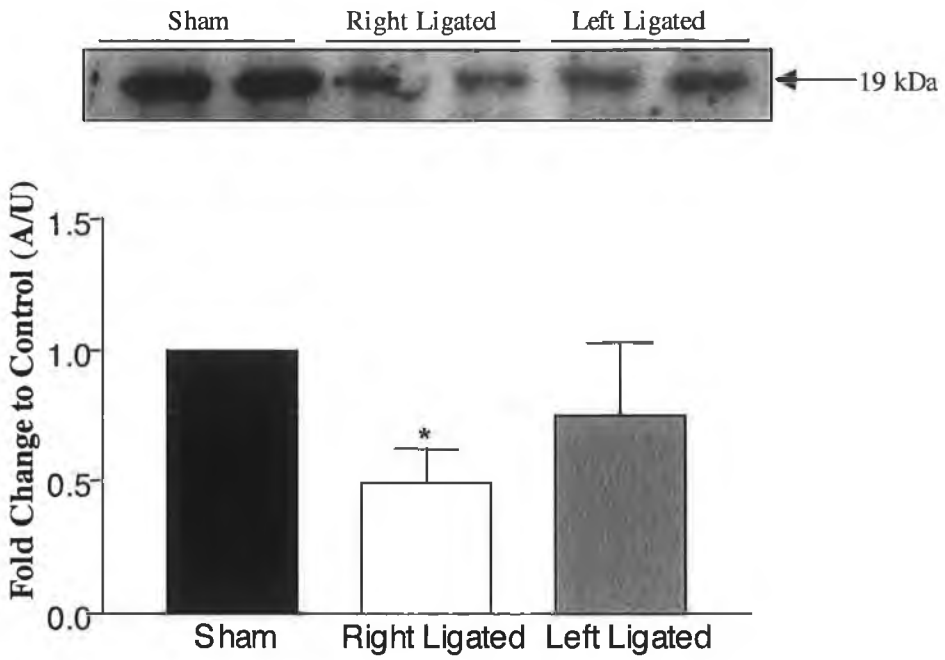
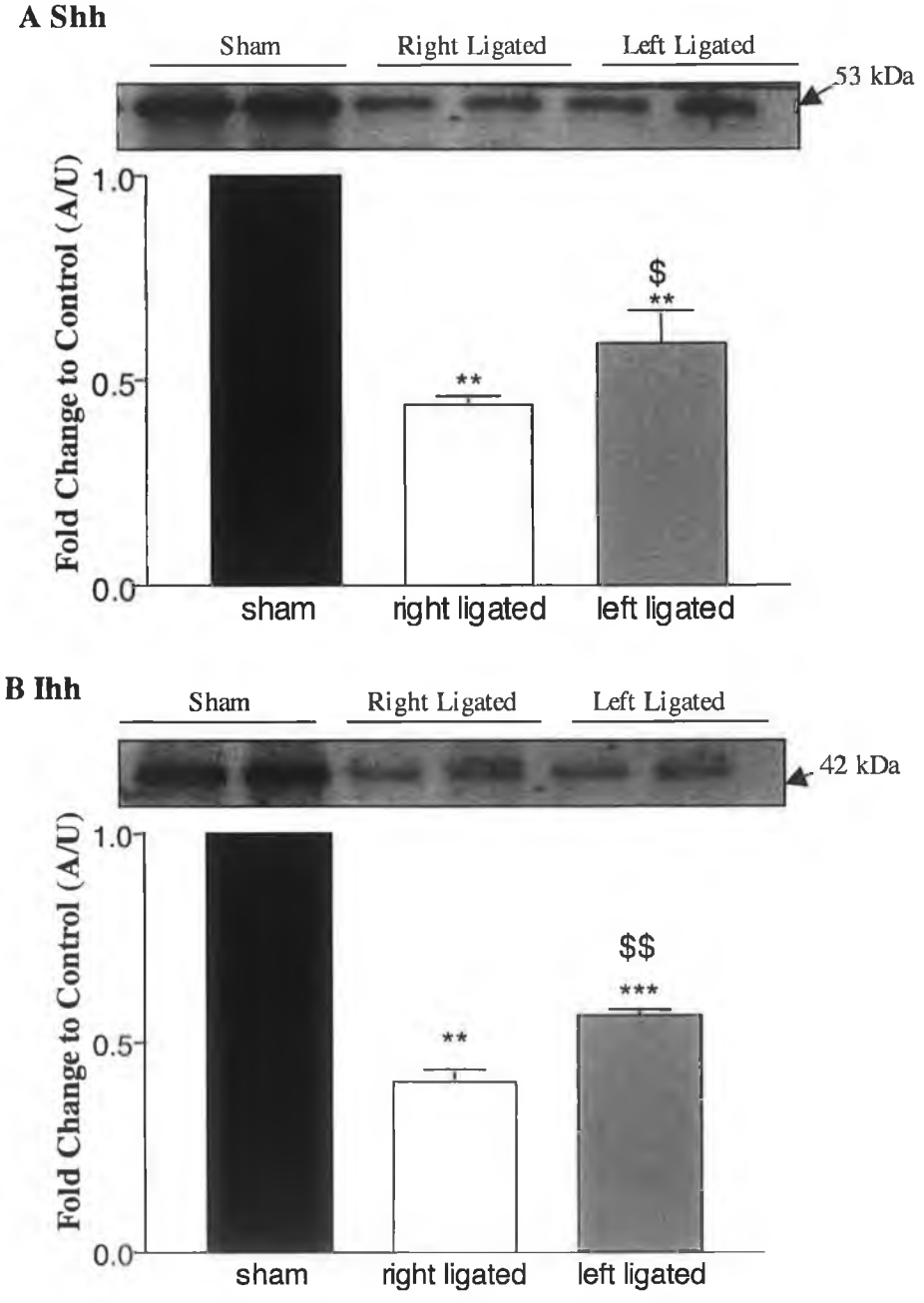


Fig 5.31 Changes in Notch Target Gene expression in the right and left carotid arteries of ligated animals compared to sham-operated controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 28 days post ligation. Protein extracted from these vessels was assayed for HRT-1 (A), HRT-2 (B), HRT-3 (C), Hes-1 (D) and Hes-5 (E) using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ as compared to sham-operated control, $^{\$}$ $p<0.05$, $^{\$\$}$ $p<0.005$ as compared to right artery of ligated animal (student's t test).

Figure 5.32 Changes in Hedgehog signaling Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation



Changes in Hedgehog signaling Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation

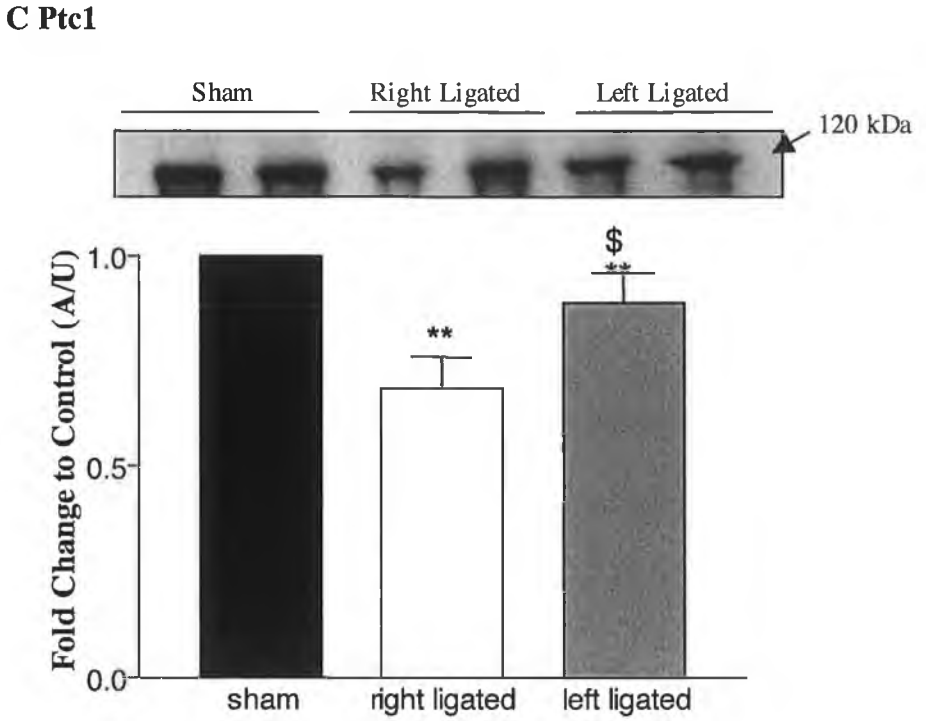


Fig 5.32 Changes in Hedgehog Gene Expression in the Right and Left Carotid Arteries of Ligated Animals Compared to Sham-operated Controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 28 days post ligation. Protein extracted from these vessels was assayed for Shh (A), Ihh (B), and Ptc1 (C) using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation, ** $p<0.005$, *** $p<0.0005$ as compared to sham-operated control, $ $p<0.05$, $$ $p<0.005$ as compared to right artery of ligated animal (student's t test).

Figure 5.33 Changes in pCNA Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation

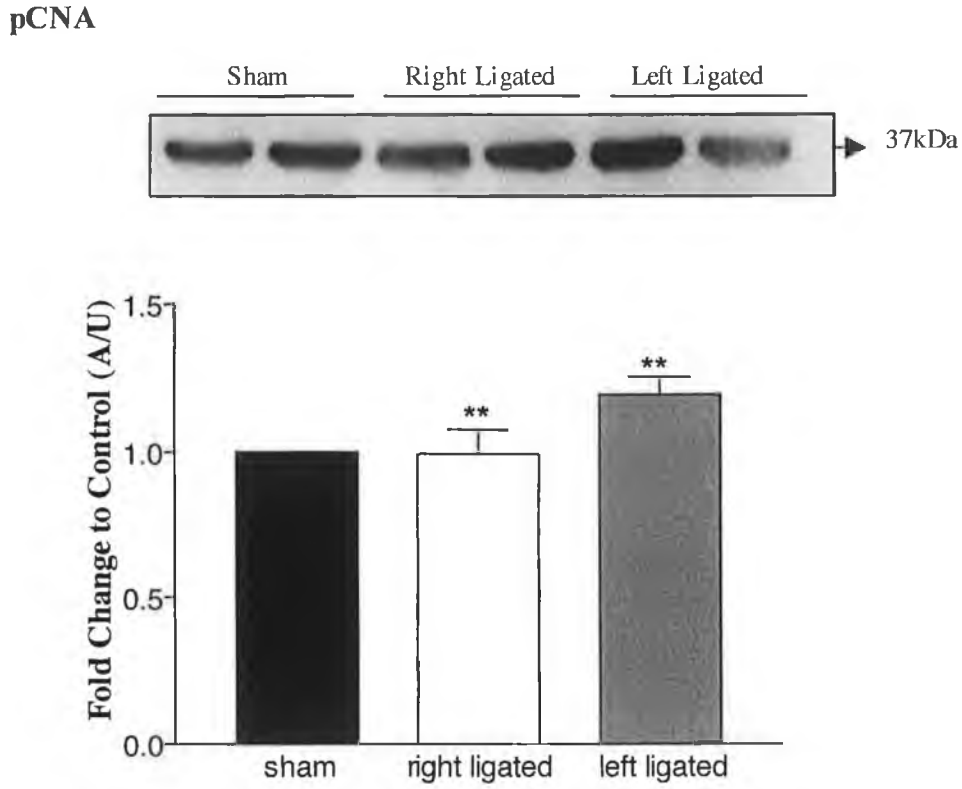
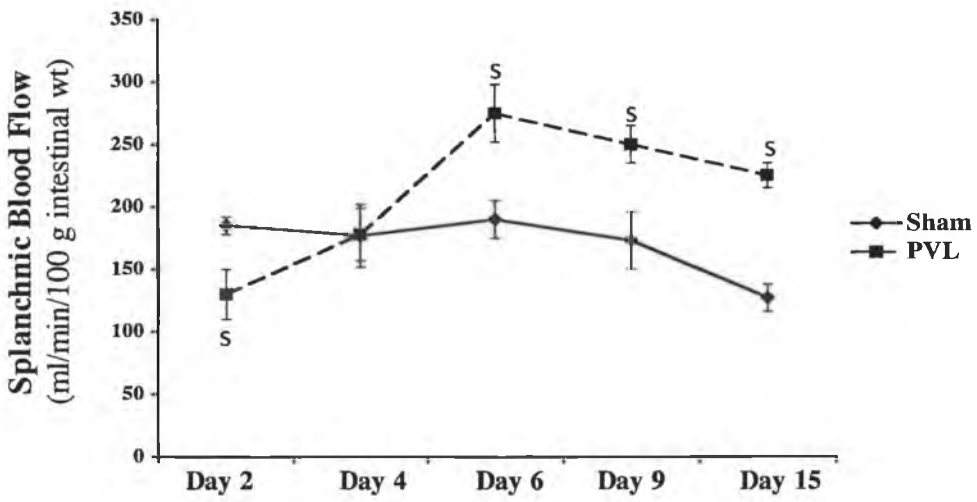


Fig 5.33 Changes in pCNA Expression in the Right and Left Carotid Arteries of Ligated Animals Compared to Sham-operated Controls. The left carotid artery of young rats were ligated at day 0. The left and right carotids of both sham-operated and ligated animals were harvested 28 days post ligation. Protein extracted from these vessels was assayed for pCNA expression using Western blot analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=2, two vessels were pooled for each protein preparation. ** $p<0.005$, as compared to sham-operated control(student's t test).

Figure 5.34 Splanchnic Blood Flow and Splenic Pressure following Portal Vein Ligation

A Blood Flow



B Splenic Pressure

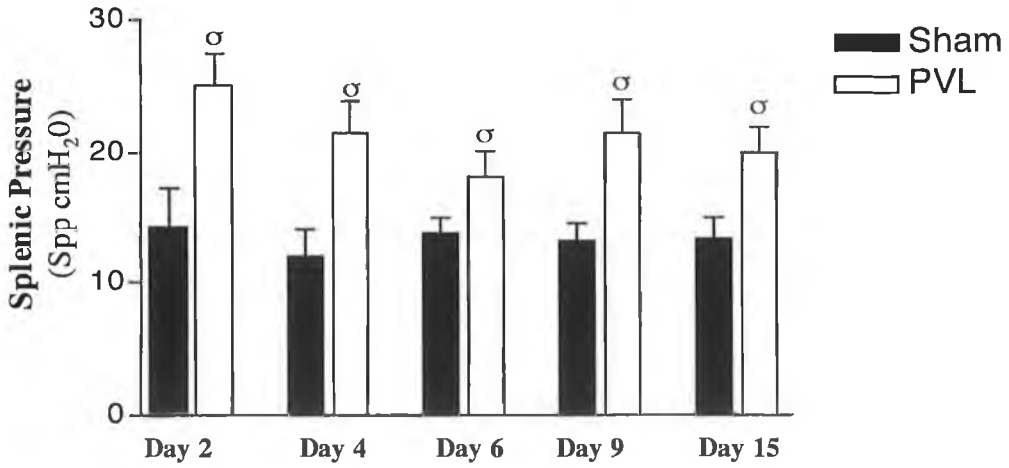


Fig 5.34 Graphs showing splanchnic blood flow and splenic pulse pressure in young rats post-PVL compared to sham-operated control animals. A) A Transonic flowprobe was used to measure splanchnic blood flow in both ligated and sham-operated aminals at at 2, 4, 6, 9 and 15 days post-ligation. B) Portal venous pressure was measured indirectly with direct splenic puncture (splenic pulse pressure using a Doppler flow probe). n=3. ^{s} $p<0.05$ as compared to sham-operated control (student's t test).

Fig 5.35 Changes in Notch 1 IC Receptor Expression in Portal Vein Ligated versus Sham-operated Animals

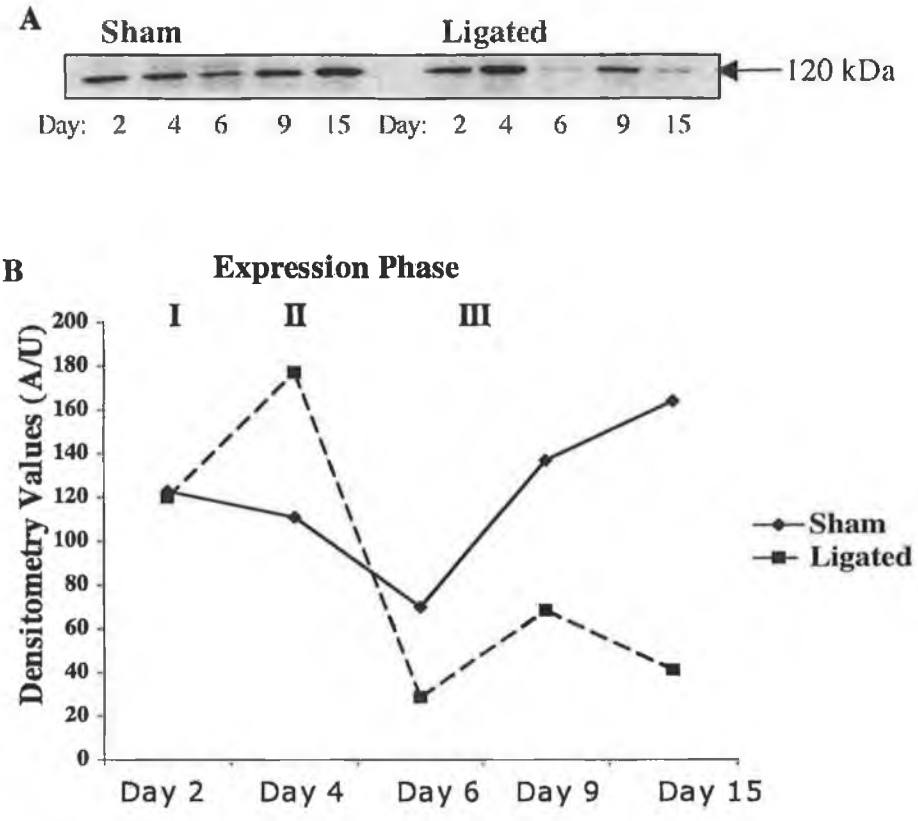


Fig 5.35 Changes in Notch 1 IC expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for Notch 1 IC using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in Notch 1 IC expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.36 Changes in Notch 3 IC Receptor Expression in Portal Vein Ligated versus Sham-operated Animals

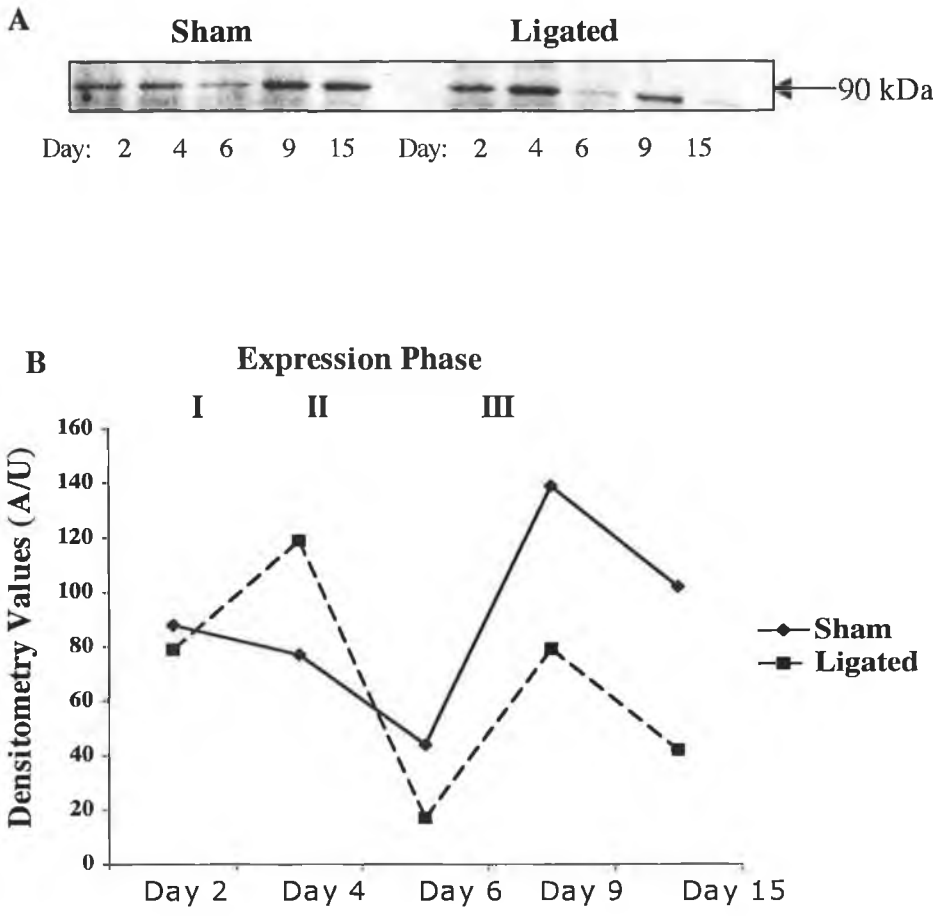


Fig 5.36 Changes in Notch 3 IC expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for Notch 3 IC using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in Notch 3 IC expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.37 Changes in Jagged Expression in Portal Vein Ligated versus Sham-operated Animals

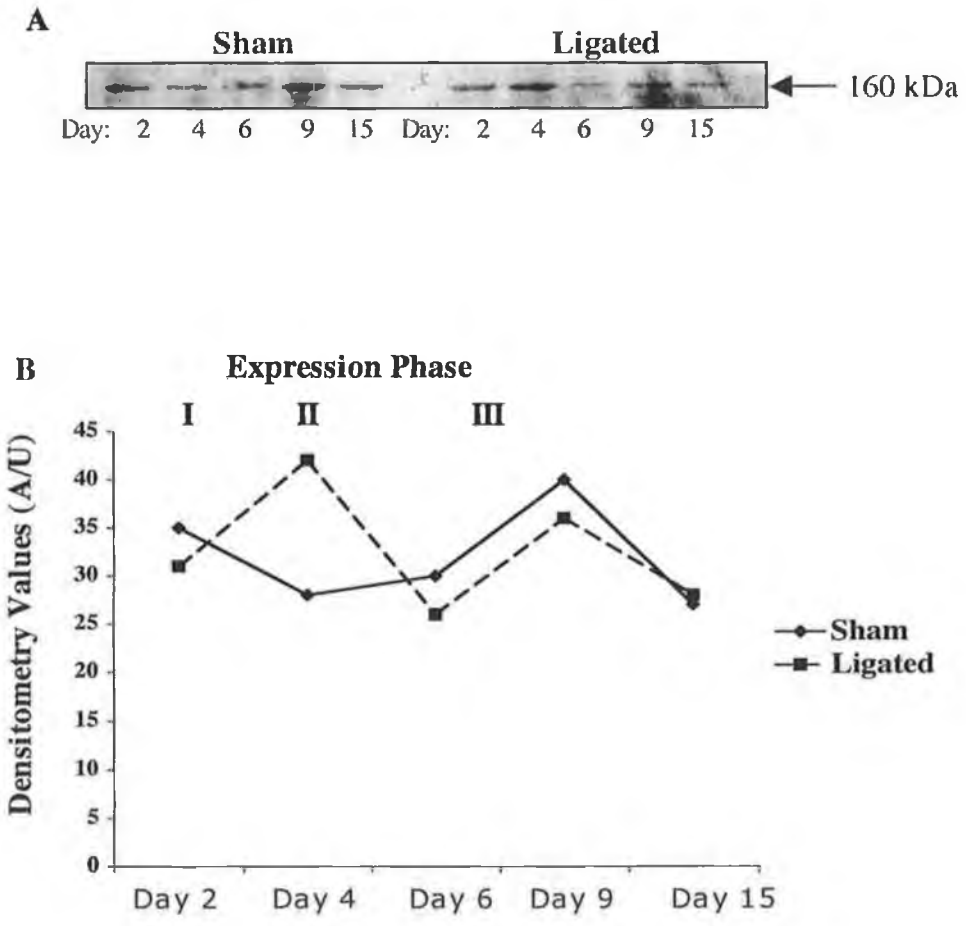


Fig 5.37 Changes in Jagged expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for Jagged using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in Jagged expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.38 Changes in HRT-1 Expression in Portal Vein Ligated versus Sham-operated Animals

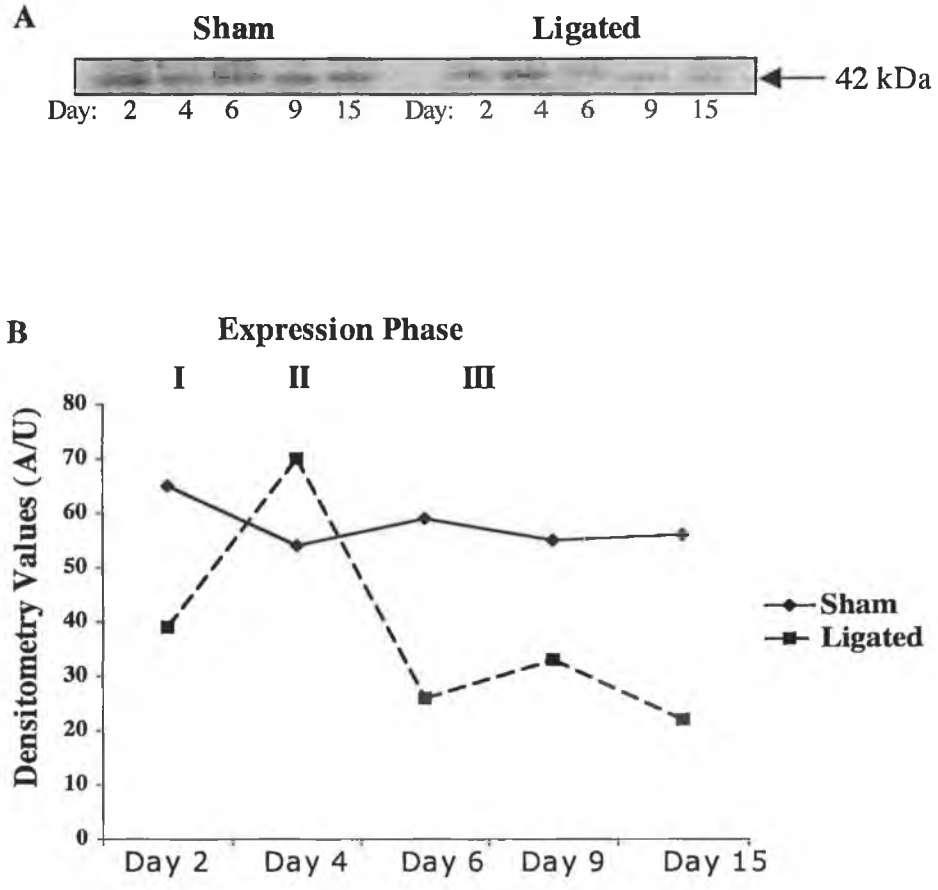


Fig 5.38 Changes in HRT-1 expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for HRT-1 using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in HRT-1 expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.39 Changes in HRT-2 Expression in Portal Vein Ligated versus Sham-operated Animals

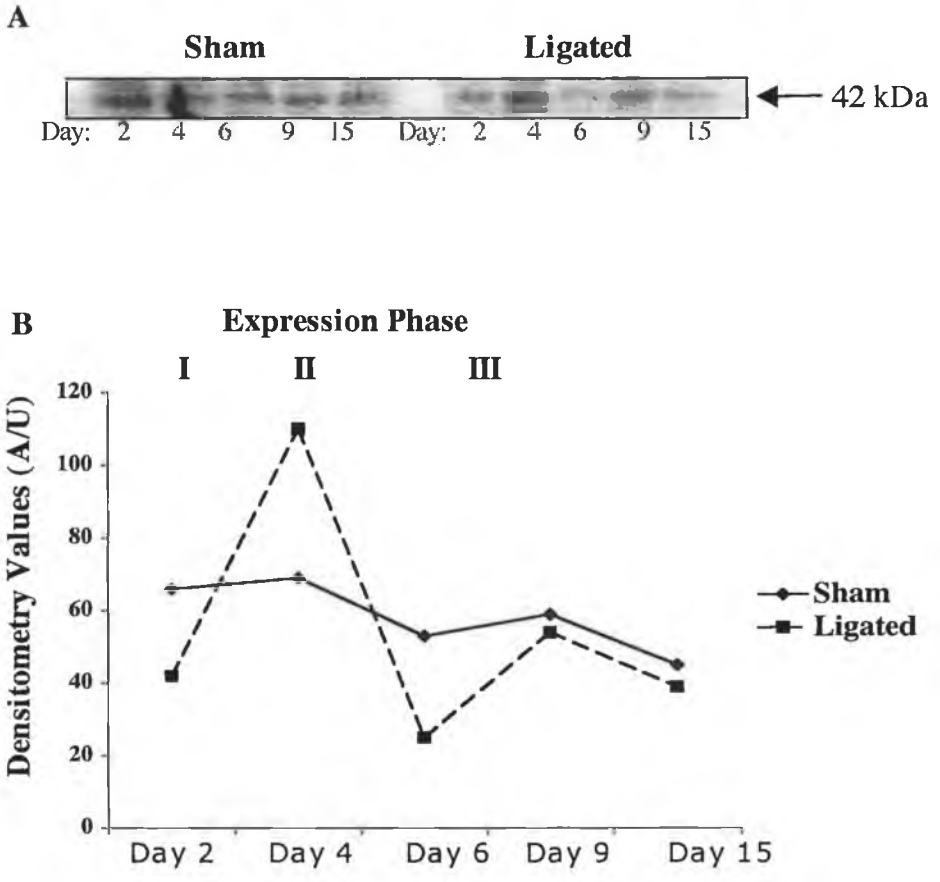


Fig 5.39 Changes in HRT-2 expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for HRT-2 using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in HRT-2 expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.40 Changes in HRT-3 Expression in Portal Vein Ligated versus Sham-operated Animals

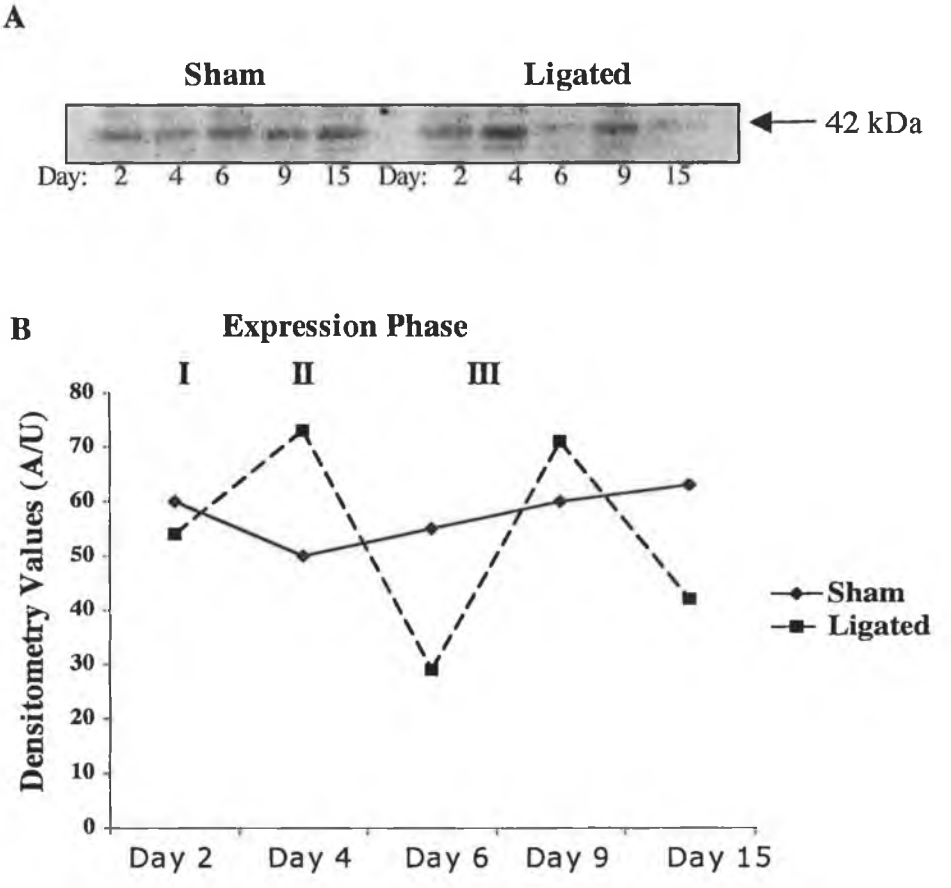


Fig 5.40 Changes in HRT-3 expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for HRT-3 using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in HRT-3 expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.41 Changes in Hes-1 Expression in Portal Vein Ligated versus Sham-operated Animals

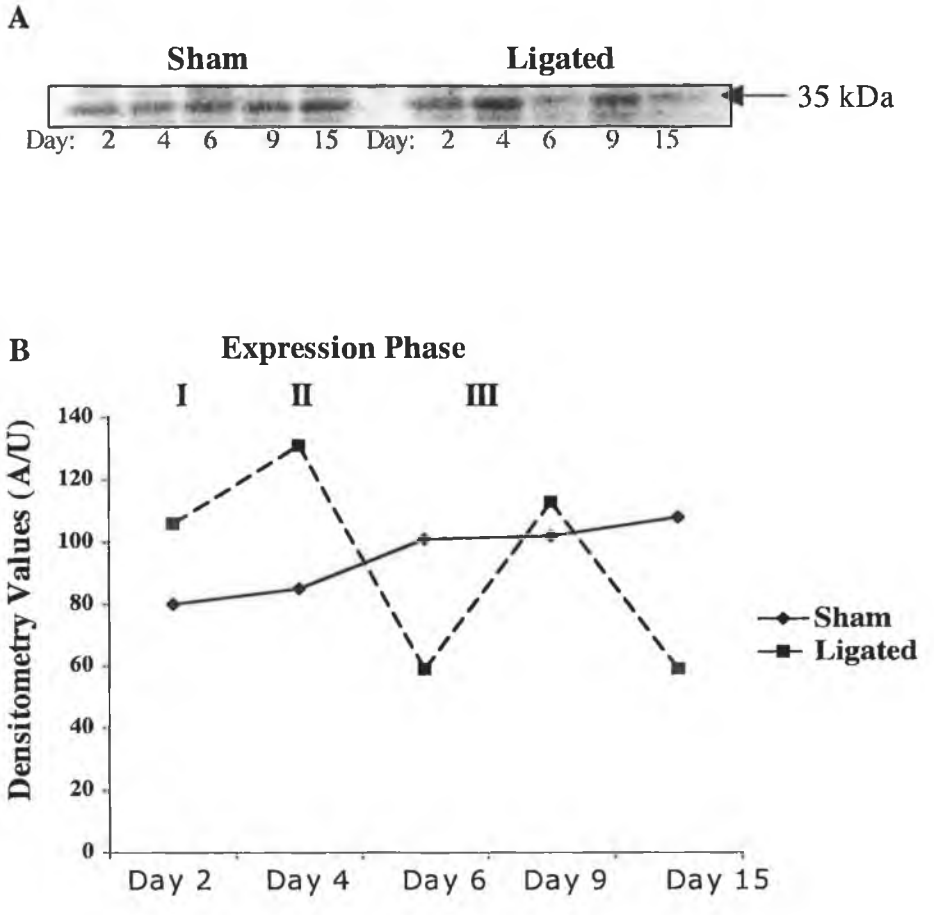


Fig 5.41 Changes in Hes-1 expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for Hes-1 using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in Hes-1 expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Fig 5.42 Changes in Hes-5 Expression in Portal Vein Ligated versus Sham-operated Animals

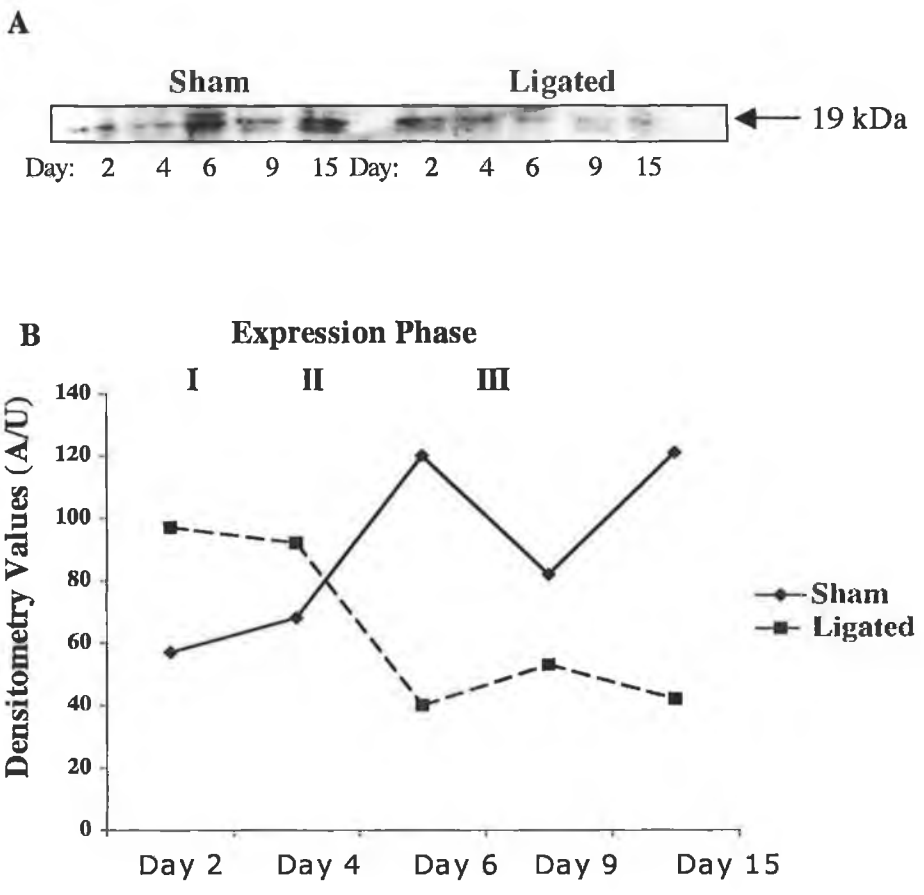


Fig 5.42 Changes in Hes-5 expression in portal vein ligated animals compared to sham-operated controls. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. Protein extracted from these vessels was assayed for Hes-5 using Western blot analysis. A) Values are expressed as fold increase over sham-operated animals for each timepoint (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation. B) Absolute densitometry values showing changes in Hes-5 expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation.

Figure 5.43 The Effect of Portal Vein Ligation (Day 2) on Notch and Hedgehog Signalling.

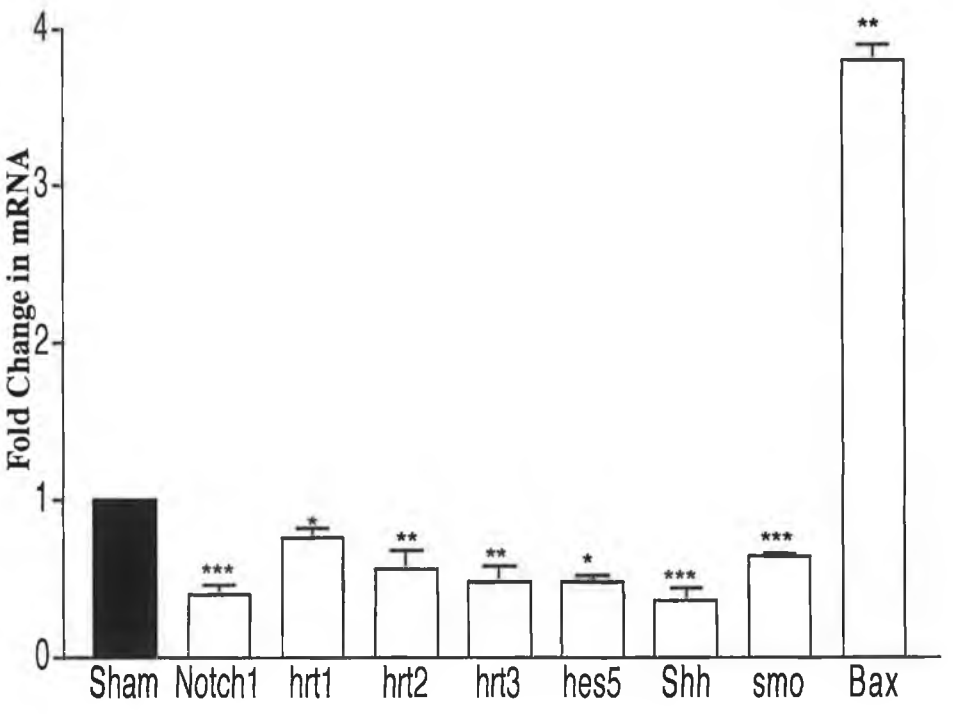


Figure 5.43 The Effect of Portal Vein Ligation (Day 2) on Notch and Hedgehog Signalling. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. RNA was extracted at Day 15 from these vessels was assayed for Notch 1, hrt1-3 ,hes-5, Shh, Smo and Bax expression using QRTPCR analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=1, 7 vessels were pooled for each RNA preparation. ** $p<0.005$, *** $p<0.0005$ as compared to sham-operated control (rank test).

Figure 5.44 The Effect of Portal Vein Ligation (Day 15) on Notch and Hedgehog Signalling.

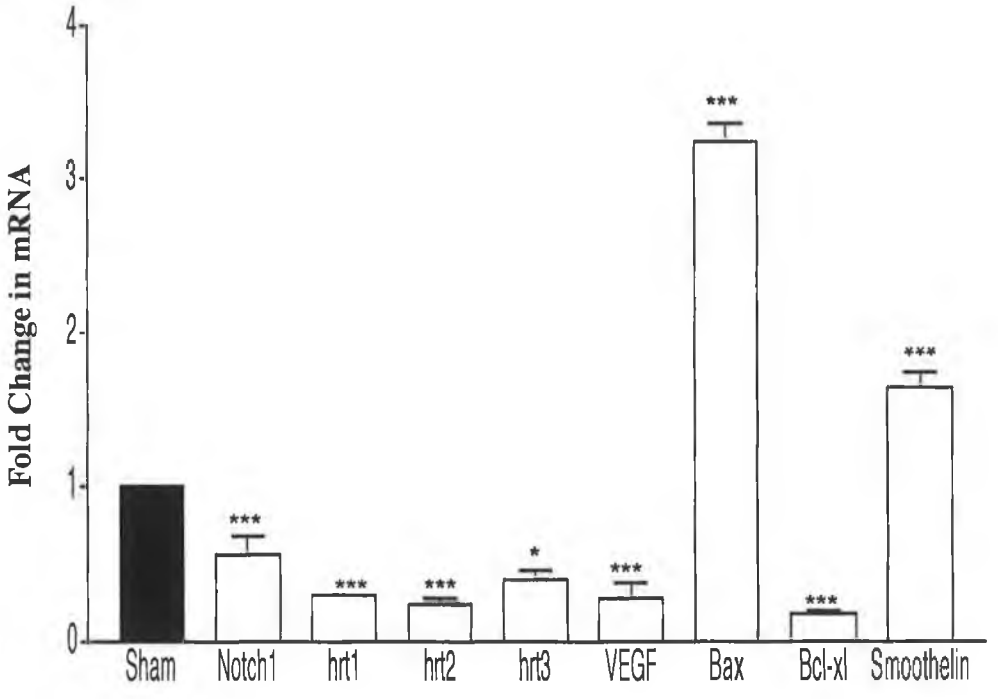


Figure 5.44 The Effect of Portal Vein Ligation (Day 15) on Notch and Hedgehog Signalling. The portal veins of young rats were ligated at day 0. The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation. RNA was extracted at Day 15 from these vessels was assayed for Notch 1, hrt1-3 , VEGF, Bax, Bcl-x_L and smoothelin expression using QRTPCR analysis. Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1), n=1, 7 vessels were pooled for each RNA preparation. ** $p<0.005$, *** $p<0.0005$ as compared to sham-operated control (rank test).

Discussion

This study examined the effect of equibiaxial cyclic strain on Notch signaling in adult SMC and determined the contributory role of Notch receptors in regulating strain-induced changes in SMC growth. Furthermore, it examined the effect of cyclic strain on the Hh signaling pathway and the contributory role of Notch receptors in regulating the strain-induced changes in Hh signaling in VSMC *in vitro.* To further investigate the role of biomechanical forces on Notch and Hh signaling and subsequently on cell growth, we utilized two models of vascular disease associated with vascular remodeling. This allowed us to extrapolate the effect of mechanical forces on these pathways to an *in vivo* scenario.

Biomechanical signals can induce a highly restricted transcriptional response in vascular SMC that can include genes that can modify structure (Osol *et al.*, 1995; Koller *et al.*, 2002). We established that cyclic strain induces a significant decrease in the pro-proliferative and anti-apoptotic effect of Notch by decreasing the expression and activity of components of the Notch 3 signalling pathway in a force and time-dependent manner. Furthermore this study determined that this decrease observed in Notch signaling pathway expression, was a Gi-protein and MAPK-dependent event. In addition, strain-induced inhibition of SMC proliferation could be reversed by over-expression of Notch 3 IC to levels comparable to unstrained cells while concurrently attenuating the strain-induced SMC apoptosis. Moreover, Notch inhibition served to potentiate the strain-induced decrease in Notch signaling while concurrently further decreasing proliferation and increasing apoptosis. This decrease in Notch signaling was also mirrored by a similar decrease in Hh signaling. Furthermore, the strain-induced decreases in Hh signaling could be reversed by over-expression of Notch 3 IC, again further highlighting the functional relationship between the Notch and Hh signaling pathways in adult SMC. Collectively this study suggests for the first time that biomechanical stimulation of SMC inhibits both endogenous Notch and Hh signaling resulting in fundamental changes in vascular SMC proliferation and apoptosis *in vitro.* Further understanding of the response of these pathways to biomechanical forces, may provide new insights into the pathogenesis and treatment of vascular diseases such as atherosclerosis and intimal hyperplasia.

In the previous chapter we reported that by inhibiting the Notch signalling pathway we could down regulate the Hh pathway downstream, and as a result,

decrease VSMC growth. Furthermore by over-expressing the Notch and Hh pathways we could achieve a level of protection against serum-deprivation induce apoptosis while concurrently promoting serum–induced proliferation. In this study we aimed to investigate whether this pro-proliferative and anti-apoptotic effect of Notch was conserved against cyclic-strain-induced changes in VSMC growth. This was studied through the over-expression of Notch 3 IC, as other studies by Wang *et al.,* 2002 reported that in vivo deformation of VSMC causes a greater down-regulation of Notch 3 as compared to other Notch receptors. This study demonstrated for the first time that constitutively active Notch 3 IC increased Bcl-X_L expression while concomitantly inhibiting Bax expression, an effect that was fully reversed following inhibition of CBF-1/RBP-Jκ with co-expression of RPMS-1. This data further suggests that both Bax and Bcl-X_L are downstream targets of CBF-1/RBP- Jκ for in the previous chapter we reported that specific siRNA targeted against either Hrt-1, 2 or 3, resulted in significant increases and decreases in Bax and Bcl-X_L expression respectively. This is further re-enforced by studies that demonstrate that constitutively active Notch 1 modulates the expression of Bcl-X_L (Jang *et a*l., 2004). This regulation of both Bax and Bcl-X_L by cyclic strain is somewhat unsurprising as both have been implicated in neonatal vascular remodelling and the pathogenesis of vascular disease (Pollman *et al.,* 1999; Gibbons *et al.,* 2000). The stimulus for the observed increases in Bax expression due to cyclic strain is likely due to increased p53 activity as Bax is a direct transcriptional target of p53 and recent studies have shown similar increases in Bax expression due to increases in p53 activity (Mayr *et al.,* 2002). However in the same study by Mayr, they reported an increase in Bcl-X_L expression following cyclic strain. This could perhaps be attributed to difference in VSMC phenotype. Conflicting reports on the effects of cyclic strain on SMC growth are also evident in previous studies (Schulze *et al.*, 2003; Sedding *et al.,* 2003; Hipper *et al.*, 2000; Wilson *et al.*, 1993). However, again depending on the species of SMC, the phenotype studied, the extracellular matrix environment, the cell cycle status, SMC can either increase (2003; Sedding *et al.,* 2003; Wilson *et al.*, 1993) or decrease (Schulze *et al.*, 2003; Hipper *et al.*, 2000) their proliferative capacity. In this study, cyclic strain served to decrease SMC proliferation while concomitantly increasing SMC apoptosis.

As cyclic strain can induce a highly restricted transcriptional response in vascular SMC that regulates vascular structure (Kleinstreuer *et al.*, 2001), we hypothesized

that Notch could represent a novel pathway for contributing to strain-induced changes in VSMC fate. This study demonstrates a functional role for cyclic strain-induced decreases in Notch–mediated CBF-1/RBP-Jκ-dependent signaling and subsequent changes in vascular cell fate. In addition, the decrease in the expression of Notch and Hh components is consistent with the observed changes in SMC fate following strain. Moreover, selective inhibition of Gi proteins and MAPK respectively, which are known signaling pathways involved in mechanotransduction in vascular cells (Shaw, 2004), despite decreasing baseline Notch signaling, blocked the strain-induced changes in Notch and corresponding changes in SMC fate. These findings further suggest a possible nexus by which the activation of a biomechanical signaling pathway is coupled to the Notch cellular fate programme. However the mechanisms by which cyclic strain regulates Notch signaling will require additional study.

To further elucidate the effect of biomechanical forces on the Notch and Hh signaling pathways and their contributory role in regulating strain-induced changes in SMC growth, we utilized two *in vivo* models of vascular remodeling. This study provides new evidence of alterations in Notch and Hh signaling pathway expression due to altered biomechanical forces *in vivo.* The aim of using an *in vivo* model of altered biomechanical forces and vascular remodeling was to further validate the cyclic strain induced decreases in Notch and Hh signaling pathway expression which we had determined *in vitro.* Partial ligation of the left carotid artery in the rat resulted in significant increases in blood flow in the right carotid artery at both 3 and 28 days post ligation. Contrast to this, blood flow in the left ligated carotid exhibited a significant decrease at both days 3 and 28 post ligation. These alterations in blood flow observed in our model proved consistent with Korshunov and Berk who used a similar model. However Korshunov and Berk, (2002) reported no significant changes in systolic blood pressure between sham-operated and ligated animals at both 3 and 28 days post ligation. Therefore one would suggest that the resulting vascular remodeling events, which occur in both right and left carotid arteries, is a compensation mechanism for altered blood blow rates in these vessels. Therefore, since vascular remodeling is associated with changes in cell fate, it is likely that the changes in Notch and Hh signaling pathway expression is playing a pivotal role in the vascular remodeling in both the left and right carotid arteries respectively. In the case of both the rat carotid model and in the PVL model, a bi-phasic pattern of Notch

signaling pathway component expression was observed when compared to sham-operated controls. In the rat-carotid model there is an initial upregulation of Notch and Hh signaling pathway component expression at 3 days post ligation with respect to sham-operated controls. This suggests that an up-regulation of the Notch signaling pathway and consequent upregulation of the Hh pathway is an acute response to alterations in blood flow and biomechanical stress. Furthermore, this upregulation of these signaling pathways is associated with the acute remodeling event, which occurs, in both the right and left carotid arteries, as a result of altered biomechanical forces. Analysis of Notch and Hh signaling pathway expression at 28 days post carotid ligation resulted in a decrease in Notch and Hh component expression as compared to sham-operated controls. Moreover expression levels of Notch and Hh were decreased in the high flow right carotid artery as compared to the low flow left carotid artery. This serves to complement our previous data reporting cyclic-strain-induced down regulation of Notch and Hh signalling is mirrored in an *in vivo* scenario of strain due to increased blood flow.

The observed bi-phasic pattern of Notch signaling pathway expression following both the carotid artery ligation and PVL model is somewhat expected as Campos *et al,* 2002 observed a bi-phasic pattern of Notch signalling pathway expression following balloon catheter injury. However in contrast to our study, Wang *et al* observed an initial downregulation of Jagged1, Notch 1 and Hrt1, 2 and 3 following balloon injury and an upregulation of both Jagged1 and Notch 3 expression 28 days post injury whilst no increase was observed in Hrt1, 2 and 3 expression levels. One possible explanation is that Wang and coworkers measured expression in medial SMC, whereas Campos measured expression of Notch in intimal SMC. A possible reason for these reported differences may be due to different SMC phenotype, which can often act differently to the same stimulus (Campadona *et al.,* 1999). In contrast however, our study describes a bi-pasic regulation of the Notch and Hh signaling pathways in the entire vessel wall. Analysis of the regulation of the Notch and Hh signaling pathways in this study offers a more representative model of a vascular disease state, as cells are continuously exposed to increased and decreased blood flow and subsequent biomechanical forces as compared to sham-operated controls.

Chapter 6

Discussion

Discussion

The research presented in this thesis has succeeded in its specific aims. We have clearly established the presence of Notch and Hedgehog signalling in adult VSMC and provided further insight into the regulation of cell growth by these pathways both *in vitro* and *in vivo.* Elucidating the role of Notch and Hedgehog and its role in VSMC growth will help contribute in the design of novel therapeutics for the treatment of vascular disease.

Vascular smooth muscle cell (VSMC) fate decisions (cell growth, migration and apoptosis) are the fundamental features in the pathogenesis of vascular disease. Firstly, this study investigated the role of Notch 1 and 3 receptor signalling in controlling adult SMC growth *in vitro* by establishing that hairy enhancer of split (*hes-1* and *-5*) and related *hrt's* (*hrt-1, -2* and -3*)* are direct downstream target genes of Notch 1 and 3 receptors in VSMC. Furthermore, we identified the essential role for nuclear protein CBF-1/RBP-Jκ in the regulation of these Notch target genes. In addition, by constitutively over expressing Notch 1 IC and Notch 3 IC we reported a significant up-regulation of CBF-1/RBP-Jκ dependent promoter activity and Notch target gene expression concomitant with significant increases in SMC growth. Moreover, by inhibiting endogenous Notch-mediated CBF-1/RBP-Jκ regulated gene expression by RPMS-1, and pharmacological inhibitors monensin and brefeldin A, we could significantly attenuate this Notch induced increase in cell growth. In addition, to Notch IC inhibition, by siRNA knockdown of Notch target genes *hrt-1, -2* and -3 we could significantly decrease cell growth while concomitantly increasing SMC apoptosis. These findings suggested in this study, proved that endogenous Notch receptors and downstream target genes, control vascular cell fate in *vitro.*

To further investigate the signalling mechanisms that govern cell fate decisions in VSMC, we then attempted to establish the role of the Hedgehog signalling pathway in these cells. Notch receptor-ligand interactions, vascular endothelial growth factor (VEGF) and components of the Hedgehog signalling pathway have been implicated in vascular morphogenesis and modelling of the embryonic vasculature. This study examined the specific role of a Hh/VEGF pathway in controlling VSMC growth *in vitro.* We reported that the Hh signalling pathway components *Shh, Ihh, Dhh,* the receptor *Ptc1, Smo* and the downstream target gene,

gli-2, were all constitutively expressed in adult rat SMC. In addition, activation of Hh signalling with recombinant SHh or by over-expressing full-length SHh resulted in a significant increase in Hh signalling concomitant with an increase in VEGF expression. Moreover this increase in Hh signalling resulted in a significant increase in cell growth, a decrease in apoptosis and activation of Notch target gene expression in SMC. Furthermore, inhibition of Hh signalling with the specific Hh inhibitor cyclopamine resulted in a significant decrease in Hh signalling concomitant with a decrease in SMC proliferation, an increase in apoptosis while concurrently decreasing Notch target gene expression. In addition we found that we could significantly attenuate this Shh induced increase in cell growth by inhibiting the Notch signalling pathway with RPMS-1. SHh-mediated stimulation of Notch target genes was also significantly attenuated following inhibition of VEGF expression using targeted siRNA, an effect that could be reversed by activation with recombinant VEGF. Collectively, this data suggested that Hh could control SMC growth via VEGF activation of Notch target gene expression *in vitro.*

As this study has clearly established the role of the Notch and Hh signalling pathways in regulating cell fate in adult SMC we then investigated the role of mechanical forces in modulating Notch and Hh mediated growth of SMC *in vitro.* Rat SMC cultured under conditions of cyclic strain exhibited a temporal- and force-dependent reduction in Notch 3 receptor expression concomitant with a significant reduction in CBF-1/RBP-Jκ-dependent Notch target gene expression as compared to unstrained controls. Furthermore we found this decrease in Notch signalling to be both Gi-protein- and MAPK-dependent. In parallel cultures, cyclic strain was found to inhibit SMC growth while significantly promoting SMC apoptosis. Notch 3 receptor over-expression significantly reversed the strain-induced changes in SMC growth while Notch inhibition served to further potentiate these changes in SMC proliferation and apoptosis. We then investigated the role of mechanical forces resulting from either cyclic strain or from pulse pressure on the Hh signalling pathways. As with the Notch signalling pathway, Hh signalling pathway components were significantly down-regulated with strain as compared to unstrained controls. Furthermore cyclic strain induced decreases in Hh signalling could be reversed by over expression of Notch 3 IC, again further proving the functional relationship between these pathways in VSMC. Finally to further validate this part of the study on the proposed effects of

mechanical forces on Notch and Hh signalling and subsequently on cell fate, we provided evidence of biomechanical regulation of these pathways *in vivo.* Using two models of biomechanical forces, the carotid ligation model and the portal vein ligation model, we provided evidence of the effect of biomechanical forces on Notch and Hh signalling and subsequently on cell growth. Notch and Hedgehog signalling pathway expression was decreased at 3 days and increased at 28 days post-carotid ligation. This was accompanied by a decrease and increase in cell growth respectively. In addition this pattern of an inverse relationship between Notch signalling pathway expression and cell growth was generally maintained following portal vein ligation. These results served to validate our *in vitro* data and in fact our *in vitro* model as a representation of an *in vivo* scenario.

This study has clearly provided evidence of the importance of the Notch and Hh signalling pathways in determining SMC fate decisions. Furthermore, it is these changes in SMC fate decisions which provide the framework for the development of a viable vascular system. Vascular development is typically divided into two stages, vasculogenesis, which involves the formation of endothelial tubes de novo from newly differentiated angioblasts and angiogenesis, which involves the remodelling of existing blood vessels to form the mature vasculature. With blood vessel formation being a key step in both normal embryonic development and in the growth of solid tumours, the signalling pathways that are active at the various stages of vasculogenesis and angiogenesis have become a very important focal point in vascular research. Therefore, determining how these signalling pathways interact to develop the vasculature will help contribute greatly towards the generation of therapies to both enhance and restrict vessel growth. Recent studies point to a role for Hh in blood vessel differentiation. Studies carried out in the mouse yolk sac (Byrd *et al.*, 2003), the zebrafish (Lawson *et al.*, 2002) and in adult vascular injury models (Pola *et al.*, 2003), all suggest that the target of Hh action is not the angioblast cell or endothelial cell, but rather an intermediate cell type that responds to Hh. These intermediate cell types may respond to Hh by expressing vascular specific growth factors such as VEGF. Recent studies by D'amore *et al.*, 2002 and Lawson *et al.*, 2002 have placed Hh in a signalling cascade that includes Notch, VEGF and the angiopoietins among others. However, studies by Kanda *et al.*, 2004 and Vokes *et al.*, 2004 have suggested that Hh may exert its effect during vasculogenesis independent

of the proposed Hh-VEGF-Notch cascade. This leads us to ask the question of how does the Hh signalling pathway interact with other signalling pathways in vascular development.

Pola *et al.*, 2001 reported that addition of recombinant SHh to interstitial mesenchymal cells can promote the expression of VEGF. In addition, expression of both Notch 1 and DII4 are upregulated by VEGF in human arterial endothelial cells (Liu *et al.*, 2003). These two studies together lead us to hypothesize the existence of a regulatory cascade for vascular remodelling which involves Hh promoting VEGF expression, which in turn promotes Notch expression and signalling. Furthermore Lawson *et al.*, 2001 carried out studies in the zebrafish, which revealed that the activation of Notch can compensate for the loss of VEGF activity. This placed VEGF downstream of SHh and upstream of the Notch signalling pathway in determining the arterial fate of the dorsal aorta in the zebrafish. Therefore Lawson's work proved that VEGF can restore normal arteriogenesis in the absence of SHh but not however, in the absence of Notch function.

These studies among others have implicated the essential role of Hh, VEGF and Notch signalling in vascular development. However, in the past decade there has been a greater appreciation of the fact that signalling pathways such as Notch and Hh which have been studied predominantly during embryogenesis and thought to be relatively silent during normal adult life, may be recruited postnatally in response to tissue injury. Pola *et al.*, 2003 demonstrated that SHh, IHh and ptc1 are postnatally recapitulated and that their upregulation during muscle regeneration post ischemia provides evidence of a potential regulatory role of Hh signalling in angiogenesis. In addition, dysregulation of the Hh signalling pathway has also being shown to play a major role in a variety of human tumours such as breast cancer, prostate cancer and gastric cancer (Katoh and Katoh *et al.*, 2005). Therefore with the knowledge that Hh signalling is recruited postnatally in adult life, this study aimed to investigate whether the Hh-VEGF-Notch cascade was recapitulated and playing a role of just of similar importance in VSMC growth in adult life. However, although the present study and the studies mentioned here can only offer a functional connection between SHh, VEGF and Notch in determining arterial fate, it should be noted that the molecular mechanisms by which Hh mediates VEGF and whereby VEGF mediates Notch in the vasculature, remains to be elucidated. Furthermore, though this functional relationship

of Hh-VEGF-Notch has been shown to be a potent modulator of VSMC growth, a direct link to the downstream signalling mechanisms and effectors of proliferation and apoptosis remains to be fully elucidated. Therefore the final aim of this study is to postulate possible mechanisms on how these signalling pathways may regulate VSMC growth, and therefore provide a possible platform for which future studies in the laboratory may be built on.

The regulation of Notch and Hh signalling is known to occur at multiple levels including patterns of ligand and receptor expression, receptor-ligand interactions, trafficking of the receptor and ligands, and covalent modifications including glycosylation, phosphorylation and ubiquitination (Weinstein *et al.,* 2002; Iso *et al.,* 2003; Baron *et al.,* 2002). One growing possibility on the mechanism on how Notch modulates VSMC growth is the post-translational modifications on Notch IC such as phosphorylation that may influence its transactivation capacity (Pereira *et al.,* 1999; Taylor *et al.,* 2002). One such factor, Glycogen Synthase Kinase-3 beta (GSK-3β) is known to modulate Notch signalling through phosphorylation of Notch IC (Taylor *et al.,* 2002). Moreover, it has been reported that inhibition of GSK-3β shortens the half-life of Notch IC while conversely activated GSK-3β reduces the quantity of Notch IC that is degraded by the proteosome (Espinosa *et al.,* 2002).

GSK-3β is a protein serine/threonine kinase, which phosphorylates and thereby inactivates glycogen synthase, a key enzyme in the synthesis of glycogen (Embi *et al.,* 1980). Since its discovery, research has provided insight into the pleiotropic role that GSK-3β plays in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility and cell survival (Frame *et al.,* 2001; Grimes *et al.,* 2001). It is a crucial factor in many cellular signalling pathways and regulates several transcription factors. GSK-3β phosphorylates multiple substrates, which include cyclin D1 (Alt *et al.,* 2000), GATA4 (Morisco *et al.,* 2001), c-jun (Nikolakaki *et al.,* 1993), c-myc (Saksela *et al.,* 1992) and p53 (Pap *et al.,* 1998) among others. In addition, GSK-3β is also at the nodal point of various signalling pathways such as the phosphoinositde-3-kinase pathway, the protein kinase B pathway and the Wnt (wingless and integrated) pathway (Delcommenne *et al.,* 1998; Shaw *et al.,* 1997; Oak *et al.,* 1996). It has been suggested that GSK-3β plays a significant role in several diseases including Alzheimers' disease (Aplin *et al.,* 1997), Diabetes Mellitus (Eldar-Finkelman *et al.,* 1999) and tumorigenesis (Kim *et al.,* 2000). Furthermore,

GSK-3β has recently being identified as a negative regulator of cardiac hypertrophy (Antos *et al.,* 2002) and as an important regulator of cardiac development (Hardt *et al.,* 2002). In addition, Hall *et al.,* 2002, demonstrated that during neointimal formation, VSMC apoptosis is inhibited by the upregulation of glucose metabolism and is linked to the inactivation of GSK-3β. Moreover, recent studies by Park *et al,* 2001 reported that constituted active GSK-3β gene transfer results in a significantly reduced proliferation and migration in human aortic SMC.

The phosphorylation of Notch proteins has been indirectly correlated with Notch activation and nuclear translocation as well as cellular transformation. Recent evidence has suggested that the Wnt pathway, which results in GSK-3β inhibition, cross-talks with the Notch signalling pathway. The Wnt family include several secreted glycoprotein's that are required for a variety of developmental events. The canonical Wnt pathway involves the interaction of Wnt with the frizzled receptor that results in inhibition of GSK-3β by an unclear mechanism involving the phosphoprotein Dishevelled. Inhibitory cross-talk between the Notch and Wnt signalling pathways has been reported to occur at the level of the Notch extracellular domain binding to Wnt (Wesley *et al.,* 1999), and the level of Notch intracellular domain binding to dishevelled (Axelrod *et al.,* 1996). However, both studies report evidence for synergistic cross-talk between both pathways, resulting in the activation of different sets of genes that require both Notch and wingless proteins. Espinosa *et al.,* 2002. recently reported a novel mechanism for Wnt and Notch pathway cross-talk involving GSK-3β phosphorylation. They demonstrated that GSK-3β is able to associate with and phosphorylate Notch 2 *in vivo.* Moreover, this phosphorylation occurs in the STR domain and more specifically in residues Thr-2068 and/or Ser-2070, Thr-2074, and Ser-2093, providing evidence of the importance of this region in regulating Notch function. Their study proved that full-length Notch 2 and Notch2 IC proteins are able to bind GSK-3β but only the processed fragment (Notch 2 IC) is phosphorylated. Taken together with the fact that over-expression of both full-length Notch 2 and Notch2 IC induces the accumulation of GSK-3β into the nucleus, this suggests that GSK-3β may be modulating active Notch. With this in mind they confirmed that GSK-3β is able to inhibit Notch mediated transcription of the Hes-1 promoter and this is further validated with their results showing Notch target gene upregulation following GSK-3β inhibition with its inhibitor Lithium Chloride (LiCl).

Furthermore Wnt-1 inhibits GSK-3β-dependent phosphorylation of Notch 2 and leads to HES-1 promoter upregulation.

Taken these results together, this indicates that GSK-3β activity plays an important role in regulating Notch-dependent gene transcription. With the knowledge that the Notch pathway is crucial for controlling many cell fate decisions, specific combinations of active/inactive GSK-3β and Notch may result in completely different outcomes. In addition, because GSK-3β is located at the nodal point where multiple signals such as Notch and Hh merge to control the proliferation and migration of VSMC, it may represent a pharmacological target to treat vascular disease. Studies by Park *et al.,* 2002, demonstrated or the first time *in vivo* that active GSK-3β gene transfer results in a significant reduction of neointima formation after balloon injury in rat carotid arteries. These effects were attributable, at least in part to the ability of GSK-3β to inhibit SMC proliferation and to promote sustained apoptosis. This study extends recent *in vitro* findings that GSK-3β plays an important role in VSMC proliferation and apoptosis in the process of vascular remodelling post balloon injury (Kim *et al.,* 2002). The exact role of GSK-3β in the proliferation and fate of VSMC is poorly understood but this novel regulation of Notch signalling by GSK-3β certainly contributes to the greater picture on how cell fate is determined in VSMC.

This study and others have provided insight into the molecular mechanisms of SMC growth following injury. For example, abberant SMC proliferation and extracellular matrix formation in the subintimal region of blood vessels that have been subjected to intimal injury, are responsible for restonosis following balloon angioplasty of the coronary arteries (Wang *et al.*, 2002). In addition, aberrant SMC proliferation is also responsible for accelerated atherosclerosis in a variety of pathophysiological states. The mechanisms responsible for SMC proliferation following injury remains incompletely understood. One early response proto-oncogene, c-myc is thought to play an important role in the regulation of cellular proliferation and differentiation. As activation of the Notch and Hh signalling pathways is coupled to the regulation of VSMC growth, it proves an interesting study as to whether both Notch and Hh may interact with the myc family.

Expression of c-myc in the normal cell is tightly regulated by external signals such as growth factors and extracellular matrix contacts as well as by internal clocks

such as the cell cycle. The resting cell normally expresses little c-myc, whereas cells stimulated by growth factors dramatically increase c-myc expression as an immediate early response gene. C-myc persists into the cell cycle but then returns to its basal quiescent state in resultant daughter cells. Abnormal or ectopic over expression of c-myc in primary cells activates a protective pathway through the induction of p19/p14ARF and a p53-dependent cell death pathway. Hence, normal cells that over-express c-myc are eliminated from the host organism through apoptosis, thereby protecting the organism from lethal neoplastic changes. Normal embryonic development requires regulate expression of c-myc as well as other myc family members. For example, mouse embryos in which both alleles of c-myc have been deleted by homologous recombination, die in early development due to a lack of primitive haematopoiesis. Several lines of evidence point to the interaction of the Notch signalling pathway and c-myc, which suggest a functional link in the regulation of cell growth. Similar to the Notch signalling pathway, c-myc is highly conserved from Drosophilia to humans and targeted mutations in murine c-myc gene results in widespread embryonic lethality suggesting its critical role in development (Davis *et al.,* 1993). In addition. myc proteins are helix-loop-helix (HLH) proteins (class B) as they contain a HLH motif at their c-terminus which mediates protein dimerization with other proteins containing the same motif. Notch target genes are also part of the HLH family of genes (class C), and interaction between myc and Hes proteins has been documented (Swiss institute of bioinformatics). Hrt's are known to bind to a number of E-box motifs on their target genes (Nakagawa 2000). This study therefore determined using bioinformatics and the cloned c-myc promoter sequence (Ray and Miller 1991), whether any of the Hrt binding sequences were present on the c-myc promoter. We reported that 3 Hrt E-boxes were found to be present on the c-myc promoter region, these being CAACTG, CACCTG and CACTTG. This further added to the possibility of the direct interaction between the Notch signalling pathway and c-myc.

In addition, this study among others has shown signalling by Hh family members to regulate proliferation and apoptosis in a variety of cell types. However, specific links between Hh, which has not yet been reported to activate a receptor tyrosine kinase like many other growth factors and the cell cycle, are still being elucidated. Studies by Sjostrom *et al*, has shown N-myc to be a direct target of SHh in

proliferating cerebellar granule neuron precursors. Their study reported that Hh signalling induces N-myc expression and that N-myc protein is stabilized by insulin-like growth factor-mediated suppression of GSK-3β. Therefore, it would certainly now prove an interesting study as to whether this sequence of events is mirrored in vascular cells. With this in mind, one would be tempted to speculate a pro-proliferative pathway involving Notch-Hh upregulation of myc family members where GSK-3β suppression is required. In further validation of the importance of c-myc in VSMC growth, studies by Chen and co-workers reported that inhibition of c-myc prevents proliferation of VSMC post balloon injury. Balloon injury to the arterial wall triggers the synthesis of and the release of c-myc which in turn promotes proliferation and migration through receptor specific interactions of which may include the Notch signalling pathway. Furthermore, this study by Chen suggests that c-myc participates in the proliferative response of the vascular wall to arterial injury. In addition, that the resultant over-expression of c-myc in the damaged artery if inhibited may be therapeutically useful in preventing the proliferative lesions that occur after coronary angioplasty. However, it also important to determine the possible downstream effectors of apoptosis in addition to proliferation and how these effectors are possibly coupled to the Notch and Hh signalling pathways, as a controlled balance of both cell fates' is of critical importance to vascular remodelling.

The cellular response to vascular injury involves a complex interplay between resident cells of the vessel wall such as EC, SMC and adventitial fibroblasts and circulating elements such as inflammatory cells that are recruited to participate in the healing response. Under conditions of injury VSMC switch from a contractile phenotype with slow replication into migratory cells with a high proliferation rate. Therefore it is increased total cellularity, which is a common feature of the injury response, thus focussing attention on the balance between cell proliferation and cell death. Cell growth, seen as this balance between cell proliferation and cell death is a critical feature of vessel-wall remodelling at all stages from vascular development and injury to chronic vascular lesions (Walsh *et al.,* 2000). It is now well documented that VEGF is a major regulator of both physiological and pathological neo-vascularization, a role which is conserved from development. This study has clearly shown that VEGF modulates the regulation of VSMC fate decisions through a

functional signalling cascade involving the Notch and Hh signalling pathways. Several recent reports have also implicated VEGF as a major survival factor for EC during angiogenesis and vasculogenesis along with other growth factors such as bFGF and angiopoietin-1. The activation of VEGF receptors including KDR/flk-1 induces a number of phenotypic responses which contribute to the angiogenic phenotype (Neufeld et al., 1999). Recent results have implicated VEGF as a major (anti-apoptotic) factor for newly formed EC found in immature, newly formed blood vessels (Benjamin *et al.,* 1999). These findings, including the findings of this study where Hh induced up-regulation of VEGF decreases serum-deprived induced apoptosis, asks the obvious question of how VEGF mediates its pro-survival/anti-apoptotic function. There have been several possibilities put forward, all of which are not necessarily exclusive. Gerber *et al*, and Carmeliet *et al,* reported evidence that VEGF promotes its pro-survival/anti-apoptotic function through the phosphotidyl inisotol 3 kinase (PI3K)/AKT signal transduction pathway. In addition Nor *et al,* reported that VEGF can induce high levels of anti-apoptotic *bcl-2*. This results is particularly interesting as this study has conclusively shown that over-expression of Notch and Hh signalling pathway components has shown to have a direct effect on *bcl-2* family members by both increasing anti-apoptotic *bcl-*X_L while concurrently decreasing pro-apoptotic *bax*. Furthermore, Gupta *et al*, reported evidence that VEGF suppresses apoptosis in human micro and macro vascular cells via induction and suppression of the MAPK(p42)/ERK(p44) and SAPK/JNK signalling pathways respectively. Moreover the VEGF- mediated EC survival and angiogenesis may be dependent on the adhesion molecule VE (vascular endothelial) Cadherin through both AKT kinase and bcl-2 (Carmeliet *et al.,* 1999). However, in addition to the bcl-2 family of apoptosis regulators a new different family of modulators of apoptosis has been recently discovered.

The inhibitors of apoptosis proteins (IAP) are defined by the presence of at least one "BIR" domain (Baculovirus inhibitor of apoptosis repeat), and the ability to inhibit apoptosis. Humans have eight IAP family members, NAIP, cIAP1, cIAP2, XIAP, Is-XIAP, ML-IAP, apollon and survivin. This family of anti-apoptotic proteins bind and inhibit caspase 3,7 and or 9 but not caspase 8. Whereas anti-apoptotic members of the bcl-2 family such as bcl-2 itself or bcl-X_L block upstream caspases, the IAPs and survivin appear to inhibit apoptosis by blocking the terminal effector

caspases such as 3, 7 as well as procaspase 9. Tran *et al*, reported that VEGF could inhibit apoptosis and promote survival of EC by upregulating survivin. Another interesting aspect of Trans study was the cell cycle-dependency of VEGF induced upregulation of survivin expression. Survivin, also known as Birc5, was first identified as a member of the IAP gene family by Ambrosini *et al,* and subsequently has been demonstrated to have a unique dual role in the regulation of cell proliferation and cell death. Survivin is involved in the stabilization of microtubules during mitosis at the G2/M phase (Fortugno *et al.*, 2002) and is thought to block apoptosis by direct interference with caspase 9 activation. Survivin is largely absent in normal adult tissues but is widely expressed in embryonic tissue. However, it is expressed in virtually all forms of human cancers to date and its expression has been linked to poor prognosis (Kawasaki 1998). Survivin is upregulated at the mitotic phase of the cell cycle and its activity requires phosphorylation by the mitotic kinase $p34^{cdc2}$ cyclin B1 at a unique site. From this unique function at the interface of cell proliferation and cell death, a potential role for surviving in the regulation of vascular injury has recently being postulated. Survivin up-regulation by VEGF resulting in EC survival may be a critical factor to angiogenesis. Furthermore recent studies by Blanc-Brude *et al,* has demonstrated that survivin mediates the anti-apoptotic effects of platelet derived growth factor (PDGF) in VSMC and identified survivin expression in experimental neointima formation. Moreover, survivin up-regulation has been identified in diverse forms of vascular injuries across species, including early atherosclerosis, angioplasty-induced neointima and vein-graft adaptation. Taken together, these reports all suggest a role of fundamental importance for survivin to the cellular events that determine neointima formation and vascular remodelling. With this growing evidence that VEGF up-regulates survivin, which in turn mediates cell division and cell cycle progression, a greater knowledge of VEGF-regulation of survivin and the possible role of Notch and Hh on its expression, may translate into clinically useful applications. On going investigations should seek to better identify the nature of survivin expressing cells, their contribution to vascular lesion development and the role of the Hh-VEGF-Notch signalling cascade, by which survivin may influence a SMC phenotype.

In conclusion, this study has established the presence and functional relationship between the Notch and Hh signalling pathways in the regulation of VSMC growth. However, while the regulation of both these pathways remains undefined, a number of possible molecules have been proposed which may regulate these pathways or in fact be regulated by these pathways in promoting cell growth in VSMC. As it is aberrant cell growth, which is a fundamental cause of vascular disease, a greater understanding of the signalling pathways that govern cell growth will hopefully provide greater insight into the molecular basis of this disease, which continues to be a major cause of death each year. It is a hope that this study among others can help provide a platform for which future cardiovascular research can be built on with a view to providing therapeutic solutions to an ever-growing problem.

Chapter 7

Bibliography

Alton, A. K., Fechtel, K., et al. (1989). "Molecular genetics of Delta, a locus required for ectodermal differentiation in Drosophila." Dev Genet **10** (3): 261-72.

Apelqvist, A., Li, H., et al. (1999). "Notch signalling controls pancreatic cell differentiation." Nature **400** (6747): 877-81.

Aplin, A. E., Howe, A., et al. (1998). "Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins." Pharmacol Rev **50** (2): 197-263.

Appel, B. and Eisen, J. S. (1998). "Regulation of neuronal specification in the zebrafish spinal cord by Delta function." Development **125** (3): 371-80.

Artavanis-Tsakonas, S., Delidakis, C., et al. (1991). "The Notch locus and the cell biology of neuroblast segregation." Annu Rev Cell Biol **7**: 427-52.

Artavanis-Tsakonas, S., Matsuno, K., et al. (1995). "Notch signaling." Science **268** (5208): 225-32.

Artavanis-Tsakonas, S., Rand, M. D., et al. (1999). "Notch signaling: cell fate control and signal integration in development." Science **284** (5415): 770-6.

Artavanis-Tsakonas, S. and Simpson, P. (1991). "Choosing a cell fate: a view from the Notch locus." Trends Genet **7** (11-12): 403-8.

Ashkenazi, A. and Dixit, V. M. (1998). "Death receptors: signaling and modulation." Science **281** (5381): 1305-8.

Aster, J., Pear, W., et al. (1994). "Functional analysis of the TAN-1 gene, a human homolog of Drosophila notch." Cold Spring Harb Symp Quant Biol **59**: 125-36.

Axelrod, J. D., Matsuno, K., et al. (1996). "Interaction between Wingless and Notch signaling pathways mediated by dishevelled." Science **271** (5257): 1826-32.

Bae, S., Bessho, Y., et al. (2000). "The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation." Development **127** 13): 2933-43.

Bai, H., Pollman, M. J., et al. (1999). "Regulation of vascular smooth muscle cell apoptosis. Modulation of bad by a phosphatidylinositol 3-kinase-dependent pathway." Circ Res **85** (3): 229-37.

Bailey, A. M. and Posakony, J. W. (1995). "Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity." Genes Dev **9** (21): 2609-22.

Baldwin, A. S., Jr. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annu Rev Immunol **14**: 649-83.

Banes, A. J., Tsuzaki, M., et al. (1995). "PDGF-BB, IGF-I and mechanical load stimulate DNA synthesis in avian tendon fibroblasts in vitro." J Biomech **28** (12): 1505-13.

Barolo, S., Stone, T., et al. (2002). "Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless." Genes Dev **16** (15): 1964-76.

Baron, M. (2003). "An overview of the Notch signalling pathway." Semin Cell Dev Biol **14** (2): 113-9.

Baron, M., Aslam, H., et al. (2002). "Multiple levels of Notch signal regulation (review)." Mol Membr Biol **19** (1): 27-38.

Beatus, P. and Lendahl, U. (1998). "Notch and neurogenesis." J Neurosci Res **54** (2): 125-36.

Beatus, P., Lundkvist, J., et al. (1999). "The notch 3 intracellular domain represses notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters." Development **126** (17): 3925-35.

Benedict, M. A., Hu, Y., et al. (2000). "Expression and functional analysis of Apaf-1 isoforms. Extra Wd-40 repeat is required for cytochrome c binding and regulated activation of procaspase-9." J Biol Chem **275** (12): 8461-8.

Bennett, M. R. (1999). "Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture." Cardiovasc Res **41** (2): 361-8.

Bennett, M. R., Evan, G. I., et al. (1995). "Apoptosis of rat vascular smooth muscle cells is regulated by p53-dependent and -independent pathways." Circ Res **77** (2): 266-73.

Bennett, M. R., Evan, G. I., et al. (1995). "Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques." J Clin Invest **95** (5): 2266-74.

Berechid, B. E., Kitzmann, M., et al. (2002). "Identification and characterization of presenilin-independent Notch signaling." J Biol Chem **277** (10): 8154-65.

Berliner, J., Leitinger, N., et al. (1997). "Oxidized lipids in atherogenesis: formation, destruction and action." Thromb Haemost **78** (1): 195-9.

Best, P. J., Hasdai, D., et al. (1999). "Apoptosis. Basic concepts and implications in coronary artery disease." Arterioscler Thromb Vasc Biol **19** (1): 14-22.

Bhagyalakshmi, A. and Frangos, J. A. (1989). "Mechanism of shear-induced prostacyclin production in endothelial cells." Biochem Biophys Res Commun **158** (1): 31-7.

Bigas, A., Martin, D. I., et al. (1998). "Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines." Mol Cell Biol **18** (4): 2324-33.

Bijlsma, M. F., Spek, C. A., et al. (2004). "Hedgehog: an unusual signal transducer." Bioessays **26** (4): 387-94.

Birukov, K. G., Bardy, N., et al. (1998). "Intraluminal pressure is essential for the maintenance of smooth muscle caldesmon and filamin content in aortic organ culture." Arterioscler Thromb Vasc Biol **18** (6): 922-7.

Blaumueller, C. M., Qi, H., et al. (1997). "Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane." Cell **90** (2): 281-91.

Blobel, C. P. (1997). "Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch." Cell **90** (4): 589-92.

Blokzijl, A., Dahlqvist, C., et al. (2003). "Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3." J Cell Biol **163** (4): 723-8.

Bochaton-Piallat, M. L., Gabbiani, F., et al. (1995). "Apoptosis participates in cellularity regulation during rat aortic intimal thickening." Am J Pathol **146** (5): 1059-64.

Bochaton-Piallat, M. L., Ropraz, P., et al. (1996). "Phenotypic heterogeneity of rat arterial smooth muscle cell clones. Implications for the development of experimental intimal thickening." Arterioscler Thromb Vasc Biol **16** (6): 815-20.

Boers, G. H. (2000). "Mild hyperhomocysteinemia is an independent risk factor of arterial vascular disease." Semin Thromb Hemost **26** (3): 291-5.

Borzillo, GV., Lippa, B(2005). "The Hedgehog signaling pathway as a target for anticancer drug discovery". Curr Top Med Chem **5**:147-5.

Bowling, N., Walsh, R. A., et al. (1999). "Increased protein kinase C activity and expression of Ca2+-sensitive isoforms in the failing human heart." Circulation **99** (3): 384-91.

Bray, S. (1998). "A Notch affair." Cell **93** (4): 499-503.

Brooks, AR., Lelkes, PI,, Rubanyi, GM., (2004). "Gene expression profiling of vascular endothelial cells exposed to fluid mechanical forces: relevance for focal susceptibility to atherosclerosis." Cell **11**:45-57.

Brou, C., Logeat, F., et al. (2000). "A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE." Mol Cell **5** (2): 207-16.

Bruce, L. and Nixon, G. F. (1997). "Increased sensitization of the myofilaments in rat neonatal portal vein: a potential mechanism." Exp Physiol **82** (6): 985-93.

Brulin, P., Godfraind, C., et al. (2002). "Morphometric analysis of ultrastructural vascular changes in CADASIL: analysis of 50 skin biopsy specimens and pathogenic implications." Acta Neuropathol (Berl) **104** (3): 241-8.

Byrd, N., Becker, S., Maye, P., Narasimhaiah, R., St-Jacques, B., Zhang, X., (2002). "Hedgehog is required for murine yolk sac angiogenesis". Development **129**:361-72.

Byrd, N., Grabel, L., (2004). "Hedgehog signaling in murine vasculogenesis and Angiogenesis". Trends Cardiovasc Med **14**:308-13.

Bryant, S. R., Bjercke, R. J., et al. (1999). "Vascular remodeling in response to altered blood flow is mediated by fibroblast growth factor-2." Circ Res **84** (3): 323-8.

Bush, G., diSibio, G., et al. (2001). "Ligand-induced signaling in the absence of furin processing of Notch1." Dev Biol **229** (2): 494-502.

Busseau, I., Diederich, R. J., et al. (1994). "A member of the Notch group of interacting loci, deltex encodes a cytoplasmic basic protein." Genetics **136** (2): 585-96.

Cai, J., Yang, J., et al. (1998). "Mitochondrial control of apoptosis: the role of cytochrome c." Biochim Biophys Acta **1366** (1-2): 139-49.

Campos, A. H., Wang, W., et al. (2002). "Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation." Circ Res **91** (11): 999-1006.

Cano, E. and Mahadevan, L. C. (1995). "Parallel signal processing among mammalian MAPKs." Trends Biochem Sci **20** (3): 117-22.

Cappadona, C., Redmond, E. M., et al. (1999). "Phenotype dictates the growth response of vascular smooth muscle cells to pulse pressure in vitro." Exp Cell Res **250** (1): 174-86.

Carlesso, N., Aster, J. C., et al. (1999). "Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics." Blood **93** (3): 838-48.

Castella, P., Sawai, S., et al. (2000). "HES-1 repression of differentiation and proliferation in PC12 cells: role for the helix 3-helix 4 domain in transcription repression." Mol Cell Biol **20** (16): 6170-83.

Cattaruzza, M., Berger, M. M., et al. (2002). "Deformation-induced endothelin B receptor-mediated smooth muscle cell apoptosis is matrix-dependent." Cell Death Differ **9** (2): 219-26.

Chan, S. W., Hegyi, L., et al. (2000). "Sensitivity to Fas-mediated apoptosis is determined below receptor level in human vascular smooth muscle cells." Circ Res **86** (10): 1038-46

Chen, G., Fernandez, J., et al. (1999). "A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development." Genes Dev **13** (17): 2218-30.

Chen, H., Thiagalingam, A., et al. (1997). "Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly

represses achaete-scute homolog-1 expression." Proc Natl Acad Sci U S A **94** (10): 5355-60.

Chen, J. D. and Evans, R. M. (1995). "A transcriptional co-repressor that interacts with nuclear hormone receptors." Nature **377** (6548): 454-7.

Chen, W., Burgess, S., Hopkins, N., (2001)." Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity". Development **128**:2385-2389

Cheng, G. C., Briggs, W. H., et al. (1997). "Mechanical strain tightly controls fibroblast growth factor-2 release from cultured human vascular smooth muscle cells." Circ Res **80** (1): 28-36.

Chin, M. T., Maemura, K., et al. (2000). "Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system." J Biol Chem **275** (9): 6381-7.

Chitnis, A., Henrique, D., et al. (1995). "Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta." Nature **375** (6534): 761-6.

Chomczynski, P. and Sacchi, N. (1987). "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." Anal Biochem **162** (1): 156-9.

Cludts, I. and Farrell, P. J. (1998). "Multiple functions within the Epstein-Barr virus EBNA-3A protein." J Virol **72** (3): 1862-9.

Colnot, C., de la Fuente, L., Huang S, (2005). "Indian hedgehog synchronizes skeletal angiogenesis and perichondrial maturation with cartilage development". Development. : 132:1057-67

Conlon, R. A., Reaume, A. G., et al. (1995). "Notch1 is required for the coordinate segmentation of somites." Development **121** (5): 1533-45.

Cook, S. A., Sugden, P. H., et al. (1999). "Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential." Circ Res **85** (10): 940-9.

Cornell, M., Evans, D. A., et al. (1999). "The Drosophila melanogaster Suppressor of deltex gene, a regulator of the Notch receptor signaling pathway, is an E3 class ubiquitin ligase." Genetics **152** (2): 567-76.

Corti, R., Farkouh, M. E., et al. (2002). "The vulnerable plaque and acute coronary syndromes." Am J Med **113** (8): 668-80.

D'Sa-Eipper, C. and Chinnadurai, G. (1998). "Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities." Oncogene **16** (24): 3105-14.

D'Souza, B., Rowe, M., et al. (2000). "The bfl-1 gene is transcriptionally upregulated by the Epstein-Barr virus LMP1, and its expression promotes the survival of a Burkitt's lymphoma cell line." J Virol **74** (14): 6652-8.

D'Souza, B. N., Edelstein, L. C., et al. (2004). "Nuclear factor kappa B-dependent activation of the antiapoptotic bfl-1 gene by the Epstein-Barr virus latent membrane protein 1 and activated CD40 receptor." J Virol **78** (4): 1800-16.

Dang, C. V. (1999). "c-Myc target genes involved in cell growth, apoptosis, and metabolism." Mol Cell Biol **19** (1): 1-11.

Davies, P. F. (1995). "Flow-mediated endothelial mechanotransduction." Physiol Rev **75** (3): 519-60.

Davis, A. C., Wims, M., et al. (1993). "A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice." Genes Dev **7** (4): 671-82.

Dawson, S. R., Turner, D. L., et al. (1995). "Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression." Mol Cell Biol **15** (12): 6923-31.

de Celis, J. F., Barrio, R., et al. (1993). "Genetic and molecular characterization of a Notch mutation in its Delta- and Serrate-binding domain in Drosophila." Proc Natl Acad Sci U S A **90** (9): 4037-41.

de la Pompa, J. L., Wakeham, A., et al. (1997). "Conservation of the Notch signalling pathway in mammalian neurogenesis." Development **124** (6): 1139-48.

De Martin, R., Hoeth, M., et al. (2000). "The transcription factor NF-kappa B and the regulation of vascular cell function." Arterioscler Thromb Vasc Biol **20** (11): E83-8.

De Strooper, B., Saftig, P., et al. (1998). "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein." Nature **391** (6665): 387-90.

DeBakey, M. E., Lawrie, G. M., et al. (1985). "Patterns of atherosclerosis and their surgical significance." Ann Surg **201** (2): 115-31.

Debbas, M. and White, E. (1993). "Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B." Genes Dev **7** (4): 546-54.

Deftos, M. L. and Bevan, M. J. (2000). "Notch signaling in T cell development." Curr Opin Immunol **12** (2): 166-72.

Deftos, M. L., He, Y. W., et al. (1998). "Correlating notch signaling with thymocyte maturation." Immunity **9** (6): 777-86.

Desagher, S. and Martinou, J. C. (2000). "Mitochondria as the central control point of apoptosis." Trends Cell Biol **10** (9): 369-77.

Di Somma, M. M., Somma, F., et al. (1999). "TCR engagement regulates differential responsiveness of human memory T cells to Fas (CD95)-mediated apoptosis." J Immunol **162** (7): 3851-8.

Dou, S., Zeng, X., et al. (1994). "The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor." Mol Cell Biol **14** (5): 3310-9.

Dunwoodie, S. L., Henrique, D., et al. (1997). "Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo." Development **124** (16): 3065-76.

Ellis, R. E., Yuan, J. Y., et al. (1991). "Mechanisms and functions of cell death." Annu Rev Cell Biol **7**: 663-98.

Ellisen, L. W., Bird, J., et al. (1991). "TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms." Cell **66** (4): 649-61.

Endo, Y., Osumi, N., et al. (2002). "Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development." Development **129** (4): 863-73.

Espinosa, L., Ingles-Esteve, J., Aguilera, C., Bigas A (2003) " Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways". J Biol Chem **278**:32227-32235

Eskes, R., Antonsson, B., et al. (1998). "Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions." J Cell Biol **143** (1): 217-24.

Evan, G. and Littlewood, T. (1998). "A matter of life and cell death." Science **281** (5381): 1317-22.

Falk, E., Shah, P. K., et al. (1995). "Coronary plaque disruption." Circulation **92** (3): 657-71.

Fearnhead, H. O., Rodriguez, J., et al. (1998). "Oncogene-dependent apoptosis is mediated by caspase-9." Proc Natl Acad Sci U S A **95** (23): 13664-9.

Fehon, R. G., Johansen, K., et al. (1991). "Complex cellular and subcellular regulation of notch expression during embryonic and imaginal development of Drosophila: implications for notch function." J Cell Biol **113** (3): 657-69.

Fehon, R. G., Kooh, P. J., et al. (1990). "Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila." Cell **61** (3): 523-34.

Feng, Y., Yang, J. H., et al. (1999). "Transcriptional profile of mechanically induced genes in human vascular smooth muscle cells." Circ Res **85** (12): 1118-23.

Ferguson, JE., Patterson, C., (2003). "Break the cycle: the role of cell- cycle modulation in the prevention of vasculoproliferative diseases". Cell Cycle **211**-219

Ferrara, N. and Davis-Smyth, T. (1997). "The biology of vascular endothelial growth factor." Endocr Rev **18** (1): 4-25.

Ferri, K. F. and Kroemer, G. (2001). "Organelle-specific initiation of cell death pathways." Nat Cell Biol **3** (11): E255-63.

Fleming, R. J. (1998). "Structural conservation of Notch receptors and ligands." Semin Cell Dev Biol **9** (6): 599-607.

Fleming, R. J., Gu, Y., et al. (1997). "Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc." Development **124** (15): 2973-81.

Folkow, B. (1995). "Hypertensive structural changes in systemic precapillary resistance vessels: how important are they for in vivo haemodynamics?" J Hypertens **13** (12 Pt 2): 1546-59.

Foltz, DR., Santiago, MC., Berechid , BE., Nye, JS.(2002). "Glycogen synthase kinase-3 beta modulates notch signaling and stability". Curr Biol **12**:1006-1011

Fortini, M. E., Rebay, I., et al. (1993). "An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye." Nature **365** (6446): 555-7.

Fortuno, M. A., Ravassa, S., et al. (1998). "Overexpression of Bax protein and enhanced apoptosis in the left ventricle of spontaneously hypertensive rats: effects of AT1 blockade with losartan." Hypertension **32** (2): 280-6.

Fostier, M., Evans, D. A., et al. (1998). "Genetic characterization of the Drosophila melanogaster Suppressor of deltex gene: A regulator of notch signaling." Genetics **150** (4): 1477-85.

Frise, E., Knoblich, J. A., et al. (1996). "The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage." Proc Natl Acad Sci U S A **93** (21): 11925-32.

Fryer, C. J., Lamar, E., et al. (2002). "Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex." Genes Dev **16** (11): 1397-411.

Galis, Z. S., Muszynski, M., et al. (1994). "Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion." Circ Res **75** (1): 181-9.

Gallahan, D., Jhappan, C., et al. (1996). "Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis." Cancer Res **56** (8): 1775-85.

Geng, Y. J. and Libby, P. (2002). "Progression of atheroma: a struggle between death and procreation." Arterioscler Thromb Vasc Biol **22**(9): 1370-80.

Geng, Y. J., Wu, Q., et al. (1996). "Apoptosis of vascular smooth muscle cells induced by in vitro stimulation with interferon-gamma, tumor necrosis factor-alpha, and interleukin-1 beta." Arterioscler Thromb Vasc Biol **16** (1): 19-27.

Gerber, H. P., Dixit, V., et al. (1998). "Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells." J Biol Chem **273** (21): 13313-6.

Gessler, M., Knobeloch, K. P., et al. (2002). "Mouse gridlock: no aortic coarctation or deficiency, but fatal cardiac defects in Hey2 -/- mice." Curr Biol **12** (18): 1601-4.

Ghosh, S., May, M. J., et al. (1998). "NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses." Annu Rev Immunol **16**: 225-60.

Gibbons, G. H. and Pollman, M. J. (2000). "Death receptors, intimal disease, and gene therapy: are therapies that modify cell fate moving too Fas?" Circ Res **86** (10): 1009-12.

Giniger, E. (1998). "A role for Abl in Notch signaling." Neuron **20** (4): 667-81.

Gittenberger-de Groot, A. C., DeRuiter, M. C., et al. (1999). "Smooth muscle cell origin and its relation to heterogeneity in development and disease." Arterioscler Thromb Vasc Biol **19** (7): 1589-94.

Glagov, S., Zarins, C., et al. (1988). "Hemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries." Arch Pathol Lab Med **112** (10): 1018-31.

Gonzales, D. H. and Neupert, W. (1990). "Biogenesis of mitochondrial c-type cytochromes." J Bioenerg Biomembr **22** (6): 753-68.

Goodbourn, S. (1995). "Signal transduction. Notch takes a short cut." Nature **377** (6547): 288-9.

Goping, I. S., Gross, A., et al. (1998). "Regulated targeting of BAX to mitochondria." J Cell Biol **143** (1): 207-15.

Gray, G. E., Mann, R. S., et al. (1999). "Human ligands of the Notch receptor." Am J Pathol **154** (3): 785-94.

Green, D. R. and Reed, J. C. (1998). "Mitochondria and apoptosis." Science **281** (5381): 1309-12.

Greenwald, I. (1998). "LIN-12/Notch signaling: lessons from worms and flies." Genes Dev **12** (12): 1751-62.

Greenwald, I. and Seydoux, G. (1990). "Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans." Nature **346**(6280): 197-9.

Gridley, T. (1997). "Notch signaling in vertebrate development and disease." Mol Cell Neurosci **9** (2): 103-8.

Gridley, T. (2001). "Notch signaling during vascular development." Proc Natl Acad Sci U S A **98** (10): 5377-8.

Gridley, T. (2003). "Notch signaling and inherited disease syndromes." Hum Mol Genet **12** Spec No 1: R9-13.

Grillot, D. A., Gonzalez-Garcia, M., et al. (1997). "Genomic organization, promoter region analysis, and chromosome localization of the mouse bcl-x gene." J Immunol **158** (10): 4750-7.

Guan, E., Wang, J., et al. (1996). "T cell leukemia-associated human Notch/translocation-associated Notch homologue has I kappa B-like activity and physically interacts with nuclear factor-kappa B proteins in T cells." J Exp Med **183** (5): 2025-32.

Gudi, S., Nolan, J. P., et al. (1998). "Modulation of GTPase activity of G proteins by fluid shear stress and phospholipid composition." Proc Natl Acad Sci U S A **95** (5): 2515-9.

Gudi, S. R., Lee, A. A., et al. (1998). "Equibiaxial strain and strain rate stimulate early activation of G proteins in cardiac fibroblasts." Am J Physiol **274** (5 Pt 1): C1424-8.

Gudi, T., Lohmann, S. M., et al. (1997). "Regulation of gene expression by cyclic GMP-dependent protein kinase requires nuclear translocation of the kinase: identification of a nuclear localization signal." Mol Cell Biol **17** (9): 5244-54.

Guenther, M. G., Barak, O., et al. (2001). "The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3." Mol Cell Biol **21** (18): 6091-101.

Gupta, S. (2003). "Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review)." Int J Oncol **22** (1): 15-20.

Gutkind, J. S. (1998). "The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades." J Biol Chem **273** (4): 1839-42.

Haddon, C., Smithers, L., et al. (1998). "Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis." Development **125** (3): 359-70.

Hammerschmidt, W. and Sugden, B. (1989). "Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes." Nature **340** (6232): 393-7.

Han, D. K., Haudenschild, C. C., et al. (1995). "Evidence for apoptosis in human atherogenesis and in a rat vascular injury model." Am J Pathol **147** (2): 267-77.

Han, W., Ye, Q., et al. (2000). "A soluble form of human Delta-like-1 inhibits differentiation of hematopoietic progenitor cells." Blood **95** (5): 1616-25.

Hanahan, D. (1983). "Studies on transformation of Escherichia coli with plasmids." J Mol Biol **166** (4): 557-80.

Hardy, J. and Israel, A. (1999). "Alzheimer's disease. In search of gamma-secretase." Nature **398** (6727): 466-7.

Harper, JA., Yuan, JS., Tan, JB, (2003). "Notch signaling in development and disease" . Clin Genet **64**:461-472.

Harry, LE., Paleolog, EM., (2003). "From the cradle to the clinic: VEGF in developmental, physiological, and pathological angiogenesis". Birth Defects Res C Embryo Today **69**:363-367

Hartley, D. A., Xu, T. A., et al. (1987). "The embryonic expression of the Notch locus of Drosophila melanogaster and the implications of point mutations in the extracellular EGF-like domain of the predicted protein." Embo J **6**(11): 3407-17.

Henderson, A. M., Wang, S. J., et al. (2001). "The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation." J Biol Chem **276** (9): 6169-76.

Henderson, S. T., Gao, D., et al. (1997). "Functional domains of LAG-2, a putative signaling ligand for LIN-12 and GLP-1 receptors in Caenorhabditis elegans." Mol Biol Cell **8** (9): 1751-62.

Henderson, S. T., Gao, D., et al. (1994). "lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of C. elegans." Development **120** (10): 2913-24.

Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature **407** (6805): 770-6.

Hetts, S. W. (1998). "To die or not to die: an overview of apoptosis and its role in disease." Jama **279** (4): 300-7.

Higashita, R., Li, L., et al. (1997). "Galpha16 mimics vasoconstrictor action to induce smooth muscle alpha-actin in vascular smooth muscle cells through a Jun-NH2-terminal kinase-dependent pathway." J Biol Chem **272** (41): 25845-50.

Hirata, H., Ohtsuka, T., et al. (2000). "Generation of structurally and functionally distinct factors from the basic helix-loop-helix gene Hes3 by alternative first exons." J Biol Chem **275** (25): 19083-9.

Ho, K. S. and Scott, M. P. (2002). "Sonic hedgehog in the nervous system: functions, modifications and mechanisms." Curr Opin Neurobiol **12** (1): 57-63.

Hofelmayr, H., Strobl, L. J., et al. (2001). "Activated Notch1 can transiently substitute for EBNA2 in the maintenance of proliferation of LMP1-expressing immortalized B cells." J Virol **75** (5): 2033-40.

Hofelmayr, H., Strobl, L. J., et al. (1999). "Activated mouse Notch1 transactivates Epstein-Barr virus nuclear antigen 2-regulated viral promoters." J Virol **73** (4): 2770-80.

Hooper, JE., Scott., MP, (2005). "Communicating with Hedgehogs". Nat Rev Mol CellBiol. 2005 **6**:306-17.

Horlein, A. J., Naar, A. M., et al. (1995). "Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor." Nature **377** (6548): 397-404.

Hou, M. C., Cahill, P. A., et al. (1998). "Enhanced cyclooxygenase-1 expression within the superior mesenteric artery of portal hypertensive rats: role in the hyperdynamic circulation." Hepatology **27** (1): 20-7.

Hsieh, J. J. and Hayward, S. D. (1995). "Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2." Science **268** (5210): 560-3.

Hsieh, J. J., Henkel, T., et al. (1996). "Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2." Mol Cell Biol **16** (3): 952-9.

Hsieh, J. J., Zhou, S., et al. (1999). "CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex." Proc Natl Acad Sci U S A **96** (1): 23-8.

Huang, D. C., Adams, J. M., et al. (1998). "The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4." Embo J **17** (4): 1029-39.

Hunter, J. J., Bond, B. L., et al. (1996). "Functional dissection of the human Bc12 protein: sequence requirements for inhibition of apoptosis." Mol Cell Biol **16** (3): 877-83.

Igata, E., Inoue, T., et al. (1999). "Molecular cloning and functional analysis of the murine bax gene promoter." Gene **238** (2): 407-15.

Ihling, C., Haendeler, J., et al. (1998). "Co-expression of p53 and MDM2 in human atherosclerosis: implications for the regulation of cellularity of atherosclerotic lesions." J Pathol **185** (3): 303-12.

Irmler, M., Thome, M., et al. (1997). "Inhibition of death receptor signals by cellular FLIP." Nature **388** (6638): 190-5.

Ishibashi, M., Moriyoshi, K., et al. (1994). "Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system." Embo J **13** (8): 1799-805.

Iso, T., Chung, G., et al. (2002). "HERP1 is a cell type-specific primary target of Notch." J Biol Chem **277** (8): 6598-607.

Iso, T., Hamamori, Y., et al. (2003). "Notch signaling in vascular development." Arterioscler Thromb Vasc Biol **23** (4): 543-53.

Iso, T., Kedes, L., et al. (2003). "HES and HERP families: multiple effectors of the Notch signaling pathway." J Cell Physiol **194** (3): 237-55.

Iso, T., Sartorelli, V., et al. (2001). "HERP, a new primary target of Notch regulated by ligand binding." Mol Cell Biol **21** (17): 6071-9.

Iso, T., Sartorelli, V., et al. (2001). "HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling." Mol Cell Biol **21** (17): 6080-9.

James, T. N. (1994). "Normal and abnormal consequences of apoptosis in the human heart. From postnatal morphogenesis to paroxysmal arrhythmias." Circulation **90** (1): 556-73.

Jang, M. S., Miao, H., et al. (2004). "Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways." J Cell Physiol **199** (3): 418-33.

Jarriault, S., Le Bail, O., et al. (1998). "Delta-1 activation of notch-1 signaling results in HES-1 transactivation." Mol Cell Biol **18** (12): 7423-31.

Jehn, B. M., Bielke, W., et al. (1999). "Cutting edge: protective effects of notch-1 on TCR-induced apoptosis." J Immunol **162** (2): 635-8.

Jhappan, C., Gallahan, D., et al. (1992). "Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands." Genes Dev **6**(3): 345-55.

Jockusch, B. M., Bubeck, P., et al. (1995). "The molecular architecture of focal adhesions." Annu Rev Cell Dev Biol **11**: 379-416.

Joutel, A., Andreux, F., et al. (2000). "The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients." J Clin Invest **105** (5): 597-605.

Joutel, A., Corpechot, C., et al. (1996). "Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia." Nature **383** (6602): 707-10.

Joutel, A., Monet, M., et al. (2004). "Pathogenic mutations associated with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy differently affect Jagged1 binding and Notch3 activity via the RBP/JK signaling Pathway." Am J Hum Genet **74** (2): 338-47.

Joutel, A. and Tournier-Lasserve, E. (1998). "Notch signalling pathway and human diseases." Semin Cell Dev Biol **9** (6): 619-25.

Jurgensmeier, J. M., Xie, Z., et al. (1998). "Bax directly induces release of cytochrome c from isolated mitochondria." Proc Natl Acad Sci U S A **95** (9): 4997-5002.

Kadesch, T. (2000). "Notch signaling: a dance of proteins changing partners." Exp Cell Res **260** (1): 1-8.

Kaiser, C., Laux, G., et al. (1999). "The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2." J Virol **73** (5): 4481-4.

Kajstura, J., Mansukhani, M., et al. (1995). "Programmed cell death and expression of the protooncogene bcl-2 in myocytes during postnatal maturation of the heart." Exp Cell Res **219** (1): 110-21.

Kaneta, M., Osawa, M., et al. (2000). "A role for pref-1 and HES-1 in thymocyte development." J Immunol **164** (1): 256-64.

Kang, P. M. and Izumo, S. (2000). "Apoptosis and heart failure: A critical review of the literature." Circ Res **86** (11): 1107-13.

Kao, H. Y., Ordentlich, P., et al. (1998). "A histone deacetylase corepressor complex regulates the Notch signal transduction pathway." Genes Dev **12** (15): 2269-77.

Karpen, H. E., Bukowski, J. T., et al. (2001). "The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane." J Biol Chem **276** (22): 19503-11.

Kataoka, T., Budd, R. C., et al. (2000). "The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways." Curr Biol **10** (11): 640-8.

Kelekar, A. and Thompson, C. B. (1998). "Bcl-2-family proteins: the role of the BH3 domain in apoptosis." Trends Cell Biol **8** (8): 324-30.

Kerr, J. F., Wyllie, A. H., et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26** (4): 239-57.

Khanna, A. (2004). "Concerted effect of transforming growth factor-beta, cyclin inhibitor p21, and c-myc on smooth muscle cell proliferation." Am J Physiol Heart Circ Physiol **286** (3): H1133-40.

Kieff, E. (1996). "Epstein-Barr Virus and its replication." Fields Virology **3**: 2343-2396.

Kim, H. S., Hwang, K. K., et al. (2000). "Apoptosis and regulation of Bax and Bcl-X proteins during human neonatal vascular remodeling." Arterioscler Thromb Vasc Biol **20** (4): 957-63.

Kimberly, W. T., Esler, W. P., et al. (2003). "Notch and the amyloid precursor protein are cleaved by similar gamma-secretase(s)." Biochemistry **42** (1): 137-44.

Kimble, J., Henderson, S., et al. (1998). "Notch/LIN-12 signaling: transduction by regulated protein slicing." Trends Biochem Sci **23** (9): 353-7.

Kirshenbaum, L. A. (2000). "Bcl-2 intersects the NFkappaB signalling pathway and suppresses apoptosis in ventricular myocytes." Clin Invest Med **23** (5): 322-30.

Klueg, K. M., Parody, T. R., et al. (1998). "Complex proteolytic processing acts on Delta, a transmembrane ligand for Notch, during Drosophila development." Mol Biol Cell **9** (7): 1709-23.

Kleinstreuer, C., Hyun, S., Buchanan, JR., et al (2001) GA. "Hemodynamic parameters and early intimal thickening in branching blood vessels". Crit Rev Biomed Eng **29**:1-64.

Kockx, M. M. (1998). "Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects." Arterioscler Thromb Vasc Biol **18** (10): 1519-22.

Kockx, M. M., De Meyer, G. R., et al. (1998). "Apoptosis and related proteins in different stages of human atherosclerotic plaques." Circulation **97** (23): 2307-15.

Kojika, S. and Griffin, J. D. (2001). "Notch receptors and hematopoiesis." Exp Hematol **29** (9): 1041-52.

Koller, A. (2002) . " Signaling pathways of mechanotransduction in arteriolar endothelium and smooth muscle cells in hypertension". Microcirculation. **9**:277-294.

Kono, Y., Sawada, S., et al. (2002). "Bradykinin inhibits serum-depletion-induced apoptosis of human vascular endothelial cells by inducing nitric oxide via calcium ion kinetics." J Cardiovasc Pharmacol **39** (2): 251-61.

Kopan, R. and Cagan, R. (1997). "Notch on the cutting edge." Trends Genet **13** (12): 465-7.

Kopan, R., Schroeter, E. H., et al. (1996). "Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain." Proc Natl Acad Sci U S A **93** (4): 1683-8.

Korshunov, V. A. and Berk, B. C. (2003). "Flow-induced vascular remodeling in the mouse: a model for carotid intima-media thickening." Arterioscler Thromb Vasc Biol **23** (12): 2185-91.

Kothakota, S., Azuma, T., et al. (1997). "Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis." Science **278** (5336): 294-8.

Kraemer, R. (2002). "Reduced apoptosis and increased lesion development in the flow-restricted carotid artery of p75(NTR)-null mutant mice." Circ Res **91**(6): 494-500.

Krammer, P. H. (2000). "CD95's deadly mission in the immune system." Nature **407** (6805): 789-95.

Kramsch, D. M. (1995). "Atherosclerosis progression/regression: lipoprotein and vessel wall determinants." Atherosclerosis **118** Suppl: S29-36.

Krebs, L. T., Xue, Y., et al. (2000). "Notch signaling is essential for vascular morphogenesis in mice." Genes Dev **14** (11): 1343-52.

Krishnan, V., Pereira., FA (1997). "Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase". Science; 1997; 278:1947-1950

Kuan, N. K. and Passaro, E., Jr. (1998). "Apoptosis: programmed cell death." Arch Surg **133** (7): 773-5.

Kuroda, K., Tani, S., et al. (1999). "Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis." J Biol Chem **274** (11): 7238-44.

Kurooka, H. and Honjo, T. (2000). "Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5." J Biol Chem **275** (22): 17211-20.

Lai, E. C. (2002). "Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins." EMBO Rep **3** (9): 840-5.

Lai, E. C. (2004). "Notch signaling: control of cell communication and cell fate." Development **131** (5): 965-73.

Langille, B. L. (1993). "Remodeling of developing and mature arteries: endothelium, smooth muscle, and matrix." J Cardiovasc Pharmacol **21** Suppl 1: S11-7.

Lawrence, N., Klein, T., et al. (2000). "Structural requirements for notch signalling with delta and serrate during the development and patterning of the wing disc of Drosophila." Development **127** (14): 3185-95.

Laws, A. M. and Osborne, B. A. (2004). "p53 regulates thymic Notch1 activation." Eur J Immunol **34** (3): 726-34.

Lawson, N. D., Scheer, N., et al. (2001). "Notch signaling is required for arterial-venous differentiation during embryonic vascular development." Development **128** (19): 3675-83.

Lawson, N. D., Vogel, A. M., et al. (2002). "sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation." Dev Cell **3** (1): 127-36.

Lecourtois, M. and Schweisguth, F. (1995). "The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling." Genes Dev **9** (21): 2598-608.

Lee, E. C., Yu, S. Y., et al. (2000). "The scabrous protein can act as an extracellular antagonist of notch signaling in the Drosophila wing." Curr Biol **10** (15): 931-4.

Lee, H. H., Dadgostar, H., et al. (1999). "NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes." Proc Natl Acad Sci U S A **96** (16): 9136-41.

Lee, J. S., Ishimoto, A., et al. (1999). "Murine leukemia provirus-mediated activation of the Notch1 gene leads to induction of HES-1 in a mouse T lymphoma cell line, DL-3." FEBS Lett **455** (3): 276-80.

Lehoux, S. and Tedgui, A. (1998). "Signal transduction of mechanical stresses in the vascular wall." Hypertension **32** (2): 338-45.

Leimeister, C., Dale, K., et al. (2000). "Oscillating expression of c-Hey2 in the presomitic mesoderm suggests that the segmentation clock may use combinatorial signaling through multiple interacting bHLH factors." Dev Biol **227** (1): 91-103.

Leimeister, C., Externbrink, A., et al. (1999). "Hey genes: a novel subfamily of hairy- and Enhancer of split related genes specifically expressed during mouse embryogenesis." Mech Dev **85** (1-2): 173-7.

Leimeister, C., Schumacher, N., et al. (2000). "Analysis of HeyL expression in wild-type and Notch pathway mutant mouse embryos." Mech Dev **98** (1-2): 175-8.

Leng, L., Du, B., et al. (1996). "Translocation of protein kinase C-delta by PDGF in cultured vascular smooth muscle cells: inhibition by TGF-beta 1." Artery **22** (3): 140-54.

Leung, D. Y., Glagov, S., et al. (1976). "Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro." Science **191** (4226): 475-7.

Levitan, D. and Greenwald, I. (1998). "Effects of SEL-12 presenilin on LIN-12 localization and function in Caenorhabditis elegans." Development **125** (18): 3599-606.

Lewis, J. (1998). "Notch signalling and the control of cell fate choices in vertebrates." Semin Cell Dev Biol **9** (6): 583-9.

Li, C., Hu, Y., et al. (1999). "Cyclic strain stress-induced mitogen-activated protein kinase (MAPK) phosphatase 1 expression in vascular smooth muscle cells is regulated by Ras/Rac-MAPK pathways." J Biol Chem **274** (36): 25273-80.

Li, C., Hu, Y., et al. (2000). "Ras/Rac-Dependent activation of p38 mitogen-activated protein kinases in smooth muscle cells stimulated by cyclic strain stress." Arterioscler Thromb Vasc Biol **20** (3): E1-9.

Li, C. and Xu, Q. (2000). "Mechanical stress-initiated signal transductions in vascular smooth muscle cells." Cell Signal **12** (7): 435-45.

Li, H., Zhu, H., et al. (1998). "Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis." Cell **94** (4): 491-501.

Li, L., Milner, L. A., et al. (1998). "The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1." Immunity **8** (1): 43-55.

Li, L. Y., Luo, X., et al. (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412** (6842): 95-9.

Li, Q., Muragaki, Y., et al. (1997). "Stretch-induced proliferation of cultured vascular smooth muscle cells and a possible involvement of local renin-angiotensin system and platelet-derived growth factor (PDGF)." Hypertens Res **20** (3): 217-23.

Libby, P. (1995). "Molecular bases of the acute coronary syndromes." Circulation **91** (11): 2844-50.

Libby, P. (2001). "Current concepts of the pathogenesis of the acute coronary syndromes." Circulation **104** (3): 365-72.

Lieber, T., Kidd, S., et al. (1993). "Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei." Genes Dev **7** (10): 1949-65.

Lieber, T., Kidd, S., et al. (2002). "kuzbanian-mediated cleavage of Drosophila Notch." Genes Dev **16** (2): 209-21.

Lindner, V., Booth, C., et al. (2001). "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." Am J Pathol **159** (3): 875-83.

Linke, S. P., Clarkin, K. C., et al. (1996). "A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage." Genes Dev **10** (8): 934-47.

Liu, F., Schaphorst., KL (2002). " Hepatocyte growth factor enhances endothelial cell barrier function and cortical cytoskeletal rearrangement: potential role of glycogen synthase kinase-3beta.". FASEB J. **16**:950-62.

Liu, J. and Lobe, C. (2003). "The role of the Notch signaling pathway in embryonic and adult angiogenesis using a transgenic mouse model." Endothelium **10** (6).

Liu, L., Azhar, G., et al. (1998). "Bcl-2 and Bax expression in adult rat hearts after coronary occlusion: age-associated differences." Am J Physiol **275** (1 Pt 2): R315-22.

Liu, X., Kim, C. N., et al. (1996). "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c." Cell **86** (1): 147-57.

Liu, Z. G., Hsu, H., et al. (1996). "Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death." Cell **87** (3): 565-76

Liu, Z. J., Shirakawa, T., et al. (2003). "Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis." Mol Cell Biol **23** (1): 14-25.

Logeat, F., Bessia, C., et al. (1998). "The Notch1 receptor is cleaved constitutively by a furin-like convertase." Proc Natl Acad Sci U S A **95** (14): 8108-12.

Loomes, K. M., Underkoffler, L. A., et al. (1999). "The expression of Jagged1 in the developing mammalian heart correlates with cardiovascular disease in Alagille syndrome." Hum Mol Genet **8** (13): 2443-9.

Lopez, S. L., Paganelli, A. R., et al. (2003). "Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in Xenopus." Development **130** (10): 2225-38.

Lundberg, A. S. and Weinberg, R. A. (1999). "Control of the cell cycle and apoptosis." Eur J Cancer **35** (4): 531-9.

Luo, B., Aster, J. C., et al. (1997). "Isolation and functional analysis of a cDNA for human Jagged2, a gene encoding a ligand for the Notch1 receptor." Mol Cell Biol **17** (10): 6057-67.

Luttun, A., Carmeliet, G., et al. (2002). "Vascular progenitors: from biology to treatment." Trends Cardiovasc Med **12** (2): 88-96.

Lyman, D. and Young, M. W. (1993). "Further evidence for function of the Drosophila Notch protein as a transmembrane receptor." Proc Natl Acad Sci U S A **90** (21): 10395-9.

Ma, Y. H., Ling, S., et al. (1999). "Mechanical strain increases PDGF-B and PDGF beta receptor expression in vascular smooth muscle cells." Biochem Biophys Res Commun **265** (2): 606-10.

MacDonald, I., Wang, H., et al. (1996). "Transforming growth factor-beta 1 cooperates with anti-immunoglobulin for the induction of apoptosis in group I (biopsy-like) Burkitt lymphoma cell lines." Blood **87** (3): 1147-54.

MacKenzie, F., Duriez, P., et al. (2004). "Notch4 inhibits endothelial apoptosis via RBP-Jkappa-dependent and -independent pathways." J Biol Chem **279** (12): 11657-63.

MacLellan, W. R. and Schneider, M. D. (1997). "Death by design. Programmed cell death in cardiovascular biology and disease." Circ Res **81** (2): 137-44

Maier, M. M. and Gessler, M. (2000). "Comparative analysis of the human and mouse Hey1 promoter: Hey genes are new Notch target genes." Biochem Biophys Res Commun **275** (2): 652-60.

Majesky, MW. "Vascular smooth muscle diversity: insights from developmental Biology" (2003). Curr Atheroscler Rep **5**:208-213.

Mallat, Z., Hugel, B., et al. (1999). "Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity." Circulation **99** (3): 348-53.

Mallat, Z., Ohan, J., et al. (1997). "Colocalization of CPP-32 with apoptotic cells in human atherosclerotic plaques." Circulation **96** (2): 424-8.

Manabe, I., Nagai, R. (2003). " Regulation of smooth muscle phenotype". Curr Atheroscler Rep **5**:214-222.

Mango, S. E., Maine, E. M., et al. (1991). "Carboxy-terminal truncation activates glp-1 protein to specify vulval fates in Caenorhabditis elegans." Nature **352** (6338): 811-5.

Marsden, V. S., O'Connor, L., et al. (2002). "Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome." Nature **419** (6907): 634-7.

Martelli, A. M., Sang, N., et al. (1999). "Multiple biological responses activated by nuclear protein kinase C." J Cell Biochem **74** (4): 499-521.

Martinez Arias, A., Zecchini, V., et al. (2002). "CSL-independent Notch signalling: a checkpoint in cell fate decisions during development?" Curr Opin Genet Dev **12** (5): 524-33.

Matsunami, N., Hamaguchi, Y., et al. (1989). "A protein binding to the J kappa recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif." Nature **342** (6252): 934-7.

Matsuno, K., Diederich, R. J., et al. (1995). "Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats." Development **121** (8): 2633-44.

Matsuno, K., Eastman, D., et al. (1998). "Human deltex is a conserved regulator of Notch signalling." Nat Genet **19** (1): 74-8.

Matsuno, K., Go, M. J., et al. (1997). "Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements." Development **124** (21): 4265-73.

Mayo, L. D., Turchi, J. J., et al. (1997). "Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53." Cancer Res **57** (22): 5013-6.

Mayr, M., Hu, Y., et al. (2002). "Mechanical stress-induced DNA damage and rac-p38MAPK signal pathways mediate p53-dependent apoptosis in vascular smooth muscle cells." Faseb J **16** (11): 1423-5.

Mayr, M., Li, C., et al. (2000). "Biomechanical stress-induced apoptosis in vein grafts involves p38 mitogen-activated protein kinases." Faseb J **14** (2): 261-70.

Mayr, M. and Xu, Q. (2001). "Smooth muscle cell apoptosis in arteriosclerosis." Exp Gerontol **36** (7): 969-87.

McElhinney, D. B., Krantz, I. D., et al. (2002). "Analysis of cardiovascular phenotype and genotype-phenotype correlation in individuals with a JAG1 mutation and/or Alagille syndrome." Circulation **106** (20): 2567-74.

McLarren, K. W., Lo, R., et al. (2000). "The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1." J Biol Chem **275** (1): 530-8.

Meng, X., Mavromatis, K., et al. (1999). "Mechanical stretching of human saphenous vein grafts induces expression and activation of matrix-degrading enzymes associated with vascular tissue injury and repair." Exp Mol Pathol **66** (3): 227-37.

Miele, L. and Osborne, B. (1999). "Arbiter of differentiation and death: Notch signaling meets apoptosis." J Cell Physiol **181** (3): 393-409.

Mills, I., Cohen, C. R., et al. (1997). "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." J Cell Physiol **170** (3): 228-34.

Minoguchi, S., Taniguchi, Y., et al. (1997). "RBP-L, a transcription factor related to RBP-Jkappa." Mol Cell Biol **17** (5): 2679-87.

Misao, J., Hayakawa, Y., et al. (1996). "Expression of bcl-2 protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction." Circulation **94** (7): 1506-12.

Miyashita, T. and Reed, J. C. (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell **80** (2): 293-9.

Miyazono, K., Suzuki, H., et al. (2003). "Regulation of TGF-beta signaling and its roles in progression of tumors." Cancer Sci **94** (3): 230-4.

Mochly-Rosen, D. and Gordon, A. S. (1998). "Anchoring proteins for protein kinase C: a means for isozyme selectivity." Faseb J **12** (1): 35-42.

Molloy, C. J., Taylor, D. S., et al. (1999). "Novel cardiovascular actions of the activins." J Endocrinol **161** (2): 179-85.

Moloney, D. J., Panin, V. M., et al. (2000). "Fringe is a glycosyltransferase that modifies Notch." Nature **406** (6794): 369-75.

Moreno, P. R., Bernardi, V. H., et al. (1996). "Macrophages, smooth muscle cells, and tissue factor in unstable angina. Implications for cell-mediated thrombogenicity in acute coronary syndromes." Circulation **94** (12): 3090-7.

Morimura, T., Goitsuka, R., et al. (2000). "Cell cycle arrest and apoptosis induced by Notch1 in B cells." J Biol Chem **275** (47): 36523-31.

Morishita, R., Gibbons, G. H., et al. (1993). "Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia." Proc Natl Acad Sci U S A **90** (18): 8474-8.

Moroni, M. C., Hickman, E. S., et al. (2001). "Apaf-1 is a transcriptional target for E2F and p53." Nat Cell Biol **3** (6): 552-8.

Morrissette, J. D., Colliton, R. P., et al. (2001). "Defective intracellular transport and processing of JAG1 missense mutations in Alagille syndrome." Hum Mol Genet **10** (4): 405-13.

Motoyama, N., Wang, F., et al. (1995). "Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice." Science **267** (5203): 1506-10.

Muchmore, S. W., Sattler, M., et al. (1996). "X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death." Nature **381** (6580): 335-41.

Mulvany, M. J. (1999). "Vascular remodelling of resistance vessels: can we define this?" Cardiovasc Res **41** (1): 9-13.

Mumm, J. S. and Kopan, R. (2000). "Notch signaling: from the outside in." Dev Biol **228** (2): 151-65.

Mumm, J. S., Schroeter, E. H., et al. (2000). "A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1." Mol Cell **5** (2): 197-206.

Murre, C., Bain, G., et al. (1994). "Structure and function of helix-loop-helix proteins." Biochim Biophys Acta **1218** (2): 129-35.

Muscat, J. E., Harris, R. E., et al. (1991). "Cigarette smoking and plasma cholesterol." Am Heart J **121** (1 Pt 1): 141-7.

Nagata, S. (1997). "Apoptosis by death factor." Cell **88** (3): 355-65.

Nagata, S. (1999). "Fas ligand-induced apoptosis." Annu Rev Genet **33**: 29-55.

Nagata, S. (2000). "Apoptotic DNA fragmentation." Exp Cell Res **256** (1): 12-8.

Nair, P., Somasundaram, K., et al. (2003). "Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway." J Virol **77** (12): 7106-12.

Nakagawa, O., McFadden, D. G., et al. (2000). "Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling." Proc Natl Acad Sci U S A **97** (25): 13655-60.

Nickoloff, B. J., Qin, J. Z., et al. (2002). "Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma." Cell Death Differ **9** (8): 842-55.

Nicotera, P., Leist, M., et al. (1999). "Neuronal cell death: a demise with different shapes." Trends Pharmacol Sci **20** (2): 46-51.

Nijjar, S. S., Wallace, L., et al. (2002). "Altered Notch ligand expression in human liver disease: further evidence for a role of the Notch signaling pathway in hepatic neovascularization and biliary ductular defects." Am J Pathol **160** (5): 1695-703.

Nordborg, C., Ivarsson, H., et al. (1983). "Morphometric study of mesenteric and renal arteries in spontaneously hypertensive rats." J Hypertens **1** (4): 333-8.

Nusslein-Volhard, C. and Wieschaus, E. (1980). "Mutations affecting segment number and polarity in Drosophila." Nature **287** (5785): 795-801.

Nybakken, K. and Perrimon, N. (2002). "Hedgehog signal transduction: recent findings." Curr Opin Genet Dev **12** (5): 503-11.

Oakley, F., Mann, J., et al. (2003). "Basal expression of IkappaBalpha is controlled by the mammalian transcriptional repressor RBP-J (CBF1) and its activator Notch1." J Biol Chem **278** (27): 24359-70.

Oberg, C., Li, J., et al. (2001). "The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog." J Biol Chem **276** (38): 35847-53.

Ohishi, K., Varnum-Finney, B., et al. (2000). "Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1." Blood **95** (9): 2847-54.

Ohtsuka, T., Ishibashi, M., et al. (1999). "Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation." Embo J **18** (8): 2196-207.

Okabe, M., Imai, T., et al. (2001). "Translational repression determines a neuronal potential in Drosophila asymmetric cell division." Nature **411**(6833): 94-8.

Oltvai, Z. N., Milliman, C. L., et al. (1993). "Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death." Cell **74** 4): 609-19.

Ordentlich, P., Lin, A., et al. (1998). "Notch inhibition of E47 supports the existence of a novel signaling pathway." Mol Cell Biol **18** (4): 2230-9.

Osol G. Mechanotransduction by vascular smooth muscle. *J Vasc Res*. 1995; 32:275-292

Oswald, F., Tauber, B., et al. (2001). "p300 acts as a transcriptional coactivator for mammalian Notch-1." Mol Cell Biol **21** (22): 7761-74.

Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kappaB transcription factors." Oncogene **18** (49): 6853-66.

Pan, D. and Rubin, G. M. (1997). "Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis." Cell **90** (2): 271-80.

Panin, V. M. and Irvine, K. D. (1998). "Modulators of Notch signaling." Semin Cell Dev Biol **9** (6): 609-17.

Panin, V. M., Papayannopoulos, V., et al. (1997). "Fringe modulates Notch-ligand interactions." Nature **387** (6636): 908-12.

Park, M., Yaich, L. E., et al. (1998). "Mesodermal cell fate decisions in Drosophila are under the control of the lineage genes numb, Notch, and sanpodo." Mech Dev **75** (1-2): 117-26.

Parks, A. L., Huppert, S. S., et al. (1997). "The dynamics of neurogenic signalling underlying bristle development in Drosophila melanogaster." Mech Dev **63** (1): 61-74.

Parks, A. L., Klueg, K. M., et al. (2000). "Ligand endocytosis drives receptor dissociation and activation in the Notch pathway." Development **127** (7): 1373-85.

Patrick, C. W., Jr. and McIntire, L. V. (1995). "Shear stress and cyclic strain modulation of gene expression in vascular endothelial cells." Blood Purif **13** (3-4): 112-24.

Pepicelli, C. V., Lewis, P. M., et al. (1998). "Sonic hedgehog regulates branching morphogenesis in the mammalian lung." Curr Biol **8** (19): 1083-6.

Pereira, FA, Qiu, Y. (1999). " The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development". Genes Dev **13**:1037-1049.

Perlman, H., Maillard, L., et al. (1997). "Evidence for the rapid onset of apoptosis in medial smooth muscle cells after balloon injury." Circulation **95** (4): 981-7.

Plutzky, J. (1999). "Atherosclerotic plaque rupture: emerging insights and opportunities." Am J Cardiol **84** (1A): 15J-20J.

Pola, R., Ling, L. E., et al. (2003). "Postnatal recapitulation of embryonic hedgehog pathway in response to skeletal muscle ischemia." Circulation **108** (4): 479-85.

Pola, R., Ling, L. E., et al. (2001). "The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors." Nat Med **7** (6): 706-11.

Pollman, M. J., Hall, J. L., et al. (1999). "Determinants of vascular smooth muscle cell apoptosis after balloon angioplasty injury. Influence of redox state and cell phenotype." Circ Res **84** (1): 113-21.

Pollman, M. J., Hall, J. L., et al. (1998). "Inhibition of neointimal cell bcl-x expression induces apoptosis and regression of vascular disease." Nat Med **4** (2): 222-7.

Pollman, M. J., Naumovski, L., et al. (1999). "Vascular cell apoptosis: cell type-specific modulation by transforming growth factor-beta1 in endothelial cells versus smooth muscle cells." Circulation **99** (15): 2019-26.

Powell, P. A., Wesley, C., et al. (2001). "Scabrous complexes with Notch to mediate boundary formation." Nature **409** (6820): 626-30.

Prakash, N., Hansson, E., et al. (2002). "Mouse Notch 3 expression in the pre- and postnatal brain: relationship to the stroke and dementia syndrome CADASIL." Exp Cell Res **278** (1): 31-44.

Pukac, L., Huangpu, J., et al. (1998). "Platelet-derived growth factor-BB, insulin-like growth factor-I, and phorbol ester activate different signaling pathways for stimulation of vascular smooth muscle cell migration." Exp Cell Res **242** (2): 548-60.

Qi, H., Rand, M. D., et al. (1999). "Processing of the notch ligand delta by the metalloprotease Kuzbanian." Science **283** (5398): 91-4.

Qi, R., An, H., et al. (2003). "Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis." Cancer Res **63** (23): 8323-9.

Qiao, J. H., Tripathi, J., et al. (1997). "Role of macrophage colony-stimulating factor in atherosclerosis: studies of osteopetrotic mice." Am J Pathol **150** (5): 1687-99.

Qiu, L., Joazeiro, C., et al. (2000). "Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase." J Biol Chem **275** (46): 35734-7.

Rand, M. D., Grimm, L. M., et al. (2000). "Calcium depletion dissociates and activates heterodimeric notch receptors." Mol Cell Biol **20** (5): 1825-35.

Rao, L., Perez, D., et al. (1996). "Lamin proteolysis facilitates nuclear events during apoptosis." J Cell Biol **135** (6 Pt 1): 1441-55.

Rao, R. S., Miano, J. M., et al. (1997). "The A10 cell line: a model for neonatal, neointimal, or differentiated vascular smooth muscle cells?" Cardiovasc Res **36** (1): 118-26.

Ray, R. and Miller, D. M. (1991). "Cloning and characterization of a human c-myc promoter-binding protein." Mol Cell Biol **11** (4): 2154-61.

Ray, W. J., Yao, M., et al. (1999). "Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch." J Biol Chem **274** (51): 36801-7.

Ray, W. J., Yao, M., et al. (1999). "Evidence for a physical interaction between presenilin and Notch." Proc Natl Acad Sci U S A **96** (6): 3263-8.

Rebay, I., Fehon, R. G., et al. (1993). "Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor." Cell **74** (2): 319-29.

Rebay, I., Fleming, R. J., et al. (1991). "Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor." Cell **67** (4): 687-99.

Reed, J. C. (1994). "Bcl-2 and the regulation of programmed cell death." J Cell Biol **124** (1-2): 1-6.

Reed, J. C. (1997). "Double identity for proteins of the Bcl-2 family." Nature **387** (6635): 773-6.

Rich, T., Allen, R. L., et al. (2000). "Defying death after DNA damage." Nature **407** (6805): 777-83.

Riedy, M. C., Brown, M. C., et al. (1999). "Activin A and TGF-beta stimulate phosphorylation of focal adhesion proteins and cytoskeletal reorganization in rat aortic smooth muscle cells." Exp Cell Res **251** (1): 194-202.

Rivard, A., Luo, Z., et al. (1999). "Early cell loss after angioplasty results in a disproportionate decrease in percutaneous gene transfer to the vessel wall." Hum Gene Ther **10** (5): 711-21.

Robbins, J., Blondel, B. J., et al. (1992). "Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells." J Virol **66** (4): 2594-9.

Roberts, D. J., Johnson, R. L., et al. (1995). "Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut." Development **121** (10): 3163-74.

Robertson, E. S., Lin, J., et al. (1996). "The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa)." J Virol **70** (5): 3068-74.

Robey, E. (1999). "Regulation of T cell fate by Notch." Annu Rev Immunol **17**: 283-95.

Roehl, H., Bosenberg, M., et al. (1996). "Roles of the RAM and ANK domains in signaling by the C. elegans GLP-1 receptor." Embo J **15** (24): 7002-12.

Rogers, S., Wells, R., et al. (1986). "Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis." Science **234** (4774): 364-8.

Rohrer, L., Freeman, M., et al. (1990). "Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II." Nature **343** (6258): 570-2.

Ross, D. A. and Kadesch, T. (2001). "The notch intracellular domain can function as a coactivator for LEF-1." Mol Cell Biol **21** (22): 7537-44.

Ross, R. (1993). "The pathogenesis of atherosclerosis: a perspective for the 1990s." Nature **362** (6423): 801-9.

Rowitch, DH., S-Jacques, B. (1999). "Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cell". J. Neurosci **19**:8954-8965

Rubanyi, G. M., Romero, J. C., et al. (1986). "Flow-induced release of endothelium-derived relaxing factor." Am J Physiol **250** (6 Pt 2): H1145-9.

Rubio, A., Rifkin, D., et al. (1997). "Phenotypic variability of CADASIL and novel morphologic findings." Acta Neuropathol (Berl) **94** (3): 247-54.

Rudel, T. and Bokoch, G. M. (1997). "Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2." Science **276** (5318): 1571-4.

Sakahira, H., Enari, M., et al. (1998). "Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis." Nature **391** (6662): 96-9.

Sakai, T., Taniguchi, Y., et al. (1998). "Functional replacement of the intracellular region of the Notch1 receptor by Epstein-Barr virus nuclear antigen 2." J Virol **72** (7): 6034-9.

Sakamoto, K., Ohara, O., et al. (2002). "Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification." Dev Biol **241** (2): 313-26.

Sasai, Y., Kageyama, R., et al. (1992). "Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split." Genes Dev **6** (12B): 2620-34.

Sasaki, Y., Ishida, S., et al. (2002). "The p53 family member genes are involved in the Notch signal pathway." J Biol Chem **277** (1): 719-24.

Sata, M., Suhara, T., et al. (2000). "Vascular endothelial cells and smooth muscle cells differ in expression of Fas and Fas ligand and in sensitivity to Fas ligand-induced cell death: implications for vascular disease and therapy." Arterioscler Thromb Vasc Biol **20** (2): 309-16.

Sata, M., Tanaka, K., et al. (2003). "Absence of p53 leads to accelerated neointimal hyperplasia after vascular injury." Arterioscler Thromb Vasc Biol **23** (9): 1548-52.

Saxena, M. T., Schroeter, E. H., et al. (2001). "Murine notch homologs (N1-4) undergo presenilin-dependent proteolysis." J Biol Chem **276** (43): 40268-73.

Schauerte, HE., van Eeden, FJ, Fricke, C. (1998). " Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish". Development. **125**:2983-2893

Schlaepfer, D. D., Hauck, C. R., et al. (1999). "Signaling through focal adhesion kinase." Prog Biophys Mol Biol **71** (3-4): 435-78

Schmid, I., Krall, W. J., et al. (1992). "Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry." Cytometry **13** (2): 204-8

Schroeder, A. P. and Falk, E. (1995). "Vulnerable and dangerous coronary plaques." Atherosclerosis **118** Suppl: S141-9.

Schroeter, E. H., Kisslinger, J. A., et al. (1998). "Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain." Nature **393** (6683): 382-6.

Scott, N. A., Cipolla, G. D., et al. (1996). "Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary arteries." Circulation **93** (12): 2178-87.

Schwartz, RS., Henry, TD., et al. (2002). "Pathophysiology of coronary artery restenosis". Rev Cardiovasc Med **3**:Suppl 5:S4-9.

Sedlak, T. W., Oltvai, Z. N., et al. (1995). "Multiple Bcl-2 family members demonstrate selective dimerizations with Bax." Proc Natl Acad Sci U S A **92** (17): 7834-8.

Sentman, C. L., Shutter, J. R., et al. (1991). "bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes." Cell **67** (5): 879-88.

Shanahan, C. M., Weissberg, P. L., et al. (1993). "Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells." Circ Res **73** (1): 193-204.

Sharrett, A. R., Patsch, W., et al. (1994). "Associations of lipoprotein cholesterols, apolipoproteins A-I and B, and triglycerides with carotid atherosclerosis and coronary heart disease. The Atherosclerosis Risk in Communities (ARIC) Study." Arterioscler Thromb **14** (7): 1098-104.

Shaw, A., Xu, Q.(2003). " Biomechanical stress-induced signaling in smooth muscle cells: an update". Curr Vasc Pharmacol **41**-58.

Shawber, C., Nofziger, D., et al. (1996). "Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway." Development **122** (12): 3765-73.

Shelly, L. L., Fuchs, C., et al. (1999). "Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds." J Cell Biochem **73** (2): 164-75.

Shen, J., Bronson, R. T., et al. (1997). "Skeletal and CNS defects in Presenilin-1-deficient mice." Cell **89** (4): 629-39.

Shi, Y., O'Brien, J. E., Jr., et al. (1997). "Origin of extracellular matrix synthesis during coronary repair." Circulation **95** (4): 997-1006.

Shi, Y., Pieniek, M., et al. (1996). "Adventitial remodeling after coronary arterial injury." Circulation **93** (2): 340-8.

Shichiri, M., Yokokura, M., et al. (2000). "Endothelin-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via MAP kinase pathway." Arterioscler Thromb Vasc Biol **20** (4): 989-97.

Shimizu, K., Chiba, S., et al. (1999). "Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods." J Biol Chem **274** (46): 32961-9.

Sho, E., Sho, M., et al. (2001). "Blood flow decrease induces apoptosis of endothelial cells in previously dilated arteries resulting from chronic high blood flow." Arterioscler Thromb Vasc Biol **21** (7): 1139-45.

Shutter, J. R., Scully, S., et al. (2000). "Dll4, a novel Notch ligand expressed in arterial endothelium." Genes Dev **14** (11): 1313-8.

Simpson, P. (1998). "Introduction: Notch signalling and choice of cell fates in development." Semin Cell Dev Biol **9** (6): 581-2.

Singh, N., Phillips, R. A., et al. (2000). "Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis." Exp Hematol **28** (5): 527-34.

Slomp, J., Gittenberger-de Groot, A. C., et al. (1997). "Differentiation, dedifferentiation, and apoptosis of smooth muscle cells during the development of the human ductus arteriosus." Arterioscler Thromb Vasc Biol **17** (5): 1003-9.

Smith, G. H., Gallahan, D., et al. (1995). "Constitutive expression of a truncated INT3 gene in mouse mammary epithelium impairs differentiation and functional development." Cell Growth Differ **6** (5): 563-77.

Smith, J. D., Davies, N., et al. (2001). "Cyclic stretch induces the expression of vascular endothelial growth factor in vascular smooth muscle cells." Endothelium **8** (1): 41-8.

Smith, P. R., de Jesus, O., et al. (2000). "Structure and coding content of CST (BART) family RNAs of Epstein-Barr virus." J Virol **74** (7): 3082-92.

Speir, E., Modali, R., et al. (1994). "Potential role of human cytomegalovirus and p53 interaction in coronary restenosis." Science **265** (5170): 391-4.

Spender, L. C., Cannell, E. J., et al. (1999). "Control of cell cycle entry and apoptosis in B lymphocytes infected by Epstein-Barr virus." J Virol **73** (6): 4678-88.

Spinner, N. B. (2000). "CADASIL: Notch signaling defect or protein accumulation problem?" J Clin Invest **105** (5): 561-2.

Spinner, N. B., Colliton, R. P., et al. (2001). "Jagged1 mutations in alagille syndrome." Hum Mutat **17** (1): 18-33.

Srivastava, D. and Olson, E. N. (2000). "A genetic blueprint for cardiac development." Nature **407** (6801): 221-6.

Stary, H. C., Chandler, A. B., et al. (1994). "A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association." Circulation **89** (5): 2462-78.

Steller, H. (1995). "Mechanisms and genes of cellular suicide." Science **267** (5203): 1445-9.

Stifani, S., Blaumueller, C. M., et al. (1992). "Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins." Nat Genet **2** (4): 343.

Strobl, L. J., Hofelmayr, H., et al. (2000). "Activated Notch1 modulates gene expression in B cells similarly to Epstein-Barr viral nuclear antigen 2." J Virol **74** (4): 1727-35.

Struhl, G. and Adachi, A. (1998). "Nuclear access and action of notch in vivo." Cell **93** (4): 649-60.

Struhl, G. and Greenwald, I. (1999). "Presenilin is required for activity and nuclear access of Notch in Drosophila." Nature **398** (6727): 522-5.

Stuart, R. A. and Neupert, W. (1990). "Apocytochrome c: an exceptional mitochondrial precursor protein using an exceptional import pathway." Biochimie **72** (2-3): 115-21.

Sullivan, C. J. and Hoying, J. B. (2002). "Flow-dependent remodeling in the carotid artery of fibroblast growth factor-2 knockout mice." Arterioscler Thromb Vasc Biol **22** (7): 1100-5.

Sullivan, D. C. and Bicknell, R. (2003). "New molecular pathways in angiogenesis." Br J Cancer **89** (2): 228-31.

Sun, X. and Artavanis-Tsakonas, S. (1996). "The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands." Development **122** (8): 2465-74.

Sun, X. and Artavanis-Tsakonas, S. (1997). "Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila." Development **124** (17): 3439-48.

Susin, S. A., Zamzami, N., et al. (1996). "Bcl-2 inhibits the mitochondrial release of an apoptogenic protease." J Exp Med **184** (4): 1331-41.

Suzuki, M., Naruse, K., et al. (1997). "Up-regulation of integrin beta 3 expression by cyclic stretch in human umbilical endothelial cells." Biochem Biophys Res Commun **239** (2): 372-6.

Suzuki, Y. J., Forman, H. J., et al. (1997). "Oxidants as stimulators of signal transduction." Free Radic Biol Med **22** (1-2): 269-85.

Suzuma, I., Suzuma, K., et al. (2002). "Stretch-induced retinal vascular endothelial growth factor expression is mediated by phosphatidylinositol 3-kinase and protein kinase C (PKC)-zeta but not by stretch-induced ERK1/2, Akt, Ras, or classical/novel PKC pathways." J Biol Chem **277** (2): 1047-57.

Swiatek, P. J., Lindsell, C. E., et al. (1994). "Notch1 is essential for postimplantation development in mice." Genes Dev **8** (6): 707-19.

Takke, C., Dornseifer, P., et al. (1999). "her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling." Development **126** (9): 1811-21.

Tallquist, M. D., Soriano, P., et al. (1999). "Growth factor signaling pathways in vascular development." Oncogene **18** (55): 7917-32.

Tamura, K., Taniguchi, Y., et al. (1995). "Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H)." Curr Biol **5** (12): 1416-23.

Tanaka, M., Suda, T., et al. (1995). "Expression of the functional soluble form of human fas ligand in activated lymphocytes." Embo J **14** (6): 1129-35.

Tanigaki, K., Han, H., et al. (2002). "Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells." Nat Immunol **3** (5): 443-50.

Taylor, KL., Henderson, AM., Hughes, CC. (2002). "Notch activation during endothelial cell network formation in vitro targets the basic HLH transcription factor HESR-1 and downregulates VEGFR-2/KDR expression" . Microvasc Res; 64:372-83.

Thompson, C. B. (1995). "Apoptosis in the pathogenesis and treatment of disease." Science **267** (5203): 1456-62.

Thubrikar, M. J. and Robicsek, F. (1995). "Pressure-induced arterial wall stress and atherosclerosis." Ann Thorac Surg **59** (6): 1594-603.

Tournier-Lasserve, E., Joutel, A., et al. (1993). "Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy maps to chromosome 19q12." Nat Genet **3** (3): 256-9.

Touyz, R. M., Deng, L. Y., et al. (1999). "Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of extracellular signal-regulated kinases." J Hypertens **17** (7): 907-16.

Tran, J., Rak, J., et al (1999). " Marked Induction of the IAP Family Antiapoptotic Proteins Survivin and XIAP by VEGF in vascular endothelial cells" Biochemical and Biophysical Research Comm **264**, 781-788.

Traub, O. and Berk, B. C. (1998). "Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force." Arterioscler Thromb Vasc Biol **18** (5): 677-85.

Tucker, K. L., Beard, C., et al. (1996). "Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes." Genes Dev **10** (8): 1008-20.

Utley RT., Cote J., et al. (2003) "The MYST family of histone acetyltransferases." Curr Top Microbiol Immunol **274**:203-236.

Uyttendaele, H., Closson, V., et al. (2000). "Notch4 and Jagged-1 induce microvessel differentiation of rat brain endothelial cells." Microvasc Res **60** (2): 91-103.

Uyttendaele, H., Ho, J., et al. (2001). "Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium." Proc Natl Acad Sci U S A **98** (10): 5643-8.

Uyttendaele, H., Marazzi, G., et al. (1996). "Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene." Development **122** (7): 2251-9.

Veikkola, T. and Alitalo, K. (1999). "VEGFs, receptors and angiogenesis." Semin Cancer Biol **9** (3): 211-20.

Veis, D. J., Sorenson, C. M., et al. (1993). "Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair." Cell **75** (2): 229-40.

Villa, N., Walker, L., et al. (2001). "Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels." Mech Dev **108** (1-2): 161-4.

Villavicencio, E. H., Walterhouse, D. O., et al. (2000). "The sonic hedgehog-patched-gli pathway in human development and disease." Am J Hum Genet **67** (5): 1047-54.

Vinters, H. V. and Berliner, J. A. (1987). "The blood vessel wall as an insulin target tissue." Diabete Metab **13** (3 Pt 2): 294-300.

Wagner, A. J., Kokontis, J. M., et al. (1994). "Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1." Genes Dev **8** (23): 2817-30.

Walsh, K., Smith, R. C., et al. (2000). "Vascular cell apoptosis in remodeling, restenosis, and plaque rupture." Circ Res **87** (3): 184-8.

Wang, B. Y., Ho, H. K., et al. (1999). "Regression of atherosclerosis: role of nitric oxide and apoptosis." Circulation **99** (9): 1236-41.

Wang, J., Frost, J. A., et al. (1999). "Reciprocal signaling between heterotrimeric G proteins and the p21-stimulated protein kinase." J Biol Chem **274** (44): 31641-7.

Wang, J., Shelly, L., et al. (2001). "Human Notch-1 inhibits NF-kappa B activity in the nucleus through a direct interaction involving a novel domain." J Immunol **167** (1): 289-95.

Wang, S., Sdrulla, A. D., et al. (1998). "Notch receptor activation inhibits oligodendrocyte differentiation." Neuron **21** (1): 63-75.

Wang, W., Campos, A. H., et al. (2002a). "Coordinate Notch3-hairy-related transcription factor pathway regulation in response to arterial injury. Mediator role of platelet-derived growth factor and ERK." J Biol Chem **277** (26): 23165-71.

Wang, W., Prince, C. Z., et al. (2002b). "Notch3 signaling in vascular smooth muscle cells induces c-FLIP expression via ERK/MAPK activation. Resistance to Fas ligand-induced apoptosis." J Biol Chem **277** (24): 21723-9.

Weed, M., Mundlos, S., et al. (1997). "The role of sonic hedgehog in vertebrate development." Matrix Biol **16** (2): 53-8.

Weinmaster, G. (1997). "The ins and outs of notch signaling." Mol Cell Neurosci **9** (2): 91-102.

Weinmaster, G. (1998). "Notch signaling: direct or what?" Curr Opin Genet Dev **8** (4): 436-42.

Weinmaster, G. (2000). "Notch signal transduction: a real rip and more." Curr Opin Genet Dev **10** (4): 363-9.

Weinmaster, G., Roberts, V. J., et al. (1992). "Notch2: a second mammalian Notch gene." Development **116** (4): 931-41.

Weinstein, BM., Lawson, ND. (2002). " Arteries, veins, Notch, and VEGF". Cold Spring Harb Symp Quant Biol **67**:155-162.

Wernig, F., Mayr, M., et al. (2003). "Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by beta1-integrin signaling pathways." Hypertension **41** (4): 903-11.

White, S. M., Constantin, P. E., et al. (2004). "Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function." Am J Physiol Heart Circ Physiol **286** (3): H823-9.

Wilcox, J. N., Smith, K. M., et al. (1989). "Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque." Proc Natl Acad Sci U S A **86** (8): 2839-43.

Williams, K. J. and Tabas, I. (1998). "The response-to-retention hypothesis of atherogenesis reinforced." Curr Opin Lipidol **9** (5): 471-4.

Wilson, E., Mai, Q., et al. (1993). "Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF." J Cell Biol **123** (3): 741-7.

Wilson, E., Sudhir, K., et al. (1995). "Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions." J Clin Invest **96** (5): 2364-72.

Wilson-Rawls, J., Molkentin, J. D., et al. (1999). "Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C." Mol Cell Biol **19** (4): 2853-62.

Wong, P. C., Zheng, H., et al. (1997). "Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm." Nature **387** (6630): 288-92.

Wu-Wong, J. R., Chiou, W. J., et al. (1997). "Endothelin attenuates apoptosis in human smooth muscle cells." Biochem J **328** (Pt 3): 733-7.

Xu, Q. (2000). "Biomechanical-stress-induced signaling and gene expression in the development of arteriosclerosis." Trends Cardiovasc Med **10** (1): 35-41.

Xue, Y., Gao, X., et al. (1999). "Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1." Hum Mol Genet **8** (5): 723-30.

Yang, C. C., Lin, H. P., et al. (2003). "Bcl-xL mediates a survival mechanism independent of the phosphoinositide 3-kinase/Akt pathway in prostate cancer cells." J Biol Chem **278** (28): 25872-8.

Yang, X., Klein, R., et al. (2004). "Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway." Dev Biol **269** (1): 81-94.

Yao, R. and Cooper, G. M. (1995). "Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor." Science **267** (5206): 2003-6.

Ye, Y., Lukinova, N., et al. (1999). "Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants." Nature **398** (6727): 525-9.

Yeh, E. T. (1997). "Life and death in the cardiovascular system." Circulation **95** (4): 782-6.

Yokoyama, Y., Baveja, R., et al. (2001). "Hepatic neovascularization after partial portal vein ligation: novel mechanism of chronic regulation of blood flow." Am J Physiol Gastrointest Liver Physiol **280** (1): G21-31.

Zagouras, P., Stifani, S., et al. (1995). "Alterations in Notch signaling in neoplastic lesions of the human cervix." Proc Natl Acad Sci U S A **92** (14): 6414-8.

Zamzami, N. and Kroemer, G. (2001). "The mitochondrion in apoptosis: how Pandora's box opens." Nat Rev Mol Cell Biol **2** (1): 67-71.

Zardoya, R., Abouheif, E. and Meyer, A (1996). "Evolution and orthology of hedgehog genes". Trends Genet **12**:496-497

Zavadil, J., Cermak, L., et al. (2004). "Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition." Embo J **23** (5): 1155-65.

Zecchini, V., Brennan, K., et al. (1999). "An activity of Notch regulates JNK signalling and affects dorsal closure in Drosophila." Curr Biol **9** (9): 460-9.

Zhang, J., Chen, H., et al. (2001). "Epstein-Barr virus BamHi-a rightward transcript-encoded RPMS protein interacts with the CBF1-associated corepressor CIR to negatively regulate the activity of EBNA2 and NotchIC." J Virol **75** (6): 2946-56.

Zheng, W., Seftor, E. A., et al. (2001). "Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-beta." Am J Physiol Heart Circ Physiol **280** (2): H909-17.

Zhong, T. P., Rosenberg, M., et al. (2000). "gridlock, an HLH gene required for assembly of the aorta in zebrafish." Science **287** (5459): 1820-4.

Zhou, S., Fujimuro, M., et al. (2000). "SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function." Mol Cell Biol **20** (7): 2400-10.

Zimber-Strobl, U., Strobl, L. J., et al. (1994). "Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless." Embo J **13** (20): 4973-82.

Zou, Y., Hu, Y., et al. (1998). "Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells (review)." Int J Mol Med **1** (5): 827-34.