# The Role of the Notch Signalling Pathway in Vascular Smooth Muscle Cell Apoptosis

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.

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**Abstract**

The Notch signalling system is a highly conserved method of cell-to-cell communication involved in cell fate decisions in many cell types. Until recently, the importance of the Notch signalling pathway was recognized in the embryonic but not in the adult vasculature. This project has successfully identified the presence of components of this pathway in adult vascular smooth muscle cells (VSMC). The effect of this pathway on serum deprivation- and cyclic strain-induced VSMC apoptosis has also been determined. This study reports that over-expression of components of the Notch signalling pathway results in a decrease in serum deprivation-induced VSMC apoptosis, whereas endogenous inhibition of the pathway increases apoptosis in these cells. The effect of the Notch signalling pathway on VSMC apoptosis is mediated, at least in part, in a CBF-1-dependent manner. A possible mechanism of the Notch signalling pathway regulation of apoptosis is through interaction with members of the Bcl-2 family of apoptotic proteins. Notch over-expression decreases pro-apoptotic *bax*, and increases anti-apoptotic *bcl-x*$_L$ expression in VSMC in a CBF-1-dependent manner. In addition, this study has shown previously unreported interaction of the Notch and NFκB signalling pathways in VSMC. Additionally, the effect of cyclic strain, a biomechanical force increased in many vascular disease states, was determined on components of the Notch signalling pathway and also on VSMC apoptosis. Increased cyclic strain results in decreased Notch signalling pathway component expression, and increased apoptosis. Over-expression of the Notch receptor, Notch 3, attenuates the cyclic strain-induced apoptosis in a *bcl-x*$_L$-caspase 3-dependent manner. *In vivo* validation of the effects of altered biomechanical forces was performed through analysis of carotid artery, and hepatic portal vein, ligation models. In correlation with the *in vitro* results obtained, increased alterations in cyclic strain results in an inverse relationship between the Notch signalling pathway and apoptosis in the vessel. These findings may be relevant in the future management of vascular diseases.

**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 |
| ATP | Adenosine tri-phosphate |
| A/U | Arbitary units |
| AV | Annexin V |
| BART | BamHI-A rightward transcript |
| BCA | Bicinchoninic acid |
| bFGF | basic fibroblast growth factor |
| α-gal | α-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 |
| CHAPSO | 3[(3-cholamidopropyl)dimethylamminonio]-2-hydroxypropanesulphonic acid |
| 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-α-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 | Dithiotheitol |
| EBNA-2 | EBV nuclear antigen-2 |
| EBV | Epstein Barr Virus |
| EC | Endothelial cells |
| ECM | Extracellular matrix |
| EDTA | Ethyldiamine |
| EGF | Epidermal growth factor |
| EGTA | Ethyleneglycol-bis-(b-amino-ethyl ether) 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-Jα | Recombination signal binding protein of the Jα 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-α | Transforming growth factor-α |
| 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 |
| $cm^2$ | Centimetre squared |
| °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**

**Sweeney, C** , Morrow, D , Coyle S , Hennessy, C , Scheller, A , Birney, Y , Cummins, P , Walls, D , Redmond, E , and Cahill, PA "Notch 1 and Notch 3 Receptor Signalling Modulates Vascular Smooth Muscle Cell Growth, Apoptosis and Migration" *FASEB Journal, 2004, in press*

Birney, Y , **Sweeney, C** , Cappadona, C , Sitzmann, J , Cummins, P , Redmond, E , and Cahill, PA " Pulsatile Flow Increases Bovine Aortic Smooth Muscle Cell Apoptosis in a Mitogen Activated Protein Kinase-dependent Manner" *Journal of Vascular Research, 2004, in press*

**Sweeney, C** , Morrow, D , Birney, Y , Cummins, P , Redmond, E , and Cahill, PA "Cyclic Strain Inhibits Notch Receptor Signalling in Rat Vascular Smooth Muscle Cells" *(submitted Arterioscler Thromb Vasc Biol, May 2004)*

Morrow, D , **Sweeney, C** , Birney, Y , Cummins, P , Redmond, E , and Cahill, PA "Hedgehog-Notch Interactions in Rat Vascular Smooth Muscle Cells" *(submitted Biochem Biophys Res Commun, May 2004)*

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**Poster Presentations**

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

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**Table of Contents**

# Chapter 1

# Introduction

# Chapter 1 Introduction

## 1 1 Cardiovascular Disease

Cardiovascular diseases are any disease of the circulatory system, chief among which are myocardial infarction, cerebrovascular disease, and other peripheral vascular diseases Changes in the structure, function and integrity of arterial blood vessels is central to the pathogenesis of many cardiovascular diseases The arterial blood vessel in an active, integrated organ composed of endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts divided into three structural layers, termed the tunicas intima, media and adventita The innermost layer, the tunica intima, is a simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibres The underlying tunica media is primarily comprised of SMC, which play a key role in maintaining vascular tone and function SMC are orientated circumferentially in the outer media, but closer to the lumen they present in a more random fashion (Gittenberger-de Groot *et al*, 1999) Several studies have demonstrated a marked heterogeneity of SMC phenotypes in the vessel wall of both human and animal models These phenotypes are classified as synthetic and contractile, and are evident embryonically but become more prominent as the vessel matures Whilst both the intimal and medial layers contain a mixture of both phenotypes, the synthetic phenotype is most commonly associated with the intimal layer and with vascular remodelling These cells resemble immature, dedifferentiated SMC, with lower levels of contractile proteins (ε -actin, myosin, calpomn, smoothelin) and fewer myofilaments than their medial counterparts (Shanahan *et al*, 1993, Bochaton-Piallat *et al*, 1995, Gittenberger-de Groot *et al*, 1999) These intimal cells are said to be similar in both morphology and gene expression to foetal or embryonic SMC, and have been shown to be more prone to both physiological and pathophysiological apoptosis than the medial SMC (Bochaton-Piallat *et al*, 1995, Rao *et al*, 1997, Slomp *et al*, 1997) The contractile SMC, on the other hand, are most commonly associated with the tunica media, and express differentiated cell markers associated with contractile function, and are involved in the synthesis and maintenance of extracellular components of the vessel wall The outermost layer, which attaches the vessel to the surrounding tissue, is termed the tunica adventita This is a layer of connective tissue, with varying amounts of elastic and collagenous fibres The connective tissue is dense adjacent to the tunica media, but changes to loose connective tissue near the periphery of the vessel

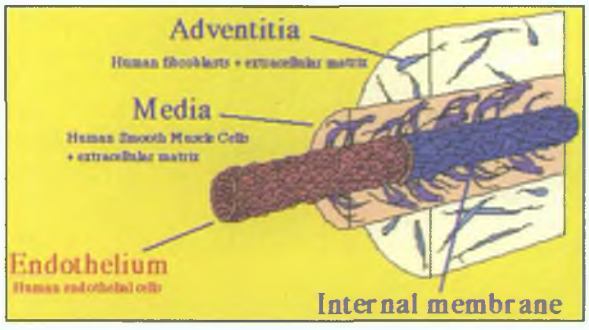



**Figure 1: Arterial blood vessel.** Diagrammatic representation of arterial blood vessel structure.

Cardiovascular diseases are the leading cause of death in the western world, accounting for 40% of all deaths in the United States. An estimated 17 million people die due to cardiovascular disease per annum, which translates into one death every 30 seconds. The incidence of cardiovascular disease in Ireland is the highest in the European Union (EU), with 53 deaths per 100,000 population compared to the EU average of 32. In addition, it is estimated that one quarter of people in the western world live with cardiovascular disease, resulting in a significant economic impact, both in terms of health care expenditures and lost productivity *(World Health Organisation, American Heart Foundation)*. Therefore, an increased understanding of the mechanisms underlying the pathology of cardiovascular diseases is imperative both in the prevention and management of this condition.

A number of risk factors are associated with the development of cardiovascular disease, these include genetic pre-disposition, gender, age and race. Many factors, however, are lifestyle determined, including poor nutrition, obesity, lack of exercise and smoking. A diet high in saturated fat and cholesterol can increase the risk of developing certain cardiovascular disorders. Whilst cholesterol has important functions in the body, such as the formation of cellular membranes, it is important that the correct ratio of cholesterols within the body is maintained. Cholesterol is insoluble in blood and is transported via lipoproteins, these are high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL), the ratio of HDL to LDL is an important factor in determining the risk that cholesterol presents. Elevated LDL and low HDL levels are key risk factors in the development of atherosclerosis (Sharrett *et al.*, 1994). In addition, an increased level of homocysteine in the blood is also a major risk factor for cardiovascular disease, and can be caused due to dietary deficiencies in vitamins B6, B12 and folic acid (Boers 2000). Obesity is also a key risk factor in the development of cardiovascular disease, and is a considerable problem in

Ireland, where 47% of people are considered overweight or obese Regular exercise can help combat or prevent obesity, in addition to increasing HDL levels in the circulation *(Irish Heart Foundation)* Smoking directly damages the endothelium, increases vascular tone, increases platelet activation, and promotes LDL oxidation (Muscat *et al*, 1991) Smoking increases the risk of developing cardiovascular disease by 50% and contributes to one fifth of all cardiovascular related deaths *(American Heart Association website)*

## 1 2 Hypertension

Hypertension (high blood pressure) can develop due to a genetic pre-disposition, or may be environmentally induced due to poor diet, obesity, smoking and lack of exercise In addition, the development of hypertension is itself a major risk factor in the development of cardiovascular diseases, such as arteriosclerosis Normal blood pressure is considered to be 120/80 mm Hg, whereas blood pressure of above 140/90 mm Hg is considered to be high, and can result in vascular SMC (VSMC) hypertrophy and hyperplasia, and a subsequent increase in peripheral vascular resistance (Molloy *et al*, 1999)

## 1 3 Atherosclerosis

Atherosclerotic vascular disease, the primary cause of all cardiovascular diseases, is a systemic disease involving the intima of large and medium arteries, including the aorta, and the carotid, coronary and peripheral arteries (Corti *et al*, 2002) Atherosclerosis is characterized by a dysfunctional endothelium, chronic inflammation, lipid accumulation, aberrant regulation of VSMC fate decisions, and often calcification, which clinically manifests as an atherosclerotic plaque The generation of obstructive plaques can result in additional cardiovascular disorders, for example, an obstructive coronary plaque can cause a critical reduction in coronary blood flow, and subsequent myocardial ischaemia (Schroeder and Falk 1995) Atherosclerotic plaques occur preferentially at bifurcations and curvatures of arterial blood vessels (Glagov *et al*, 1988)

### 1.3.1 Molecular Process of Atherosclerosis

Normal endothelial function plays a pivotal role in vascular homeostasis, and limits the development of atherosclerosis. Endothelial dysfunction, therefore, is an initial pathological sign of atherosclerosis. Cardiovascular risk factors and surgical interventions, such as balloon angioplasty and vascular reconstruction, can impair endothelial function. EC function as a semi-permeable barrier, and also as a dynamic paracrine and endocrine organ, exerting considerable influence on the underlying VSMC, or on circulating blood elements such as platelets. EC are, for example, involved in maintaining the non-thrombogenic blood-tissue interface by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow. This is achieved through the production of thrombogenic (thromboxane) and anti-thrombogenic (thrombomodulin) factors, and pro- and anti-inflammatory mediators, such as leukocyte adhesion molecules and nitric oxide respectively. In addition, the endothelium can release substances that act as SMC promoters and vasoconstrictors, such as angiotensin II, or as SMC inhibitors and vasodilators, such as prostacyclin (Corti *et al.*, 2002). Therefore, endothelial dysfunction can result in the accumulation of cellular components, lipids and extracellular matrix (ECM) to yield a fibrofatty plaque, which can ultimately narrow the arterial lumen (Geng and Libby 2002).

**Figure 2: Initiation, Progression and Complication of Human Atherosclerotic Plaque**

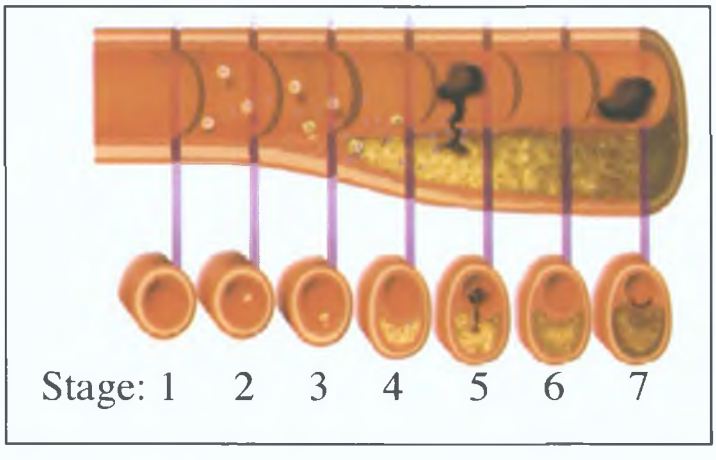



**Figure 2: Human atherosclerotic plaque progression.** Refer to text (Libby 2001).

Damage to the normal endothelium (Figure 2: stage 1) results in the initiation of an inflammatory response. The endothelium expresses adhesion and chemoattractant

molecules, that act to recruit inflammatory leukocytes, such as monocytes and T-lymphocytes, in addition, extra-cellular LDL begins to accumulate in the tunica intima, in part by binding to proteoglycans, and undergoes oxidative modification (Figure 2 stage 2) (Berliner *et al*, 1997, Williams and Tabas 1998) Accumulated monocytes in the arterial wall subsequently express scavenger receptors, bind to oxidized-LDL (ox-LDL), and transform to lipid-laden foam cells, facilitated in part by macrophage colony-stimulating factor (Rohrer *et al*, 1990, Qiao *et al*, 1997) In addition, leukocytes and endogenous cells of the vascular wall can secrete inflammatory cytokines and growth factors than further amplify leukocyte recruitment and cause VSMC migration and proliferation (Figure 2 stage 3) The formation of this mtimal macrophage-rich fatty streak, the precursors of atherosclerotic lesions, appears to be ubiquitous in humans, and can develop over many years, in fact, these possible pre-cursors to atherosclerosis have been found in the intima of infants (Stary *et al*, 1994) Lesion progression (Figure 2 stage 4) can occur due to the expression of tissue factor, a potent coagulant, and matrix-degrading proteinases that can act to weaken the integrity of the plaque If the plaque subsequently ruptures (Figure 2 stage 5), coagulation factors in the blood gain access to the thrombogenic, tissue factor-containing lipid core resulting in thrombosis Depending on the balance between pro-thrombogenic and fibrinolytic mechanisms, an occlusive thrombus could occur at this stage, with deleterious clinical consequences or death Alternatively, resorption of the thrombus (Figure 2 stage 6) results in the release of thrombin and other mediators from de-granulating platelets These include platelet-derived growth factor (PDGF) and transforming growth factor-ε (TGF-ε) This results in collagen and SMC accumulation, and the progression of the lesion from a fibrofatty to an advanced fibrous and often calcified plaque, oftentimes causing significant stenosis of the vessel In some cases, occlusive thrombi arise from superficial erosion of the endothelial layer (Figure 2 stage 7), and often complicate advanced stenotic lesions (Libby 2001)

**Figure 3 Schematic Representation of Molecular Processes of Atherosclerosis**

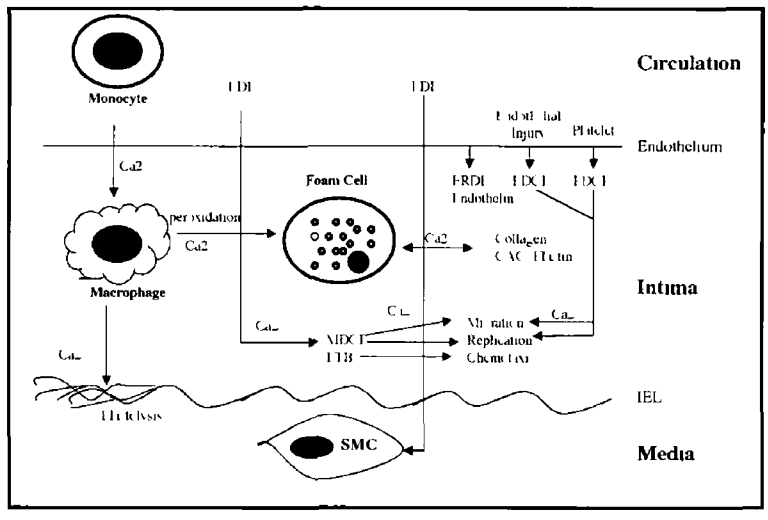



**Figure 3 Schematic representation of molecular processes of atherosclerosis** Calcium acts as a second messenger in atherogenic processes EDRF, endothelium-derived relaxing factor, EDGF, endothelium-derived growth factor, PDGF, platlet-derived growth factor, MGDF, macrophage-derived growth factor, GAG, glycosaminoglycans, PG, proteoglycans, GP, glycoprotein, IEL, internal elastic lamina, LDL, low density lipoprotein, LTB, leukotriene B (adapted from Kramsch 1995)

However, not all fatty streaks evolve into advanced atherosclerotic lesions The balance between pro- and anti-atherogenic factors determines whether a fatty streak progresses to form an atherosclerotic lesion or regresses In addition to inflammatory cell infiltration, the progression of an atherosclerotic lesion requires the participation of VSMC, which are a principal source of the ECM that constitutes a large volume of an advanced atheroma (Geng and Libby 2002) Proliferation and migration of SMC play an important role in the regulation of SMC number within an atherosclerotic plaque, however, the importance of SMC apoptosis in the atherosclerotic plaque is being increasingly recognized By counterbalancing proliferation, apoptosis may limit the cell accumulation in the intimal compartment, elimination of the lipid-laden foam cell, and a decrease in overall cell number, can lead to a regression of the fatty streak (Geng and Libby 2002)

### 1 3 2 Apoptosis in the Atherosclerotic Plaque

Apoptosis has been observed at many stages of the development of an advanced atherosclerotic plaque (Kockx *et al*, 1998) Atherosclerotic plaques typically consist of a lipid-rich core in the central portion of a thickened tunica intima, containing lipids, connective tissue, and dead cells or cell debris The lipid-core is bound on its luminal aspect by a fibrous cap, at its edges by what is termed the "shoulder" region, and

on its abluminal side by the base of the plaque (Libby 1995) Apoptosis is important in the progression of atherosclerotic lesions, resulting in the formation of a mature lesion containing a dense ECM and a relatively sparse cell population Apoptosis is concentrated in the lipid-rich core of the plaque, and occurs in both macrophage/foam cells and VSMC However, the 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 Several mechanisms may be responsible for this, for example, intracellular accumulation of lipids may decrease the ability of macrophages and SMC to phagocytose apoptotic cells, increased apoptosis of macrophages in the lesion decreases the population of apoptotic scavenging cells, and cross-linking of macromolecules can result in the stabilization of apoptotic cells within the lesion (Aeschhmann and Thomazy 2000) The presence of apoptotic cells or bodies has recently been shown to increase calcification and fibrosis of atherosclerotic lesions (Geng and Libby 2002)

Atherosclerosis is considered to be a relatively benign disease as long as complicating thrombosis can be prevented Thrombosis underlies the most acute complication of atherosclerosis, notably unstable angina and acute myocardial infarction in the coronary circulation The lipid-rich core of an atherosclerotic lesion contains large amounts of tissue factor, which is a powerful coagulant that stimulates thrombus formation when in contact with the blood (Wilcox *et al*, 1989, Schroeder and Falk 1995, Libby 2001) 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 the integrity of the fibrous cap fundamentally determines the stability of a plaque, and its clinical implications

In 1995, Libby *et al*, dubbed VSMC the "guardians of the integrity of the fibrous cap" As its name implies, the fibrous cap is made up of a dense fibrous ECM, primarily composed of collagens and elastin It is now well established that the fibrous cap can undergo continuous remodelling, which is largely influenced by VSMC VSMC synthesize and assemble the interstitial collagen, which accounts for the bulk of the ECM of the fibrous cap Therefore, apoptosis of VSMC and a subsequent reduction in cell number can seriously compromise the integrity of the fibrous cap In addition, the integrity of the fibrous cap can be weakened by the breakdown of existing ECM proteins through induction of protcolytic enzymes, such as matrix metalloproteinases, by inflammatory cells (Libby 2001) SMC of the arterial plaque also produce matrix metalloproteinases, however, these enzymes primarily effect SMC migration and

vascular remodelling (Plutzky 1999)

The realization that the probability of the atherosclerotic plaque rupture, rather than the severity of plaque stenosis, determines its clinical implications led to the classification of atherosclerotic plaques as stable or vulnerable plaques Most myocardial infarctions, for example, occur in lesions of less than 70% stenosis, with plaque rupture being the underlying pathological event (Falk *et al*, 1995)

**Figure 4 Characteristics of Vulnerable and Stable Atherosclerotic Plaques**

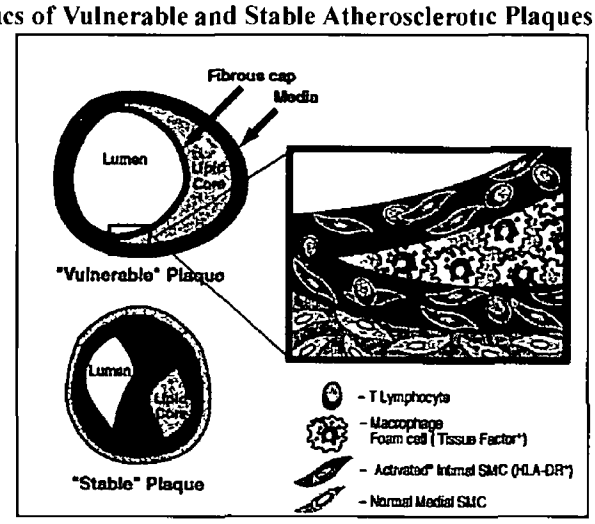



**Figure 4 Schematic representation of vulnerable and stable atherosclerotic plaques** (Kramsch 1995)

The stable atherosclerotic plaque is not considered likely to rupture, and as such, is clinically not as serious as its "vulnerable" counterpart The stable plaques are described as hard, sclerotic plaques, with a high VSMC and collagen content, a subsequently thick fibrous cap, and a limited amount of lipid-laden inflammatory cells In contrast, the vulnerable plaque usually has a large lipid-core, a thin fibrous cap, a high density of inflammatory cells, particularly at the "shoulder region" where rupture often occurs (see Figure 4) and a paucity of VSMC An atherosclerotic plaque is considered vulnerable when its lipid-rich core accounts for more than 40% of the total plaque volume (Schroeder and Falk 1995, Plutzky 1999, Corti *et al*, 2002)

Three major factors determine the vulnerability of the fibrous cap, these are lesion characteristics (location, size and consistency), blood flow characteristics, and consequent vessel wall stress or "cap fatigue" Inflammation is also a key factor in plaque disruption Inflammatory cells are a source of plaque tissue factor, and lesion thrombogemcity correlates with its tissue factor content (Moreno *et al*, 1996) In addition, inflammatory cells can secrete cytotoxic substances and proteolytic enzymes that induce VSMC apoptosis and degradation of the fibrous ECM Mechanical forces

experienced by blood vessels are critical in determining both plaque formation and disruption (Schroeder and Falk 1995).

## 1.4 Mechanical Forces

### 1.4.1 Introduction

All tissues in the body are subjected to physical forces, which can originate either from environmental factors, or from tension created by the cells themselves (Traub and Berk 1998; Zou *et al.,* 1998; Xu 2000). Almost all vessels carrying fluids within the body are distensible, and interactions between internal blood flow and vessel wall deformation contribute both to a vessels biological function and dysfunction. The vascular wall is an integrated functional component of the circulatory system that is constantly exposed to mechanical forces of haemodynamic origin. *In vivo*, the vessel walls are exposed to two principal haemodynamic forces, shear stress and cyclic circumferential strain (Davies 1995; Patrick and McIntire 1995).

### 1.4.2 Haemodynamic Forces

**Figure 5: Haemodynamic Forces Experienced by Arterial Blood Vessels**

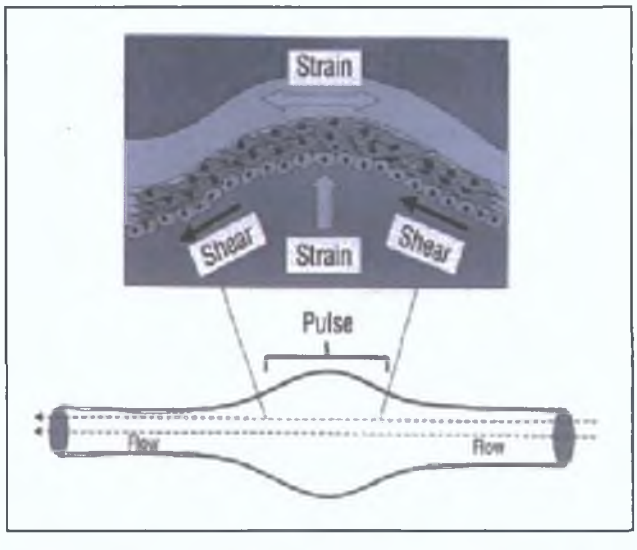



**Figure 5: Haemodynamic Forces Experienced by Arterial Blood Vessels.** Diagrammatic representation of the force exerted by shear stress and cyclic strain on arterial blood vessels.

Shear stress is described as the dragging frictional force resulting from blood flow. Under normal physiological conditions, EC are primarily subjected to this haemodynamic shear stress. However, under conditions of endothelial dysfunction or

denudation, shear stress can also exert its affect on the underlying VSMC In arterial circulation, based on altering vessel wall diameters, the mean wall shear stress is 10 – 70 dyn/cm$^2$ Changes in shear stress can result from changes in pulse pressure, which is defined as the difference between peak systolic and diastolic blood 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 (Davies 1995, Traub and Berk 1998)

Another well characterized haemodynamic force associated with the vasculature is cyclic circumferential strain (cyclic strain) Cyclic strain in arterial vessels is due to the repetitive pulsatile force on the vessel wall due to arterial blood pressure Cyclic strain can be multi-dimensional as the pulsatile force acts perpendicular to the blood vessel, resulting in "stretching" of the vascular cells in multiple planes All cells of the vessel wall experience cyclic strain under normal physiological conditions VSMC, which constitute the major component of the vessel wall, together with elastin and collagenous components, absorb most of the pressure-induced cyclic strain Normal blood pressure is considered to be 120/80 mm Hg, whereas blood pressures of above 140/90 mm Hg and below 90/60 mm Hg are considered high and low respectively Factors ranging from physical exertion to psychological stress can result in a transient rise in blood pressure, and a consequent transient increase in cyclic stress Genetic predisposition to hypertension can lead to a chronic increase in cyclic stress, resulting in potentially serious clinical manifestations Conversely, factors such as electrolyte imbalance, ischaemic heart disease and systemic sepsis can result in transient hypotension, and a consequent transient decrease in cyclic stress *(American Heart Association website)*

### 1 4 3 Role of Haemodynamic Forces

Haemodynamic forces are known to exert both physiological and non-physiological effects of the vascular wall Haemodynamic forces play a critical role in the regulation of vascular tone, remodelling, and influence vascular cell homeostasis (Folkow 1995, Thubrikar and Robicsek 1995, Zou *et al*, 1998, Li and Xu 2000) The arterial wall is continuously exposed to such forces, changes in which directly regulate vascular structure Shear stress, for example, stimulates the release of nitric oxide and prostacyclin from EC resulting in vessel relaxation (Rubanyi *et al*, 1986, Bhagyalakshmi and Frangos 1989) The effect of cyclic strain on blood vessel structure

and function is mediated primarily by VSMC VSMC are a major constituent of blood vessels, and as such, contribute prominently to the regulation of tissue strength A certain level of mechanical strain due to blood flow is essential to develop and maintain a differentiated and functional SMC phenotype (Birukov *et al*, 1998) VSMC contribute to the regulation of vascular tone by synthesizing and organizing the extra-cellular matrix, and regulating its degradation (Schlumberger *et al*, 1991, Galis *et al*, 1994) Many studies have shown that changes in cyclic strain results in changes in both gene expression and protein synthesis of many components of the VSMC Increases in cyclic strain also results in increased synthesis of matrix components *in vitro* (Leung *et al*, 1976, Cheng *et al*, 1997)

### 1 4 4 Clinical Implications of Altered Biomechanical Forces

A common feature of vascular disease is altered or elevated biomechanical stress Spontaneous atherosclerotic lesions, for example, occur preferentially at bifurcations and curvatures of arterial blood vessels, where haemodynamic forces are disturbed (DeBakey *et al*, 1985, Thubrikar and Robicsek 1995) In additon, 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 the increased biomechanical force on the venous vessel (Xu 2000) Alterations in VSMC fate decisions have been associated with numerous vascular disease states Proliferation of VSMC contributes 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) Mechanical forces associated with blood flow have been implicated in the regulation of vascular cell fate as a fundamental feature in the pathogenesis of vascular disease An alteration in the balance between VSMC proliferation and apoptosis, or an increase in VSMC migration, can result in remodelling of the vasculature (Wang *et al*, 1999a) In addition, mechanical strain has 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

### 1.4.5 Vascular Remodelling

Remodelling is both a physiological and pathological process in the vasculature. Remodelling can be defined as a change in the calibre of the vessel, and can be sub-divided into hypertrophic, hypotrophic or eutrophic remodelling, due to an increase, decrease or no change in the overall tissue mass respectively. Remodelling can result in a decrease or increase in both wall:lumen ratio and vessel luminal diameter, termed inward and outward remodelling respectively (Mulvany 1999). The biological processes that can affect vascular remodelling include VSMC proliferation, apoptosis and migration, adventitial fibrosis and migration of adventitial fibroblasts (Scott *et al.*, 1996; Shi *et al.*, 1996; Shi *et al.*, 1997). These processes can exert major changes in arterial architecture either alone, or in co-ordination with each other (Bennett 1999).

**Figure 6: Vascular Remodelling**

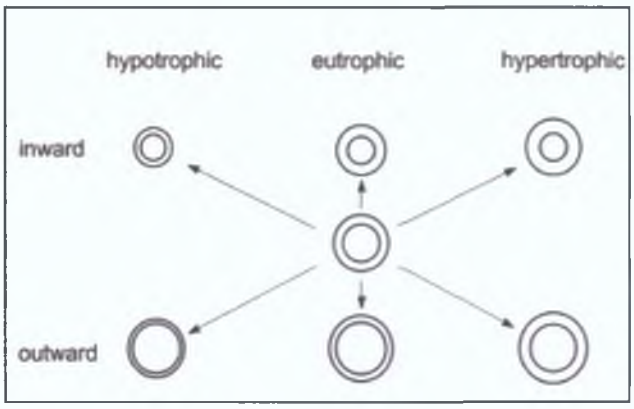



**Figure 6: Vascular Remodelling.** The starting point for vascular remodelling is represented by the vessel at the centre of the figure. Hypotrophic, eutrophic and hypertrophic remodelling are illustrated. This remodelling can be inward or outward, resulting in a reduced or increased luminal diameter respectively (Mulvany 1999).

Physiological remodelling is evident during vascular remodelling after birth. Closure of the ductus arteriosus, for example, is due to vascular remodelling accompanied by increased VSMC apoptosis, and concomitant changes in VSMC proliferation and matrix synthesis (Slomp *et al.*, 1997). Inappropriate vascular remodelling, including its absence, is a prominent feature of the pathogenesis of many vascular diseases. Many studies have demonstrated that changes in intravascular forces that occur in disease states, such as hypertension, result in a decreased lumen and increased media:lumen ratios in arterial blood vessels, as seen in essential hypertensive as compared to normotensive patients (Nordborg *et al.*, 1983).

### 1 4 6 Mechanical Force-induced Apoptosis

Apoptosis plays a pivotal role both in normal vasculogenesis and in the pathobiology of the vascular system Apoptosis is virtually absent in normal adult vessels, but is a prominent feature of pathological conditions involving vascular remodelling This suggests a direct relationship between mechanical force and the regulation of apoptosis In recent years, many studies have independently proven the relationship between increased mechanical forces and increased levels of VSMC apoptosis Analysis of atherosclerotic lesions in both human and animal models reveal high levels of VSMC apoptosis (Kockx 1998, Mayr and Xu 2001) Isolated human SMC from atherosclerotic plaques display a higher tendency to undergo both spontaneous and induced apoptosis than VSMC isolated from normal vessels (Bennett 1999) In addition, a study of biomechanical-induced apoptosis in the development of vein graft arteriosclerosis revealed that the number of apoptotic VSMC in the vein wall increased for at least eight weeks following grafting to an artery, but not to any vein, thus proving the effect of increased mechanical forces on apoptosis (Xu 2000, Mayr *et al*, 2002) Apoptotic cell death following vascular injury is a highly regulated process governed, at least in part, by activation of the mitogen-activated protein kinase (MAPK) signalling pathway and the relative expression of apoptotic genes (Xu 2000) However, the mechanisms of signal transduction converting alterations in biomechanical force to alterations in apoptosis have not been fully elucidated Recent studies have implicated both the endothelin and $\varepsilon_1$-integrin receptors as possible mediators of VSMC apoptosis due to increased cyclic strain *in vitro* (Cattaruzza *et al*, 2002, Wernig *et al*, 2003)

### 1 4 7 Mechanosensors

The transduction of alterations in mechanical forces to either physiological or pathological alterations in VSMC morphology and function, involves sensing of the physical stimulus by the VSMC, which is achieved by a mechanosensor Mechanical stress may initiate signalling by physically perturbing the cell surface or altering receptor conformation in a non-biochemical manner This can result in activation of signalling pathways, and altered gene expression in the cell (Li and Xu 2000) Mechanosensors in VSMC include integrins, receptor tyrosine kinases, G proteins and G protein-coupled receptors, and ion channels In addition, both the protein kinase C (PKC) and MAPK pathways are activated in VSMC in response to mechanical stress

(Li and Xu 2000, Xu 2000)

**Figure 7 Model for Mechanical Stress-induced Signal Transduction in VSMC**

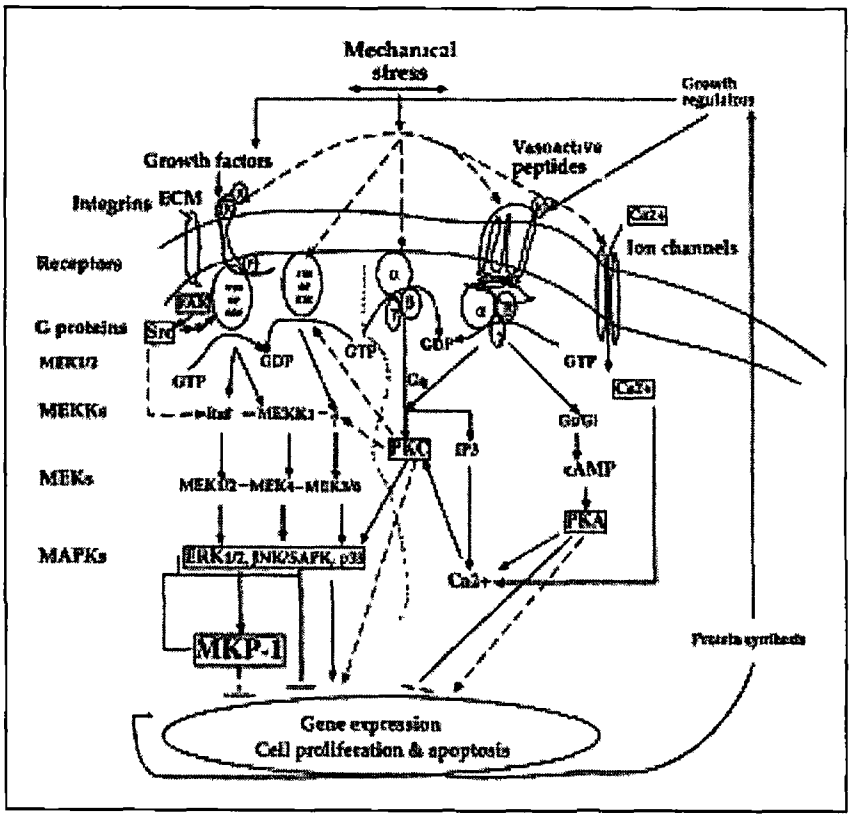



**Figure 7 Model for mechanical stress-induced signal transduction in VSMC** Mechanical stress elongates the cell membrane, resulting in exposure of the kinase domain of receptors, or altered G protein receptor conformation Integrins and ion channels are also involved in the mechanical response to stress in VSMC Subsequently, activation of Raf, MAPK kinases (MEKs) and PKCs results in MAPK activation, which mediates gene expression, protein synthesis and VSMC fate decisions In addition, MAPK can mediate MAPK phosphatase-1 (MKP-1) or protein kinase A (PKA) activation, which can serve as negative regulators for MAPKs and cyclins (Li and Xu 2000)

### 1 4.7.1 Integrins

VSMC are surrounded by basement membranes largely consisting of ECM Integrins are a family of transmembrane receptors that mediate VSMC attachment to ECM proteins at sites called focal attachments (Jockusch *et al*, 1995) Integrins are heterodimers composed of non-covalently bound transmembrane α and β subunits A diverse family of over twenty different integrin receptors can be formed through the heterodimerization of different combinations of the 15 α and 8 β subunits

Integrins are increasingly recognized as playing an important role as mediators of vascular strain Cyclic strain causes the up-regulation of β3 integrin expression in

human umbilical vein EC (Suzuki *et al*, 1997a), and mechanical stretching of human saphenous vein grafts results in the up-regulation of αv integrin, a receptor for active MMP-2 (Meng *et al*, 1999) In addition, the VSMC response to strain was attenuated by antibodies to both β3 and αvβ5 integrins (Wilson *et al*, 1995) The expression of the αvβ3 integrin appears to be significant in the regulation of vascular remodelling This receptor not only acts as a receptor for numerous ECM ligands to mediate cell adhesion, it can also transmit extracellular stimuli into intracellular signalling events (Ross 1993, Aplin *et al*, 1998) These include increases in intracellular calcium and tyrosine phosphorylation of a number of cellular proteins, including the adaptor protein Shc This results in a number of downstream events, including cytoskeletal re-organization leading to VSMC migration, and activation of the MAPK pathways resulting in mitogenic responses (Schlaepfer *et al*, 1999) Therefore, integrin-mediated signal transduction appears to be an important event in response to mechanical stretch

### 1 4 7 2 Receptor Tyrosine Kinases

A growing number of receptor tyrosine kinases are recognized as being regulated by mechanical forces both in EC and VSMC Mechanical stress, for example, has been shown to increase both vascular endothelial growth factor (VEGF) expression and phosphorylate the VEGF receptor in VSMC (Smith *et al*, 2001) This has recently been described as being mediated by phosphatidylinositol 3-kinase (PI3K) and an atypical PKC, PKC-zeta (Suzuma *et al*, 2002) In addition, PDGF receptors α and β are reported to be up-regulated by mechanical stress (Ma *et al*, 1999, Li and Xu 2000) Mechanical stress-dependent increases in the endothelin B receptor in VSMC have also been identified in venous graft models This mechanically-induced increase in gene expression is dependent on PKC, and may contribute to graft failure due to endothelin B receptor-mediated SMC hyperplasia and graft occlusion (Riedy *et al*, 1999)

### 1 4 7.3 G Proteins and G Protein Coupled Receptors

A family of G proteins, which consist of α, β and γ subunits, play a central role in coupling receptors to a variety of intracellular enzymes and ion channels In the cardiovascular system these receptors include those for adrenalin, nor-adrenalin,

endothelin and angiotensin II, which facilitate the regulation of adenylyl cyclases, phosphohpases and ion channels The G protein Gq is thought to be important in the process of remodelling in the cardiovascular system This is due to the fact that activators of Gq-coupled receptors, such as angiotensin II and endothelin I, can trigger hypertrophy and hyperplasia in cultured VSMC (Bruce and Nixon 1997, Higashita *et al*, 1997, Touyz *et al*, 1999) In addition, over-expression of Gq proteins and Gq coupled receptors in transgenic mouse models result in the development of cardiac hypertrophy (Li and Xu 2000) Whilst no reports to date investigate Gq protein expression in VSMC following exposure to mechanical stress, an activation of Gq proteins in EC due to shear stress has been shown (Gudi *et al*, 1998a) The importance of G proteins as mechanosensors in VSMC is further highlighted as treatment with the G protein inhibitor, pertussis toxin, results in an attenuation of the cyclic strain-induced activation of the p38 MAPK pathway (Li *et al*, 2000)

#### 1 4 7 4 Ion Channels

Another type of mechanosensor identified on VSMC are cyclic strain-sensitive ion channels These include $Ca^{2+}$ and $Na^{+}$ channels, which increase their frequency of opening following cyclic strain, resulting in a transient increase in intracellular $Ca^{2+}$, $Na^{+}$ and other divalent cations, and membrane depolarization This can contribute to vascular remodelling due to subsequent implications on VSMC cell fate decisions (Higashita *et al*, 1997) The strain-induced increase in $Ca^{2+}$ primarily results from an extra-cellular source due to strain-dependent depolarization of L-type $Ca^{2+}$ channels, in addition, the existence of a novel strain-sensitive intracellular store of $Ca^{2+}$ has been suggested, but as yet is not fully investigated (Li and Xu 2000)

#### 1 4 7 5 Mechanical Stress-induced Signal Transduction in VSMC

PKC activation plays a central role in cellular signal processing in many cell types, including vascular cells PKC is a large family of serine/threonine kinases, and can be divided into three sub-groups, the conventional, the novel and the atypical PKCs, denoted cPKC, nPKC and aPKC respectively The PKC family are activated by a large number of extra-cellular signals and, in turn, modify the activity of numerous targets such as cytoskeletal proteins, MAPK and transcription factors (Li and Xu 2000) Exposure of VSMC to cyclic strain has been shown to result in a significant increase in

PKC activity, with a concomitant increase in VSMC proliferation (Mills *et al*, 1997) In addition, PKC-ε1 and -ε2 expression and activity are increased in cardiac myocyte hypertrophy, which occurs, at least partly, as a result of altered biomechanical stresses and can lead to congestive heart failure (Li and Xu 2000) Numerous studies have shown that PKC is required for MAPK activation in response to several stimuli, such as growth factors, hormones, and environmental stress, leading to cell hypertrophy, proliferation, migration, growth inhibition or apoptosis (Leng *et al*, 1996, Pukac *et al*, 1998, Bowling *et al*, 1999, Martelli *et al*, 1999)

In addition to PKC, the MAPK signalling pathway plays a central role in the vascular response to altered biomechanical stress The MAPK signalling pathway can be divided into three possible sub-families, extracellular-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK MAPKs are phosphorylated (activated) by MAPK kinases (MEKs), these are primarily dual specific kinases that catalyze the phosphorylation of MAPKs on both tyrosine and threonine residues MEKs are subject to phosphorylation by MEK kinases (MEKKs), which are serine/threonine kinases, and are themselves subject to regulation by MEKK kinases (MEKKKs), many of which have been identified as small G proteins, such as Ras and Rac (Xu 2000)

Many studies have documented the up-regulation and activation of components of the MAPK signalling pathways due to alteration in biomechanical stress ERK 1/2 over-expression and activation, for example, has been documented both *in vivo* and *in vitro* in response to increases in both shear stress and cyclic strain Atherosclerotic lesions show increased expression of activated ERK 1/2 (Li *et al*, 1999), and ERK 1/2 is activated by cyclic stretch in VSMC in a time- and strength-dependent manner In addition, increased levels of shear stress activate ERK 1/2 in EC and cardiac myocytes (Suzuki *et al*, 1997a, Wang *et al*, 1999b) Both hypertension and balloon angioplasty induce expression and activation of ERK 1/2 and JNKs in rat vascular models (Wilson *et al*, 1995, Suzuki *et al*, 1997a) In addition, cyclic strain can rapidly induce activation of JNKs in VSMC (Gudi *et al* 1998b, Wang *et al*, 1999b), suggesting that mechanical forces also influence JNK activation and expression Cyclic strain also results in a rapid p38 MAPK phosphorylation in VSMC in a time- and strength-dependent manner This is apparently mediated by PKC-Ras/Rac pathways, and results in increased cellular proliferation and apoptosis (Gudi *et al*, 1997, Mochly-Rosen and Gordon 1998, Mayr *et al*, 2000, Mayr *et al*, 2002) In addition, this group has also revealed that inhibition of p38 activation in VSMC results in an attenuated cyclic strain-

induced increase in VSMC migration, presumably as p38 mediates cytoskeletal reorganization within the cell Therefore, biomechanical stress-induced activation of the MAPK pathway can have a profound effect on VSMC fate decisions, and subsequently on vascular remodelling and cardiovascular disease

## 1 5 Apoptosis

### 1 5 1 Introduction

Apoptosis was first described by Kerr *et al*, the term originating from Greek meaning falling (ptosis) off (apo) (Kerr *et al*, 1972) Apoptosis is described as a physiologically relevant and active form of cell death whose control and mediation is cell-specific and contextual, and is highly conserved throughout evolution (Hetts 1998, Bai *et al*, 1999) 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 both quickly and efficiently

Cells of multi-cellular organisms generally die in one of two well-characterized ways, depending on the context and cause of death These two forms of cell death, apoptosis and necrosis, can be defined and contrasted on the basis of their individual mechanisms, biochemistry, and altered cellular morphology (Hetts 1998) Necrosis is a passive form of cell death, resulting from external noxious stimuli, and is pathologic in that localized injury and inflammation is produced A classic example of necrosis is ischemic necrosis of the cardiomyocyte during acute myocardial infarction (Yeh 1997) The necrotic process is characterized by severe cell swelling, breakdown of the membrane barrier and resulting release of the cellular components into the extracellular space, and random degradation of nuclear DNAs The release of extracellular components such as kinins incites localized inflammation, edema, capillary dilation and macrophage aggregation (Yeh 1997, Hetts 1998, Kuan and Passaro 1998) The inflammatory response is lengthy and unpredictable in its timecourse, often taking hours to days to occur and subside (Kuan and Passaro 1998) Although necrosis may be important in acute injury and certain acute inflammatory responses, it is not the mechanism whereby cells normally die (Hetts 1998)

In contrast, apoptosis is an active, contained process, resulting from either external or internal stimuli (Kuan and Passaro 1998) The morphology of an apoptotic

cell is clearly distinct to that of a necrotic cell. 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 (Steller 1995; Yeh 1997). Apoptotic bodies are formed due to cleavage of the membrane, these are phagocytosed and digested by macrophages or neighbouring cells, or undergo secondary necrosis. As no cytosolic components are released into the extracellular space, an inflammatory response is not initiated (Hetts 1998; Kuan and Passaro 1998). Unlike necrosis, this process is relatively rapid, reaching completion in approximately two hours (Kuan and Passaro 1998).

The genetics and molecular mechanisms of apoptosis were first characterized during studies in *C. elegans*. Programmed cell death during the development of this nematode is highly precise and predictable, of the 1090 cells produced during development, 131 are destined to die (Ellis *et al.*, 1991). Such studies have identified four sequential steps during the process of apoptosis, commitment to cell death induced by extracellular or intracellular triggers, activation of intracellular proteases, engulfment of the apoptotic bodies by other cells, and degradation of the apoptotic bodies within the lysosomes of the phagoctyotic cells (Steller 1995). Genetic analysis of factors involved in apoptosis in *C. elegans* implicated three main genes, cell death defective 3 (ced 3), ced 4 and ced 9. As the genetic control of apoptosis is conserved throughout evolution, human homologues of these genes have been identified, these are caspase 8, Apaf-1, and Bcl-2 respectively (Hetts 1998).

**Figure 8: The Apoptotic Program is conserved in Evolution**

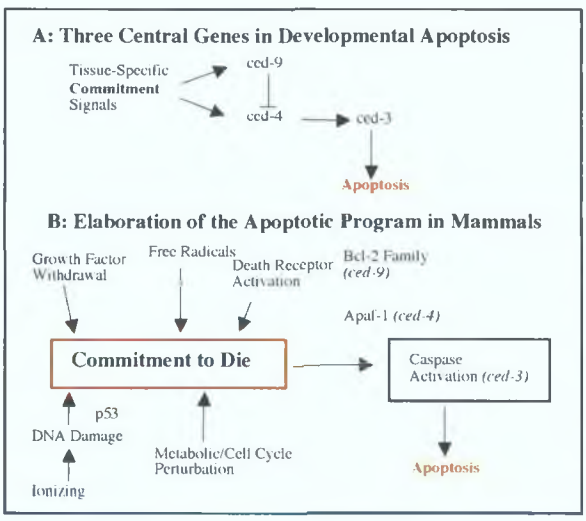



**Figure 8: The apoptotic program is conserved in evolution.** A: Tissue-specific signals activate ced-4, which activates ced-3 resulting in apoptosis. If activated, ced-9 can inhibit apoptosis by inhibiting ced-4-mediated activation of ced-3. B: A wide array of factors commit a mammalian cell to die, but the downstream apoptotic machinery is conserved (*C. elegans* genes homologous to mammalian apoptotic genes shown in italics). Arrows indicate positive interactions; blunted arrows, negative interactions. Adapted from Hetts 1998.

### 1.5 2 Triggers of Apoptosis

Apoptosis-inducing stimuli can be either extrinsic or intrinsic, and can cause apoptosis through the activation of a number of different pathways In most cases, however, these pathways converge on the caspase system of enzymes to execute their function Extrinsic triggers of apoptosis include activation of receptor-mediated death-signalling pathways, for example Fas ligand activation, exposure to substances that cause DNA damage including chemotherapeutic agents and ionizing radiation (Hetts 1998, Rich *et al*, 2000) Apoptosis can also be induced due to the removal of death-inhibiting (or survival-promoting) ligands, for example, vascular smooth muscle cells undergo apoptosis due to the withdrawal of growth factors, such as insulin-like growth factor and PDGF (Best *et al*, 1999) In addition, intrinsic signals such as increased intracellular oxidative stress, can cause the initiation of apoptosis within the cell, in which the mitochondria play a pivotal role (Desagher and Martinou 2000)

#### 1.5 2.1 Receptor-Mediated Death Signalling Pathways

Death receptors belong to the tumour necrosis factor (TNF) superfamily, and play 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 initiation of the apoptotic signal by these receptors TNF receptor 1 and Fas (CD95) are two such receptors that initiate apoptosis when activated either 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 forming a death inducing signalling complex (DISC), which recruits and activates the caspase cascade and can culminate in apoptosis of the cell (Yeh 1997, Gupta 2003)

*Fas and Fas Ligand*

Apoptosis following a cellular immune response is primarily mediated by Fas-Fas ligand (Fas-L) interaction (Krammer 2000) Fas is a type I transmembrane receptor, that is abundantly expressed in many tissues, including the heart, EC and VSMC (Gibbons and Pollman 2000) Fas-L, however, displays a more restricted expression Fas-L is a type II membrane protein, but is capable of undergoing cleavage by a

metalloproteinase to generate a soluble, but less biologically active form of the receptor (Tanaka *et al.,* 1995). Both Fas and Fas-L expression can be upregulated by cytokines and stressful stimuli via nuclear factor-κB (NFκB) dependent mechanisms (Nagata 1999).

**Figure 9: Fas-mediated Signalling Pathway of Apoptosis.**

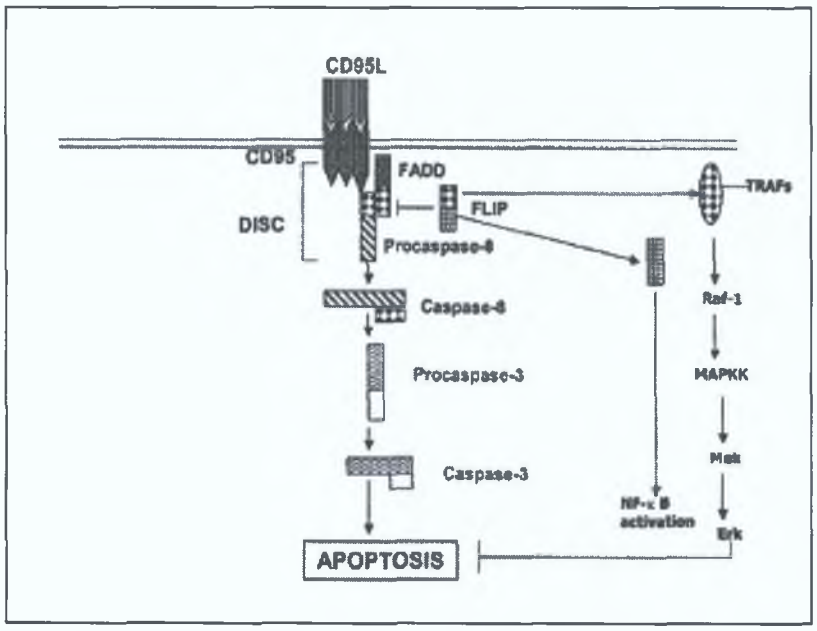



**Figure 9: Fas-mediated signalling pathway of apoptosis.** Following ligation of Fas receptor with Fas antibody, death domains (DD) of Fas receptor are oligomerized and recruit Fas-associated death domain (FADD) and pro-caspase 8 to form a DISC. Pro-caspase 8 is autolytically cleaved, activated and released from the DISC into the cytoplasm where it activates caspase 3 to induce apoptosis. Flice inhibitory protein (FLIP) with homologous death effector domain (DED) prevents the association between FADD and pro-caspase 8 resulting in an inhibition of activation of pro-caspase 8, therefore inhibiting apoptosis (Gupta 2003).

Fas receptor activation results in the trimerization of the receptor, and the formation of a signalling complex of molecules linked to the cytoplasmic portion of the receptor, as the cytoplasmic domain of the receptor does not have intrinsic enzymatic activity. The adaptor protein FADD is recruited to Fas through interactions between their respective death domains. In addition, FADD also contains a DED at its N-terminus, which is responsible for binding to pro-caspase 8 (Flice) to form a DISC. Subsequently, pro-caspase 8 is auto-proteolytically cleaved, triggering the cellular apoptosis cascade (Ashkenazi and Dixit 1998; Gibbons and Pollman 2000; Gupta 2003). In some cells, the levels of pro-caspase 8 is low, therefore the caspase cascade in this case must be amplified by the mitochondria to result in apoptosis (Li *et al.,* 1998a).

Fas-mediated apoptosis is regulated by FLIP, a protein containing two DEDs.

FLIP is present within the cell in two alternatively spliced isoforms, the long form ($FLIP_L$) and the short form ($FLIP_S$), and stable over-expression of these results in resistance to receptor-mediated apoptosis $FLIP_L$ resembles caspase 8 and caspase 10, but lacks protease activity (Irmler *et al*, 1997), therefore effectively functioning as an endogenous inhibitor of apoptosis In addition, FLIP also promotes the activation of the NFεB and ERK signalling pathways through the recruitment of adaptor proteins including RIP and Raf (Kataoka *et al*, 2000)

The induction of Fas-mediated apoptosis in VSMC has been the subject of a number of conflicting reports Sata *et al*, (2000) report that soluble Fas-L and agonistic Fas antibodies fail to induce VSMC apoptosis In contrast, however, viral over-expression of membrane-tethered Fas-L has been shown to promote VSMC apoptosis (Gibbons and Pollman 2000, Sata *et al*, 2000) This discrepancy in VSMC response to Fas may be a consequence of differing VSMC phenotype Chan *et al*, (2000) has shown that the Fas-L resistant VSMC exhibit normal levels of receptor expression and receptor engagement mechanisms However, these cells display decreased expression of FADD, caspase 8 and caspase 3, and increased expression of FLIP when compared to Fas-L susceptible VSMC (Chan *et al*, 2000) This highlights the fact that the VSMC population is heterogenous, and as such, may respond differently to stimuli governing cell fate decisions

### 1 5 2 2 Apoptosis due to DNA Damage

Growth arrest, repair and apoptosis are all legitimate cellular responses to DNA damage The choice of cell fate in each instance will depend on cell type, location, environment, and extent of damage

p53 is a transcription factor that has been implicated in cell cycle arrest and in some, but not all, forms of apoptosis (MacLellan and Schneider 1997) The level of p53 activity within the cell is maintained at a low level, under normal conditions, due to interaction with the Mdm-2 protein, which marks it for ubiquitin-mediated destruction (Mayo *et al*, 1997) DNA damage-induced phosphorylation of either p53 or Mdm-2 prevents these two proteins from interacting, thus stabilizing and activating p53 (Evan and Littlewood 1998) p53 levels are reported to increase within minutes of DNA damage, resulting in growth arrest or apoptosis of the cell (Lundberg and Weinberg 1999)

**Figure 10 Central Role of p53 in Controlling Cell Growth and Apoptosis**

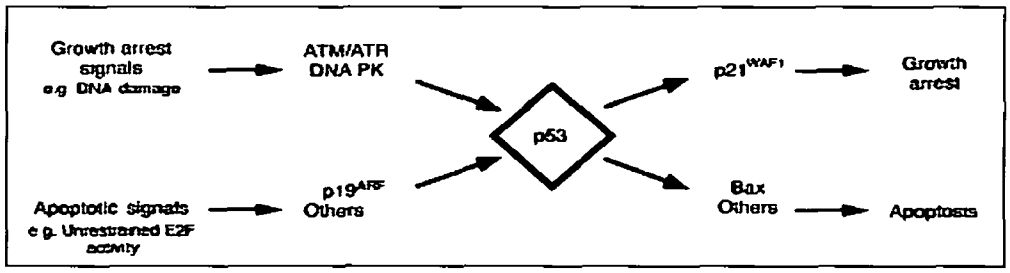



**Figure 10 Schematic representation of the central role of p53 in the regulation of cellular growth and apoptosis** Intermediate steps have been omitted for simplicity (Lundberg and Weinberg 1999)

Several cell-cycle regulators are induced by p53, including the cyclin-dependent kinase inhibitor p21, GADD 45 and members of the 14-3-3 family, resulting in growth arrest, followed by either DNA repair or cell death (Rich *et al*, 2000) p53 alone will not induce apoptosis, but acts as a transcription factor, activating the expression of numerous apoptosis-mediating genes (Bennett *et al*, 1995b) These include Fas molecules and members of the Bcl-2 family of apoptosis regulators, among others Increased levels of p53 result in the up-regulation of Fas expressed on cell surfaces, 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 the anti-apoptotic Bcl-2 protein within the cell p53 also regulates the expression of several proteins that regulate the redox state of the cell The up-regulation of these proteins results in damage to the mitochondria of the cells, causing leakage of mitochondrial components, such as cytochrome c, and subsequent activation of the caspase cascade (Bennett *et al*, 1995b, Gupta 2003)

The regulation of p53 expression within the cell has been linked to cellular myc (c-myc) and E1A proteins, which have both been implicated in the regulation of apoptosis and proliferation (Evan and Littlewood 1998) c-Myc, a transcription factor of the basic helix-loop-helix-zip family, and E1A, a protein encoded by adenovirus has been shown to cause transcriptional up-regulation of p53 (Bennett *et al*, 1995b) In addition, both of these proteins are potent inducers of apoptosis (Samuelson and Lowe 1997)

p53 is also implicated in apoptosis that does not involve obvious DNA damage, including metabolite deprivation, physical damage, heat shock and hypoxia (Debbas and White 1993, Wagner *et al*, 1994, Linke *et al*, 1996)

### 1 5 2 3 Mitochondrial Pathway of Apoptosis

A number of stimuli, including UV radiation, stress molecules (reactive oxygen and reactive nitrogen species), and growth factor withdrawal, mediate apoptosis via the mitochondrial pathway (Gupta 2003) During the process of apoptosis the mitochondria undergo morphological and cellular re-distribution changes The mitochondria undergo a reduction in size and an increase in matrix density (mitochondrial pyknosis) In addition, the mitochondria which are normally dispersed throughout the entire cell, display perinuclear clustering (Desagher and Martinou 2000)

Mitochondria are organelles comprising of a matrix surrounded by an inner membrane (IM), an inter-membrane space, and an outer membrane (OM) The IM contains molecules that contribute to the formation of an electrochemical gradient or membrane potential, these include adenosine tri-phosphate (ATP) synthase and adenine nucleotide translocator The OM contains a voltage-dependent anion channel, whilst the inter-membrane space contains proteins that, when activated, contribute to apoptosis, including holocytochrome c, some pro-caspases, and apoptosis-inducing factor (AIF) (Gupta 2003) At least three mechanisms contribute to mitochondrial-mediated apoptosis These include disruption of electron transport, oxidative phosphorylation and ATP production, alteration of the cellular redox potential, and release of proteins, such as cytochrome c, that trigger activation of the caspase family of proteases (Green and Reed 1998)

## 1 5 3 Effectors of Apoptosis

### 1 5 3 1 The Caspase Cascade

Caspases are an evolutionally conserved family of cysteine proteases, which are viewed as the "central executioners" in apoptotic cell death Caspases are synthesized as enzymatically inert zymogens, requiring proteolytic cleavage at an internal aspartate residue to induce their activation (Gibbons and Pollman 2000, Hengartner 2000) These zymogens are composed of three domains, an N-terminal pro-domain, and two domains termed p10 and p20 The activation of caspases generally results in a serial sequence of caspase activation, known as the caspase cascade, which is a common end pathway in apoptosis induced by many different stimuli Three general mechanisms of caspase activation have been described to date, these are proximity-induced activation,

processing by an upstream caspase, and association with a regulatory subunit (Hengartner 2000)

Proximity-induced activation occurs in the case of caspases 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) Most caspases are activated by cleavage between the p10 and p20 domains, and between the p20 and N-terminal pro-domain Activation of caspase 8 and caspase 9, known as initiator caspases, results in subsequent cleavage and activation of downstream effector caspases, such as caspase 3, caspase 6 and caspase 7 The effector caspases are responsible for the induction of the biochemical and morphological changes associated with apoptosis, and are usually more abundant and active than the initiator caspases (Gibbons and Pollman 2000, Hengartner 2000)

Caspase 9 is activated through 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 (Hengartner 2000, Gupta 2003) Cytochrome c is a nuclear DNA encoded protein, its precursor, apocytochrome c, is synthesized on free ribosomes within the cytoplasm, and can spontaneously insert into the mitochondrial outer membrane (Gonzales and Neupert 1990, Stuart and Neupert 1990) This protein then incorporates a heme group, the protein re-folds, and is inserted into the inter-membrane space The release of functional cytochrome c is reported to be an essential component for the formation of the apoptosome, and subsequent activation of caspases 9 and 3 (Liu *et al*, 1996a) Apaf-1 is another essential component of the apoptosome, and appears to be activated by p53 and adenoviral 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) The binding of cytochrome c to the WD-40 repeats allows a conformational change of Apaf-1, thus exposing the CARD domain This conformational change is stabilized with the binding of ATP or dATP to the Walkers boxes Pro-caspase 9 subsequently binds to the CARD domain, resulting in its activation As Apaf-1 does not have caspase activity, it is proposed that it facilitates caspase 9 auto-catalysis (Cai *et al*, 1998)

Figure 11 Activation Cascade Initiated by Mitochondrial Cytochrome c Release

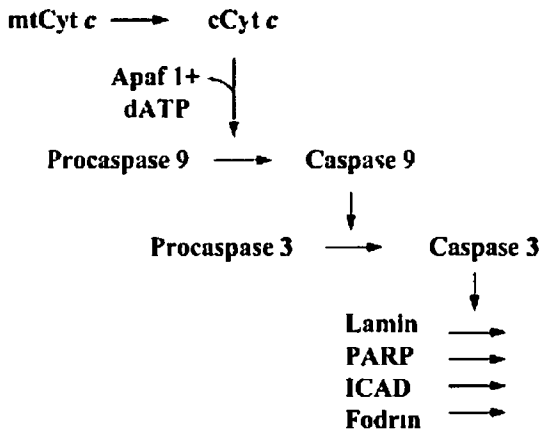



**Figure 11 Activation cascade initiated by mitochondrial cytochrome c release** Release of mitochondrial cytochrome c (mtCyc c) into the cytoplasm (cCyt c) allows interaction with Apaf 1 which, in the presence of dATP, results in the proteolytic activation of pro-caspase 9 Caspase 9 subsequently activates pro-caspase 3 Caspase 3 cleaves a variety of substrates, resulting in the characteristic morphological changes associated with apoptosis (Cai *et al*, 1998)

Caspase 9 subsequently cleaves and activates caspase 3, caspase 6, and a number of other substrates resulting in the biochemical and morphological characteristics of an apoptotic cell These substrates include caspase-activated DNase (CAD), nuclear laminins, cytoskeletal proteins, and p21-activated kinase 2, among others Activation of CAD within the cell results from the caspase 3-mediated cleavage of the CAD inhibitory subunit This active nuclease is subsequently responsible for the characteristic "DNA laddering" of apoptosis Cleavage of cytoskeletal proteins, such as fodrin and gelsolin, results in an overall loss of cellular shape (Kothakota *et al*, 1997), whereas nuclear laminin cleavage is responsible for the characteristic nuclear shrinkage and budding seen in apoptosis (Rao *et al*, 1996) In addition, caspase-mediated cleavage of PAK 2, a member of the p21-activated kinase family, appears to mediate the distinctive blebbing of apoptotic cells (Rudel and Bokoch 1997)

Whilst caspase activation undoubtedly plays an important role in the initiation and execution of apoptosis, a number of caspase-independent inducers of apoptosis have also been identified Reactive oxygen species (ROS), for example, are associated with apoptosis (Suzuki *et al*, 1997b) The generation of oxidants is involved in changes in mitochondrial permeability, and release of molecules, other than cytochrome c, involved in the execution of apoptosis AIF, apoptosis inducing factor, is one such molecule that is released from the mitochondria, and can induce caspase-independent apoptosis AIF is transported to the nucleus where it causes ATP-independent large DNA fragmentation and chromatin condensation (Susin *et al*, 1996, Gupta 2003) In addition, the release of Endo G nuclease from the mitochondrial inter-membrane space is thought to mediate nuclear DNA fragmentation (Li *et al*, 2001)

### 1 5 3 2 The Bcl-2 Family

Bcl-2 was initially identified as a frequent translocation occurring in human B-cell follicular lymphoma, and was found to function by promoting cell survival (Kirshenbaum 2000) Bcl-2 is now recognized as being part of a large family of homologous proteins that can either promote or suppress apoptosis, known as 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)

At least fifteen members of the Bcl-2 family have been identified, and these can be divided into two functional groups, pro-apoptotic and anti-apoptotic Bcl-2 family members Examples of family members that prevent apoptosis are Bcl-2, Bcl-$x_L$ and Bfl-1 among others, whilst Bcl-2 family members that promote apoptosis include Bad, Bax, Bid and Bik, (see table 1)(Reed 1994, Sedlak *et al*, 1995, Green and Reed 1998)

**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

Table 1 Pro- and anti-apoptotic members of the Bcl-2 family of proteins
Table adapted from Mc Lellan and Schneider, 1997

Structural analysis of the Bcl-2 family of proteins has identified four conserved regions within the family, known as the Bcl-2 homology domains (BH1-BH4) (Muchmore *et al*, 1996) All members of the Bcl-2 family contain at least one of these domains, which are formed by ε-helices and thus enable different members of the family to form either homo- or heterodimers and regulate each other (Oltvai *et al*, 1993, Kelekar and Thompson 1998) The majority of Bcl-2 family members share sequence homology at the C-terminal region, with a ε20-residue hydrophobic domain, which targets the Bcl-2 family of proteins to intracellular membranes The principal membrane to which the Bcl-2 family members are directed is the outer mitochondrial membrane, therefore this C-terminal region is critical for the function of both the pro- and anti-apoptotic Bcl-2 family members (Goping *et al*, 1998, Kirshenbaum 2000) Variable sequence homology, however, exists between the BH1 to BH4 domains,

which implies that this variation in homology may determine whether the given Bcl-2 family member acts to promote or prevent cell death (Kirshenbaum 2000). The anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-$x_L$, contain at least three BH domains, and all contain the N-terminal BH4 domain. The BH4 domain is restricted to Bcl-2 family members with anti-apoptotic properties, therefore it is postulated that this domain is critical in preventing apoptosis. This is supported in a number of studies in which the deletion of the BH4 domain rendered the anti-apoptotic Bcl-2 protein defective in suppressing apoptosis (Hunter *et al.*, 1996; Huang *et al.*, 1998). Pro-apoptotic Bcl-2 family members, such as Bax and Bak, have been identified as closely resembling Bcl-2, containing BH1-BH3 domains. Other pro-apoptotic members of this family are described as "BH3 only" as they contain the BH3 domain alone, which is therefore sufficient for the anti-apoptotic activity of these proteins (Kelekar and Thompson 1998). Cell fate is determined by the ratio of pro- and anti-apoptotic members of the Bcl-2 family within any given cell (Sedlak *et al.*, 1995; Reed 1997).

The Bcl-2 family can delay or prevent apoptosis by a diverse number of death signals, thus suggesting that it influences a number of signalling factors that can lead to cell death. Bcl-2 has been shown, for example, to increase the anti-apoptotic NFεB transactivation, which can, in turn, up-regulate anti-apoptotic Bfl-1 and Bcl-$x_L$ expression (Lee *et al.*, 1999a; Kirshenbaum 2000).

**Figure 12: The Major Apoptotic Pathways in Mammalian Cells**

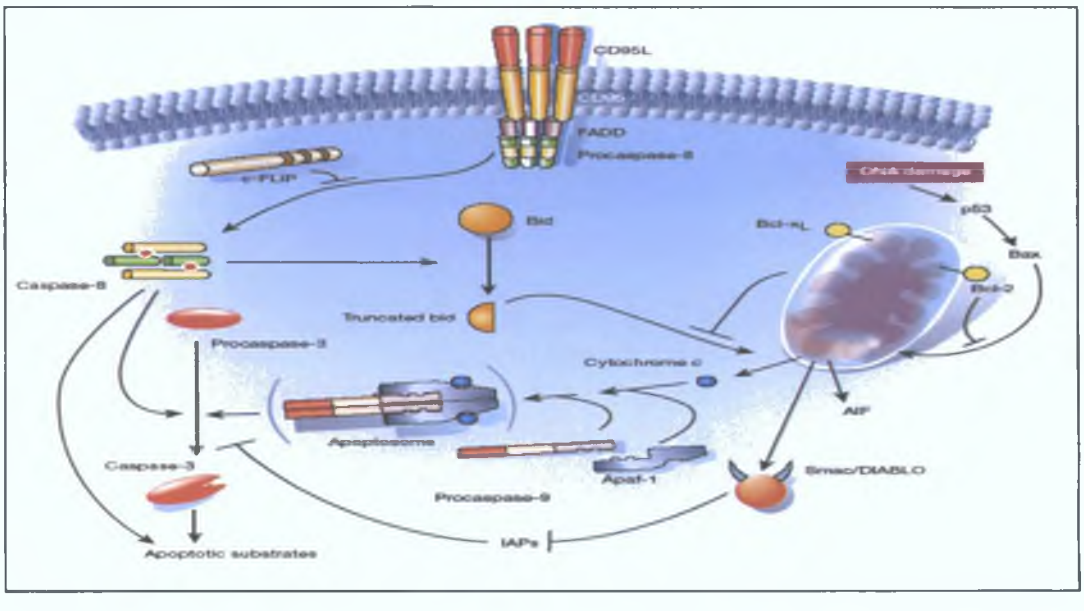



**Figure 12: The major apoptotic pathways in mammalian cells.** Schematic diagram showing the death-receptor and the mitochondrial pathways of apoptosis. Crosstalk between the pathways is facilitatied by Bid, as caspase 8-mediated cleavage of Bid increases its pro-apoptotic activity, and results in its translocation to the mitochondria (Hengartner 2000).

However, members of the Bcl-2 family primarily exert their pro- or anti-apoptotic influence through regulation of mitochondrial membrane potential, and the corresponding cytochrome c release Upon stimulation of apoptosis, many members of the pro-apoptotic Bcl-2 family translocate from the cytoplasm to the mitochondria Following a conformational change, these proteins can insert into the mitochondrial membranes, disrupting membrane integrity and increasing mitochondrial membrane potential This results in the release of several mitochondrial proteins involved in caspase activation and other apoptotic events (Goping *et al*, 1998, Zamzami and Kroemer 2001) The pro-apoptotic protein, Bax, for example, is normally present in the cell cytoplasm Following stimulation of apoptosis Bax migrates to the mitochondria where it inserts into the mitochondrial membrane and forms a homodimer, resulting in an increase in mitochondrial membrane potential, thus facilitating apoptosis Similarly, the pro-apoptotic protein Bid is cleaved by caspase 8, and the resulting C-terminal fragment, tBid, translocates to the mitochondria Bid therefore mediates crosstalk between the death receptor and mitochondrial pathways of apoptosis tBid facilitates insertion of other pro-apoptotic proteins into the mitochondrial membrane, and promotes Bax dimerization (Eskes *et al*, 1998, Jurgensmeier *et al*, 1998, Ferri and Kroemer 2001) Conversely, many of the anti-apoptotic Bcl-2 family proteins are associated with the mitochondrial membrane, where they act to inhibit increases in mitochondrial membrane potential, and prevent apoptosis by maintaining membrane integrity Both the pro- and anti-apoptotic members of the Bcl-2 family appear, at least in part, to regulate each other Bcl-2, for example, 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, for example, binds to Bcl-$x_L$ thus inhibiting its anti-apoptotic function (Desagher and Martinou 2000, Ferri and Kroemer 2001, Zamzami and Kroemer 2001) In addition, many factors within the cell regulate the level of expression of Bcl-2 family of proteins, increased levels of p53 tumour suppressor protein can, for example, increase Bax expression (Miyashita and Reed 1995)

Bcl-2 family members also appear to modulate other cellular processes in addition to apoptosis, however, this appears to be restricted to certain family members Bfl-1 is an anti-apoptotic member of the Bcl-2 family that also exhibits proliferative and potent oncogene transforming activities (D'Sa-Eipper and Chinnadurai 1998) Bfl-1, therefore, communicates with both the apoptotic and proliferation cell machineries, suggesting a link between these two cellular processes

The importance of the Bcl-2 family of proteins in normal physiology is highlighted by the fact that Bcl-2- and Bcl-$x_L$-deficient mice die either at an embryonic stage or immediately post-natal due to increased apoptosis in multiple organs and tissues of the body (Veis *et al*, 1993, Motoyama *et al*, 1995)

**1.5 3 3 NFκB**

NFκB is a family of transcription factors that was originally identified in B cells, and is now recognized as being ubiquitously expressed in mammals Family members include p65 (RelA), p50 (NFκB 1), p52 (NFκB 2), RelB and c-Rel, and the inhibitory subunits IκBα, IκBβ and IκBε NFκB subunits form either homo- or heterodimers, with the most predominant form of NFκB being the p50/p65 heterodimer (De Martin *et al*, 2000, Gupta 2003) In un-stimulated cells, NFκB is sequestered in the cytoplasm through interactions with inhibitor κB (IκB) proteins, however, NFκB can be rapidly activated in response to a variety of inflammatory and other stimuli, such as TNF-α, interleukin (IL)-1, oxidized lipids, shear stress, and hypoxia/reperfusion (Pahl 1999) Cell stimulations result in rapid phosphorylation of IκB at serine 32 and serine 36, which is mediated by IκB kinases Phosphorylated IκB dissociates from NFκB, and is targeted for ubiquitinylation and proteolytic degradation (Kirshenbaum 2000) Free NFκB dimers then translocate to the nucleus where they activate transcription of target genes Activation of NFκB is generally transient, as NFκB stimulates the transcription of the IκB gene, resulting in a rapid replenishment of the inhibitor The newly synthesized IκB molecules then enter the nucleus, where they bind to the NFκB dimers resulting in their dissociation from the DNA and return to the cytoplasm Perturbation of IκB control of NFκB has been associated with many pathological conditions, including chronic inflammatory conditions and cancer (Oakley *et al*, 2003)

NFκB generally mediates cell survival signals and opposes apoptotic processes by regulating the expression of anti-apoptotic proteins This is supported by the embryonic lethality of p65 negative mice due to excessive apoptosis, suggesting that NFκB is crucial for regulating apoptosis during embryonic development (Beg *et al*, 1995) In addition, NFκB has repeatedly been shown to inhibit apoptosis in adult cells (Baldwin 1996, Liu *et al*, 1996b, Ghosh *et al* 1998) NFκB activation results in the up-regulation of a number of anti-apoptotic proteins, including the regulator of Fas-mediated apoptosis, FLIP, and also inhibitors of apoptosis proteins (IAP), which results

in inhibition of caspase activity (Nicotera *et al*, 1999) In addition, NFκB also up-regulates the expression of the anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2, Bcl-$x_L$, and Bfl-1 (Nicotera *et al*, 1999, Benedict *et al*, 2000), however the pro-apoptotic protein Bax also appears to be regulated by NFκB (Bentires-Alj *et al*, 2001) NFκB activity is typically transient, its activity can be down-regulated by removal of the activation stimulus, or due to NFκB-dependent transcriptional activation and re-synthesis of IκB, resulting in the re-sequestration of NFκB in the cytoplasm (De Martin *et al*, 2000)

### 1 5.4 Role of Apoptosis in the Vasculature

Apoptosis is a normal physiological process, playing important roles in both development and maintenance of a wide variety of tissues, including the vasculature It also is an essential component in the vascular response to tissue insult or injury The critical role of apoptosis is evident throughout life, and consequently dysfunctions in apoptosis manifest themselves not only in developmental abnormalities, but also in a wide range of adult pathologies

Apoptosis plays a critical role in normal vasculogenesis, and aberrations in this process can result in embryonic lethality Decreased apoptosis can, for example, result in increased development of endocardial cushions, and subsequently increased cardiac valve formation, whilst overabundance of apoptosis in the heart of developing mice has been shown to result in emybryonic lethality (White *et al*, 2004) Apoptosis is also one of the mechanisms of neonatal vascular remodelling during the transition from foetal to neonatal circulation Apoptosis is involved in the regression of the human umbilical vessels and the ductus arteriosus, and in the remodelling of the branching great arteries during the neonatal period (Kim *et al*, 2000) In addition, apoptosis has been shown to be involved in the post-natal morphogenesis of the atrioventricular node and Bundle of His, and aberrations in this process could predispose to tachyarrhythmias or bradyarrythmias in adulthood (James 1994)

The pathogenesis of various forms of vascular diseases involves dysregulation of both apoptosis and proliferation within the cells of the vasculature Many vascular diseases involve an accumulation of cells within the intimal space This was initially accredited to perturbations in cellular proliferation, however, the importance of apoptosis in vascular remodelling and lesion formation is now recognized (Bai *et al*,

1999, Pollman *et al*, 1999)

Apoptosis can contribute to the pathogenesis of vascular disease through several potential mechanisms The transition of a fatty streak to an atherosclerotic plaque, for example, is characterized by the appearance of focal and diffuse regions of cell death The SMC of these fatty acid streaks express increased levels of the pro-apoptotic protein Bax, which increases the susceptibility of the cells to undergo apoptosis (Kockx *et al*, 1998) Apoptosis also occurs in advanced atherosclerotic lesions resulting in the formation of hypocellular fibrous zones and a lipid-rich core (Geng *et al*, 1996) It has also been extensively documented that SMC isolated from atherosclerotic arteries undergo apoptosis more frequently than their counterparts isolated from healthy vessels (Bennett *et al*, 1995a) Apoptosis can influence the structure of the atherosclerotic plaque, causing thinning of the fibrous cap, rendering the plaque more prone to rupture (Walsh *et al*, 2000) In addition, increased apoptosis can also cause activation of tissue factor, which may increase the thrombogenicity of the lesion (Mallat *et al*, 1999) It is postulated that the development of atherosclerotic lesions are regulated by forces governing cellular proliferation and migration, in addition to plaque remodelling due to apoptotic cell death Similarly, the development of restenotic lesions following balloon angioplasty or atherectomy of diseased arteries is thought to be due, at least in part, to impaired apoptotic signalling (Kraemer 2002) Many studies have documented increased VSMC apoptosis following balloon injury This increase in apoptosis occurs both early after injury, with a 70% increase in apoptosis evident 30 minutes post-injury, and can also occur in the neointima from 7 – 30 days post-injury (Han *et al*, 1995, Bochaton-Piallat *et al*, 1996, Perlman *et al*, 1997) It is postulated that apoptosis plays an important role in the development of vascular lesions, as exuberant balloon injury-induced apoptosis has been found to result in enhanced neointimal formation (Rivard *et al*, 1999) However, the mechanisms that regulate neointimal SMC apoptosis remain incompletely understood

Cardiac myocyte apoptosis is a feature of many pathological disorders, including myocardial infarction and congestive heart failure (Cook *et al*, 1999, Kang and Izumo 2000) The Bcl-2 family of proteins are becoming increasingly recognized as important modulators of cardiac myocyte apoptosis Bcl-2, for example, which is expressed in both the developing and adult hearts, is up-regulated following coronary occlusion in rat hearts and myocardial infarction in human hearts (Kajstura *et al*, 1995, Misao *et al*, 1996, Liu *et al*, 1998) The pro-apoptotic protein Bax is also up-regulated following coronary occlusion and is over-expressed in spontaneously hypertensive rats,

which may contribute to increased apotosis (Fortuno *et al*, 1998, Liu *et al*, 1998) Therefore, the possibility of limiting cardiac myocyte loss by inhibiting apoptosis, possibly through interference with the level of expression of members of the Bcl-2 family, may have important therapeutic implications in the treatment of heart failure

## 1 6 Notch Signalling Pathway

### 1 6.1 Introduction to the Notch Signalling Pathway

The Notch gene of *Drosophila melanogaster* (*Drosophila*) was first described by Morgan in 1916 (Simpson 1998), and was so named as haploinsufficiencies of the gene causes "notches" at the wing margin in these flies Further studies in *Drosophila, C elegans*, and subsequently in mammals and humans, have identified and characterized a super-family of Notch receptors that show a high degree of evolutionary conservation Notch was originally identified in humans as a gene involved in the chromosomal translocations in T-cell leukaemias (Kojika and Griffin 2001) The receptors exhibit a high degree of structural conservation both within, and across, species as diverse as flies and humans The Notch receptor family includes Notch in *Drosophila*, LIN-12 and GLP-1 in *C elegans*, mNotch 1 and mNotch 2 in mouse, and Notch 1-4 in humans (Weinmaster 1997)

### 1.6 2 Biological Consequences and Methods of Notch Signalling

Further analysis of Notch signalling pathway mutants revealed that Notch function is important for the proper development of many tissues during embryogenesis (Miele and Osborne 1999) In *Drosophila*, for example, Notch is required for normal neurogenesis, myogenesis, wing formation, eye development, oogenesis, leg formation and heart formation (Park *et al*, 1998) The importance of this signalling pathway is highlighted as targeted disruption of components of the pathway can lead to embryonic lethality Targeted disruption of the *notch-1* gene, for example, leads to embryonic lethality at 11 5 days post-conception (Swiatek *et al*, 1994, Conlon *et al*, 1995) A number of studies present evidence that Notch signalling acts as an important "switch" controlling cell fate decisions during embryogenesis (Greenwald *et al*, 1998) Indeed, Artavanis-Tsakonas *et al*, (1995) aptly described Notch as a "gatekeeper of cell fate", as Notch signalling critically influences cell proliferation, differentiation and apoptosis

Notch signalling has, for example, been shown to affect cellular differentiation programs in many experimental models The classic paradigm for this is *Drosophila* neurogenesis, in which activation of the Notch signalling pathway prevents neuronal precursors from differentiating towards the neuronal lineage (Artavanis-Tsakonas *et al*, 1991, Artavanis-Tsakonas and Simpson 1991)

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 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)

In addition to its role as an arbiter of cell fate decisions in the developing organism, a growing body of evidence has implicated the Notch signalling pathway in determining cell fate decisions in the mature organism (Kojika and Griffin 2001, Wang *et al*, 2002b), which may be due to the fact that most tissues are renewed throughout life from reserves of uncommitted stem cells (Artavanis-Tsakonas *et al*, 1995) Notch, for example, is involved in the control of cell fate decisions during haematopoiesis (Bigas *et al*, 1998) and inhibits the differentiation of murine myoblast cells (Bush *et al*, 2001) In addition, the Notch signalling pathway has been shown to affect other cell fate decisions, such as proliferation and apoptosis in the mature organism Notch has

been shown to promote proliferation in certain cell types, for example, activation of the receptor and transfection of a constitutively active form of the receptor in bone marrow stem cells and promyelocytic leukaemia cells, respectively, cause accelerated progression through $G_1$ (Carlesso *et al*, 1999)

Notch is reported to have anti-apoptotic properties in many cell types The anti-apoptotic properties of Notch in T-cell systems has, for example, been documented by Deftos *et al*, (1998) and Jehn *et al*, (1999) showing that Notch 1 activation inhibits glucocorticoid- and Nur-77-dependent apoptosis respectively As human malignant cells display a resistance to both physiologically- and therapeutically-induced apoptosis, and Notch has been implicated in the control of cell fate decisions, it was postulated that alterations in Notch signalling or expression may contribute to tumorigenesis (Miele and Osborne 1999) Several lines of evidence support this Increased expression of Notch receptor and ligands is apparent in many malignancies, including cervical carcinomas, leukaemias, neuroblastomas, and pleural mesotheliomas, among others (Zagouras *et al*, 1995, Daniel *et al*, 1997) Notch over-expression is, for example, evident in 100% of the cervical cancer specimens studied (Zagouras *et al*, 1995) Notch receptor and ligands were both found to be over-expressed, and the sub-cellular distribution of Notch 1 changed in cervical cancer cells, with strong nuclear immunoreactivity observed only in carcinomas (Daniel *et al*, 1997) Constitutively active forms of the Notch receptor cause transforming activity in many animal models, over-expression of the active form of Notch 4 for instance, causes breast cancer in mice (Jhappan *et al*, 1992, Robbins *et al*, 1992, Smith *et al*, 1995, Gallahan *et al*, 1996) In addition, Notch 3 signalling has been shown to promote vascular smooth muscle cell survival in response to the pro-apoptotic Fas ligand (Wang *et al*, 2002b)

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 This is the case, for example, with deletion of *notch 1* and *jagged 1* in mice, which causes defects in angiogenic vascular remodelling and defects in vascular morphogenesis respectively (Kojika and Griffin 2001)

The distribution of components of the Notch signalling pathway in the developing vasculature is primarily restricted to arterial blood vessels Villa *et al*, (2001) have reported that Notch 1, Notch 3, Notch 4, Delta 4, Jagged 1 and Jagged 2 are all expressed in arteries and not in veins However, other groups have noted a few exceptions, for example expression of Jagged 1 in portal and hepatic veins (Nijjar *et al*, 2002), Notch 3 in cerebral veins (Joutel *et al*, 2000), and Notch 4 in the cardinal vein (Uyttendaele *et al*, 1996) This suggests that the Notch signalling pathway may also play a role in venous development

A growing body of evidence has indicated that the Notch signalling pathway plays a role in the maintenance of vessel homeostasis in the adult (Gridley 2001) In addition, components of the Notch signalling pathway are present primarily in the adult arterial vasculature Notch 3 expression is, for example, localized specifically to smooth muscle cell, and is absent from endothelial cells (Joutel *et al*, 2000), similarly, the expression of Notch 4 is reported to be endothelial cell-specific (Uyttendaele *et al*, 1996) It is postulated that the Notch signalling pathway plays a role in determining cell fate decisions following vascular injury Two independent studies have shown changes in the expression of the components of this pathway following balloon catheter denudation of the rat carotid artery Lindner *et al*, (2001) demonstrated that the expression of Notch receptors (Notch 1-4) and ligands (Jagged 1-2) in both endothelial cells and smooth muscle cells of the vasculature was increased following injury This report also suggests that the level of Notch receptor expression may be related to endothelial cell/smooth muscle cell interaction In contrast, Wang *et al*, (2002a) reported that Notch 1, Notch 2, Notch 3, and the effector genes HRT 1, HRT 2 and HRT 3 were coordinately down-regulated following balloon injury The discrepancies between these reports may be accounted for as Lindner *et al*, used in situ hybridization thus limiting the study to the inner face of the artery Wang *et al*, however, extracted RNA from the artery following removal of the intimal and adventitial layers,

representing the smooth muscle layer of the artery While further work is needed to elucidate the specific role of the Notch signalling pathway in maintaining vascular homeostasis, the importance of this pathway, and its potential for therapeutic intervention is not disputed

### 1 6 3 Components of the Notch Signalling Pathway

The Notch signalling pathway is an evolutionarily preserved mechanism that serves to regulate cell fate decisions (Fleming 1998, Baron *et al*, 2002) 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, which are detailed in table 2 The Notch receptor and its ligands were first identified in *Drosophila*, and the Notch gene was first cloned by Wharton *et al*, in 1985 (Baron *et al*, 2002)

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 smooth muscle cells (Joutel *et al*, 2000), while Notch 4 (Uyttendaele *et al*, 1996) and Delta 4 (Shutter *et al*, 2000) have been described as endothelial cell 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)

**Table 2 Notch Signalling Molecules**

| Gene | Chromosome | *Drosophila* | Expression/Function | Related disease |
|---|---|---|---|---|
| Notch1 | 9q34 | Notch | T cells, macrophages, precursor cells | t(7 9)(q34,q34.3) T ALL |
| Notch2 | 1p13-p11 | Notch | T cells, B cells, precursor cells | |
| Notch3 | 19p13 2 p13 1 | Notch | broad | CADASIL |
| Notch4 | 6p21 3 | Notch | macrophages | mouse mammary tumor |
| Delta | 6p23 | Delta | broad | schizophrenia? |
| Jagged1 | 20p12.1 p11.23 | Serrate | mast cells, megakaryocytes stromal cells | Alagille syndrome |
| Jagged2 | 14q32 | Serrate | T cells | |
| CBF1/RBPJκ | 9p13-p12 | Su(H) | transcriptional repressor | |
| HES1 | 3q28-q29 | E(spl) | bHLH transcription factor | |
| HES5* | Not identified | E(spl) | bHLH transcription factor | |
| Manic fringe | 22q13 1 | fringe | precursor cells neutrophil macrophages B cells | |
| Radical fringe | 17q25 | fringe | broad | |
| Lunatic fringe | 7p22 | fringe | enhance Notch/Delta interaction | |
| Presenilin1 | 14q24 3 | Not identified | γ-secretase? | Alzheimer s disease |
| Mastermind | 5q | mastermind | co-activator | |
| TLE | 19p13 3 | groucho | co-repressor | |
| Deltex | 12q24 | deltex | co-activator | |
| Numb | 14q24 3 | numb | inhibitor of Notch | |

ALL, acute lymphoblastic leukemia CADASIL, cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy Su(H) suppressor of hairless E(spl) enhancer of split, bHLH basic helix loop-helix, TLE, transducin-like enhancer of spli. *Identified in mouse but not in human.

### 1 6 3.1 Structure of Notch Signalling Pathway Receptors and Ligands

The structure and spatial arrangement of Notch Signalling Pathway receptors and ligands are highly conserved from *Drosophila* to humans However, although the general architecture is conserved, large variations in specific domain size can be observed both within and among species, as illustrated in figure 13

**Figure 13 Schematic Representation of the Notch Family of Receptors**

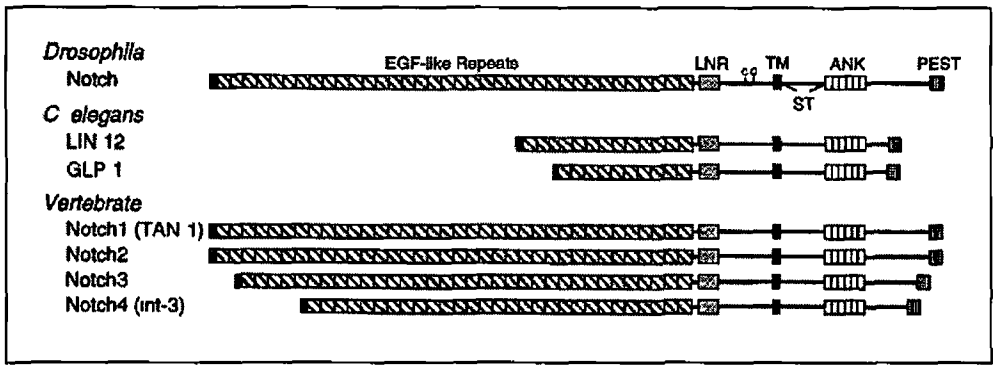



**Figure 13 Schematic representation of the Notch family of receptors** The family members have the same overall arrangement of structural motifs The extra-cellular domains contain multiple epidermal growth factor (EGF)-like repeats, in addition to LIN12/Notch-related region (LNR) and two conserved cysteines 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)

The Notch receptor family includes Notch in *Drosophila*, LIN-12 and GLP-1 in *C elegans*, mNotch 1 and mNotch 2 in mouse, and Notch 1-4 in humans (Weinmaster 1997) Notch receptors are single-pass transmembrane proteins, and as such have an

extracellular (ExC), transmembrane (TM), and intracellular (IC) domains (Baron *et al*, 2002) Notch receptors are synthesized as single (300 kDa) polypeptides, and are proteolytically processed into heterodimeric (180 kDa and 120 kDa) forms presented on the cell surface (Blaumueller *et al*, 1997, Pan and Rubin 1997, Logeat *et al*, 1998)

The predominant features of the Notch ExC domain are a number of tandemly arranged EGF-like repeats, a cysteine-rich region termed the LNR (LIN-12/Notch-related region) and a pair of conserved cysteines between the LNR and TM regions (Weinmaster 1997, Fleming 1998, Baron *et al*, 2002) Notch 1 and Notch 2 proteins contain 36 tandemly arranged EGF-like repeats, while Notch 3 and Notch 4 contain 34 and 29 respectively While the number of these EGF-like repeats is not conserved, their spatial arrangement is, suggesting that this spatial arrangement is important to the function of the receptor (Weinmaster 1997) A number of studies in *Drosophila* have elucidated the function of specific EGF-like repeats, EGF-like repeats 11 and 12, for example, are both necessary and sufficient for binding of Notch to its ligands on adjacent cells (Rebay *et al*, 1991, Baron *et al*, 2002) Additional regions between EGF-like repeats 17-20 and 24-26 have been implicated in modulating the ligand receptor interactions (de Celis *et al*, 1993), with the later region shown to be critical for Serrate-dependent signalling through Notch (Lawrence *et al*, 2000) The LNR region is located immediately downstream of the EGF-like repeat domain, and appears to negatively regulate receptor activation In support of this, studies show that either deletion of the LNR region, or missense mutations in this region produce a constitutively active receptor (Greenwald and Seydoux 1990, Lyman and Young 1993) The pair of conserved cysteines located between the LNR and TM regions have been postulated to function in receptor dimerization (Greenwald and Seydoux 1990, Lieber *et al*, 1993) In addition, a conserved valine residue has been identified within the TM domain, and sequencing studies have established this as a cleavage site of Notch during receptor activation (Schroeter *et al*, 1998)

Four functionally important regions have been identified within the Notch IC domain These are the RAM domain located in the subtransmembrane region, the ANK repeat domain, a transcriptional activator domain (TAD), and a PEST (proline, glutamate, serine, thronine-rich) sequence In addition, two nuclear localization sequences are present in the Notch IC domain, located either side of the ANK repeat domain (Stifani *et al*, 1992, Fortini *et al*, 1993) Following translocation of Notch IC to the nucleus, it exerts its effects by binding to DNA by forming a complex with CSL (CBF-1, Suppressor of Hairless, Lag-1) family of transcription factors The RAM

domain is the main site of CSL interaction (Tamura *et al*, 1995) The ANK repeat region also provides a lower affinity binding site to the CSL proteins, which facilitates the RAM/CSL interaction (Tamura *et al*, 1995, Roehl *et al*, 1996, Matsuno *et al*, 1997) These regions are essential to the function of Notch IC, studies show that expression of the RAM and ANK domains produce a similar phenotype to that of the activated Notch receptor (Roehl *et al*, 1996), and proteins lacking these regions produce dominant-negative effects (Lieber *et al*, 1993, Rebay *et al*, 1993) In addition, the ANK repeat domain is known to be a binding site for a number of positive regulators of the Notch signalling pathway, including the deltex protein (Matsuno *et al*, 1995) Therefore, this region is an essential to Notch signalling, and may mediate multiple elements of this pathway The PEST region of the Notch receptor is thought to be involved in Notch protein turnover (Rogers *et al*, 1986, Greenwald and Seydoux 1990) Deletion of the PEST domain inactivates Notch but does not produce a dominant-negative form of the receptor (Lieber *et al*, 1993, Lyman and Young 1993) The number and sequence conservation of amino acids between the ANK and PEST regions vary between the Notch proteins, accounting for the different sizes of the Notch IC domains, and may account for some of the functional differences between the Notch proteins Deletion of this amino acid sequence and the PEST region produces a dominant gain-of-function phenotype, which suggests that this region contains a negative regulatory domain (Mango *et al*, 1991, Lyman and Young 1993)

**Figure 14 Schematic Representation of the DSL Family of Notch Ligands**

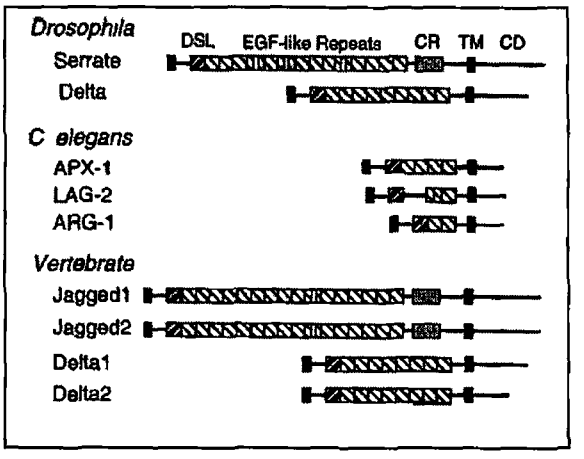



**Figure 14 Schematic Representation of the DSL Family of Notch Ligands** The family members contain the same arrangements of conserved structural motifs The extra-cellular domains contain multiple EGF-like repeats, however, some of the serrate-like ligands have inserts on some of their repeats, and contain additional cysteine-rich (CR) regions between the EGF-like repeats and the single TM domain The cytoplasmic domains do not share significant amino acid identities (Weinmaster 1997)

The Notch ligand family includes Delta and Serrate in *Drosophia*, LAG-2 in *C elegans*, and Delta 1-3 and Jagged 1-2 in vertebrates, known as the DSL family of ligands (Dunwoodie *et al*, 1997, Weinmaster 1997, Appel and Eisen 1998, Haddon *et al*, 1998) Similar in structure to the Notch receptors, the Notch ligands are also single-pass transmembrane proteins, and as such, have ExC, TM and IC domains (Artavanis-Tsakonas *et al*, 1995) Although the general architecture of the ligands is conserved, large variations in specific domain size and composition can be seen both within and among species (Fleming 1998)

The ExC domain of the Notch ligands consists of a DSL domain, multiple EGF-like repeats, and a CR region, which is present in Jagged but absent in Delta and their respective homologues All Notch ligands possess a characteristic DSL domain, located N-terminal to the EGF-like repeats (Weinmaster 1997) The DSL domain is described as a modified EGF-like repeat domain, and is believed to be the portion of the ligand that activates the receptor, point mutations that affect conserved cysteines in this domain have resulted in strong loss-of-function phenotypes (Henderson *et al*, 1994) Experiments conducted in *C elegans* suggest that these DSL domains may be functionally interchangeable (Rebay *et al*, 1991) Whilst both Jagged and Delta, and their homologues, possess an EGF-like repeat region, the structural differences between the two families are highlighted due to the difference in the number of these repeats (Fleming 1998) The importance, if any, of the number of EGF-like repeats to the function of the receptor is unknown Evidence presented to date, however, indicates that this is not an important factor, as studies in *Drosophila* have shown that Delta and Serrate are capable of activating Notch receptors interchangeably (Henderson *et al*, 1997) Both the DSL and EGF-like domains appear to be necessary for ligand function (Weinmaster 1997) The CR domain, located between the EGF-like domain and the TM domain is approx 100 amino acids in length, and is reputed to be involved in selecting specific Notch receptors (Fleming 1998) The CR region may also modulate binding of the ligand to the receptor, as Delta/Notch interactions have been shown to be more stable that those of Serrate/Notch (Fehon *et al*, 1991, Rebay *et al*, 1991)

The IC domain of the Notch ligands are relatively short, approximately 70-215 amino acids in length, and do not display significant sequence homology either within or among species (Fleming 1998) However, both the TM and IC domains are required by most ligands for proper Notch activation (Chitnis *et al* 1995, Sun and Artavanis-Tsakonas 1996, 1997), although the mechanisms remain unclear

Although Notch ligands are primarily transmembrane proteins, proteolytically

cleaved, secreted forms of the proteins have been identified (Klueg *et al*, 1998, Qi *et al*, 1999) Initial reports indicated that the secreted soluble ligands act as dominant-negative molecules, competing with membrane bound ligands and blocking Notch activation (Sun and Artavanis-Tsakonas 1997) Conflicting reports, however, have been published, describing the soluble ligands as agonists of the Notch signalling pathway (Wang *et al*, 1998, Qi *et al*, 1999, Han *et al*, 2000) A secreted form of Jagged 1, for example, can activate Notch 1 in hematopoietic cells to inhibit differentiation (Li *et al*, 1998b) The factors controlling whether the soluble form of the ligand is an agonist or antagonist on the Notch signalling pathway remain unclear

## 1 6.4 Molecular Aspects of Notch Signaling

### 1.6 4 1 Processing of the Notch Receptor

Mature Notch receptors are heterodimers derived from the cleavage of the full length Notch protein (300 kDa) into two fragments, an N-terminal fragment, Notch ExC, that contains most of the ExC domain (180 kDa), and a C-terminal fragment, cleaved N-terminal to the TM domain (120 kDa), and therefore contains the TM and IC regions (Blaumueller *et al*, 1997) The formation of this heterodimer, and subsequent activation of the Notch receptor requires at least three proteolytic cleavage events, designated S1, S2, and S3 (Mumm and Kopan 2000, Baron *et al*, 2002) Cleavage at S1 does not require ligand binding of the Notch receptor, whereas both S2 and S3 cleavage require such binding (Weinmaster 2000)

**Figure 15 Proteolytic Cleavage of a Notch Receptor**

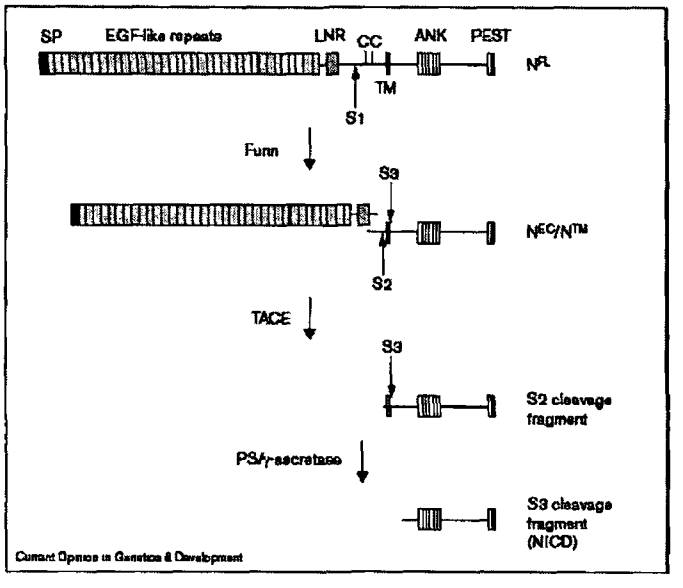



**Figure 15 Proteolytic Cleavage of a Notch Receptor** Schematic representation of proteolytic cleavage sites identified in Notch 1, putative proteases involved, and the resultant proteolytic cleavage fragments (Weinmaster 2000)

Cleavage at S1 is performed by a calcium-dependent serine protease known as furin (Bush *et al*, 2001) This cleavage event occurs within the secretory pathway, such that a processed heterodimeric form of the Notch receptor is transported to the cell surface The two subunits of the heterodimer are held together at the plasma membrane by non-covalent interactions requiring calcium (Rand *et al*, 2000) Jarriault *et al*, (1998) has shown that inhibition of furin activity interferes with heterodimer formation, and antagonizes the ability of Notch to activate its signalling intermediate, the CSL transcription factors Interaction of the heterodimeric receptor with a Notch ligand triggers two additional cleavage events, S2 and S3, adjacent to the plasma membrane (Kopan *et al*, 1996, Brou *et al*, 2000) Ligand binding to Notch induces a conformational change in the receptor that facilitates cleavage at S2 (Weinmaster 1998, Hardy and Israel 1999) The S2 cleavage site has been identified as a conserved valine located twelve amino acids N-terminal of the TM region (Brou *et al*, 2000, Mumm *et al*, 2000) Two proteins have emerged as cleavage enzymes at S2, these are TACE (TNF-ε converting enzyme) and Kuzbanian (Kuz), both members of the ADAM (a disintegrin and metalloproteinase) family (Blobel 1997), although it may emerge that other proteins are also involved in this cleavage event TACE has been shown to cleave at an S2 site in both vertebrates and *Drosophila* (Brou *et al*, 2000), suggesting that the site of action of Kuz may be distinct to that of TACE Conflicting reports surround the identification of Kuz as an S2 cleavage enzyme, however, a role for Kuz in the Notch signalling pathway is not disputed, as transgenic mice deficient in either Kuz or Notch 1 have been shown to display the same phenotype (Mumm *et al*, 2000) *Drosophila* Notch has been shown to physically interact with Kuz, and removal of Kuz activity by RNAi inhibited an S2-like cleavage *in vitro* (Lieber *et al*, 2002) However, proteolytic S2 processing has been shown in cells deficient in Kuz (Mumm *et al*, 2000), and the expression of a dominant negative form of Kuz was shown to have no effect on Notch processing (Qi *et al*, 1999), suggesting that this enzyme is not required for S2 cleavage Lieber *et al*, suggest that the subsequent S3 cleavage step may have a different efficiency depending on whether the S2 cleavage was performed by TACE or Kuz, which could have implications on the strength and duration of the Notch signal The membrane tethered Notch product produced by S2 cleavage is a substrate for the proteolytic cleavage at the S3 site, resulting in an active form of the Notch receptor, Notch IC (5-39) Presenilins, ε-secretases that play a crucial role in familial Alzheimer's disease, have been implicated in the S3 cleavage event (Levitan and Greenwald 1998, Struhl and Greenwald 1999, Ye *et al*, 1999) Two mammalian forms

of presenilins (PS) have been identified, PS 1 and PS 2 (Kojika and Griffin 2001) Several studies have shown PS 1 to be a requirement for S3 cleavage of Notch, dominant-negative mutagenesis of PS 1, for example, reduces the extent of S3 cleavage (De Strooper *et al*, 1998) In addition, Notch 1 and PS have been shown to physically interact in both *Drosophila* and mammalian cells (Ray *et al*, 1999a, Ray *et al*, 1999b), and murine Notch homologues have all been shown to undergo presenilin-dependent proteolysis (Saxena *et al*, 2001) However, Berechid *et al*, (2002) have recently documented presenilin-independent Notch signalling, suggesting that a novel mechanism supports Notch signal transduction when presenilin proteins are absent The cleavage events described appear to be mutually dependent in that cleavage at S2 requires prior cleavage at S1, similarly cleavage at S3 requires prior cleavage at S2 (Kadesch 2000)

### 1 6 4 2 Processing of the Notch Ligand

Notch ligand processing also plays an important role in the activation of the Notch receptor Parks *et al*, (2000) present evidence that endocytosis of the Delta ligand, complexed with the Notch ExC domain, into the signal-generating cell is required for the activation of Notch in *Drosophila* This endocytosis of the ligand and trans-endocytosis of the receptor is mediated by a Dynamin homologue, a GTP-ase required for the formation of clathrin-coated vesicles derived from the plasma membrane during endocytosis This study suggests that endocytosis may play a role in Notch activation, inducing cleavage of the receptor at the S2 site The removal of the Notch ExC domain relieves the LNR-mediated repression of S3 cleavage, thus promoting the release of the Notch IC domain

### 1 6 4 3 Receptor-Ligand Interactions

Ligand-dependent proteolysis generates the active form of the Notch receptor, the Notch IC domain (Kadesch 2000) Several studies have indicated that receptor-ligand interactions within the Notch signalling pathway are interchangeable (Fehon *et al*, 1990, Rebay *et al*, 1991) This suggests that the specificity of function of components of this pathway may be downstream of the receptor-ligand interaction In *C elegans*, for example, all three known ligands, LAG-2, APX-1 and ARG-1, have been shown to activate the two receptors of the Notch signalling pathway, LIN-12 and

GLP-1 (Bray 1998). However, an increasing number of studies are reporting intrinsic specificity of receptor-ligand interactions. Mammalian Jagged-1 is reported to activate both Notch 1 and Notch 2 expressed in myoblasts, whereas Delta-1 can only activate Notch 1 efficiently in the same cells (Weinmaster 1997). However, introduction of the Jagged specific CR region into Delta creates a ligand capable of activating both Notch1 and Notch 2. Similarly, removal of the CR region from the Jagged ligand results in ligand activation comparable to that of Delta. This suggests that receptor-ligand specificity can be affected by the presence or absence of the Jagged CR region (Weinmaster 1998).

Interactions between Notch ligands and receptors are predominantly intercellular (Alton *et al.*, 1989; Fehon *et al.*, 1990), however Sakamoto *et al.*,(2002) have reported that these interactions can be cell autonomous and occur predominantly in the Golgi apparatus or endoplasmic reticulum, with only full length Notch participating in their formation. The resulting dominant-negative effect on the Notch signalling pathway may be that these complexes inhibit the formation of a heterodimeric Notch receptor, or due to competition between the different forms of the Notch receptor for ligand binding.

### 1.6.4.4 Modulation of the Notch Signalling Pathway

**Figure 16: Modulators of the Notch Signalling Pathway**

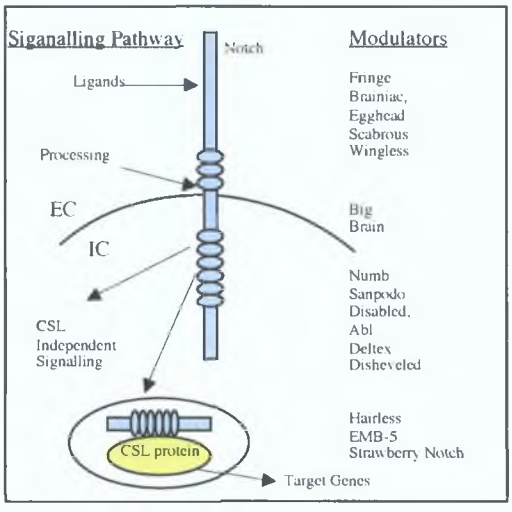



**Figure 16: Modulators of the Notch signalling pathway.** Schematic representation of sites of action of some modulators of the Notch signalling pathway (Panin and Irvine 1998).

A number of proteins have been identified that exert either a positive or negative effect on the Notch signalling pathway. These include both intracellular and extracellular modulators, such as Numb, Fringe, Scabrous and Wingless among others.

Numb, a negative regulator of Notch signalling, is a phosphotyrosine-binding (PTB) domain protein that binds via its PTB domain to two regions of Notch, the RAM domain and at the C-terminus (Baron *et al*, 2002) Numb blocks CSL protein translocation to the nucleus in response to Notch activation, but does not interfere with CSL cellular localization in the absence of Notch receptor-ligand interactions (Frise *et al*, 1996) Fringe is a glycoslytransferase that acts within the Golgi apparatus to potentiate the activating effects of Delta and impede those of Jagged (Fleming *et al*, 1997, Panin *et al*, 1997) It exerts its effects by binding to and modifying the EGF-like repeats of the Notch receptor in a cell autonomous manner (Moloney *et al*, 2000) Three vertebrate homologues of *Drosophila* fringe have been identified, Lunatic fringe, Manic fringe and Radical fringe The Wingless signalling pathway also acts as a negative regulator of Notch signalling It exerts this effect primarily through activation of Dishevelled, a cytoplasmic protein that binds to the carboxy terminus of the Notch receptor (Axelrod *et al*, 1996) Scabrous, a secreted fibrinogen-related protein, acts also acts as a negative regulator of the Notch signalling pathway by binding to the EGF-like repeats and modulating receptor-ligand binding (Lee *et al*, 2000, Powell *et al*, 2001) In addition, the Notch Signalling Pathway is regulated by a number of ubiquitin pathway associated proteins, including Sel-10, Nrarp, Neuralized and Deltex (Baron *et al*, 2002)

The modulation of the Notch signalling pathway is a complex issue, with the above list of modulators being by no means exhaustive Miele and Osborne (1999) propose that the intracellular concentrations, subcellular localization, timecourse of expression, and post-translational modifications of Notch receptors, ligands, mediators, targets, and modulators may all contribute to the biological effects of Notch activation in a specific cell type

### 1 6 5 Notch Signal Transduction

Notch signalling activates at least two different pathways, one of which involves members of the CSL family of transcription factors as an intermediary (CSL/CBF-1-dependent), and one of which does not (CSL/CBF-1-independent) (Baron 2003) These mechanisms of Notch signal transduction are widely conserved from *Drosophila* to humans (Miele and Osborne 1999) Bush *et al*, (2001) suggest that the full length (300kDa), uncleaved form of Notch is the mediator of the CBF-1-independent pathway, whereas the processed form of the receptor (120 and 180 kDa subunits) mediates the

CBF-1 dependent pathway Therefore, two isoforms of the same cell-surface receptor can mediate two distinct signalling pathways in response to ligand binding

### 1.6.5 1 CBF-1-dependent Pathway

The CBF-1 type transcription factors are the primary effectors of the Notch signalling pathway (Struhl and Adachi 1998, Lai 2002), and require presenelhn-dependent proteolysis of Notch IC for normal signal transduction (Miele and Osborne 1999) Notch IC then translocates to the nucleus (Struhl and Adachi 1998), where it acts to regulate transcription of Notch-responsive genes (Artavanis-Tsakonas *et al*, 1995, Bray 1998, Weinmaster 2000) In support of this model of Notch signalling, two nuclear localization signals have been identified in the Notch IC domain (Weinmaster 2000, Gridley 2003), and several studies have shown nuclear access and action of Notch IC both *in vivo* (Struhl and Adachi 1998) and *in vitro* (Kimble *et al*, 1998, Singh *et al*, 2000, Berechid *et al*, 2002) Additionally, Schroeter *et al* (1998) demonstrated that very low levels of Notch in the nucleus are sufficient to produce a significant response This study shows transfection of Notch IC in mammalian cells at concentrations that activate gene transcription, but that do not allow detection of nuclear Notch by immunocytochemistry Similar studies have also shown this to be the case in *Drosophila* (Weinmaster 1997, Lewis 1998, Weinmaster 2000)

Notch IC, upon nuclear localization, cannot bind directly to DNA (Goodbourn 1995) but forms a tertiary complex with a CBF-1 complex (CSL proteins) and DNA The main 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) Therefore CBF-1 acts as a "docking protein" that directs activated Notch to promoter targets, and regulates the transcription of downstream genes (Struhl and Adachi 1998)

Involvement of a CSL protein in the Notch signalling pathway was first demonstrated in *Drosophila* The *Drosophila* CBF-1 orthologue, Suppressor of Hairless (Su(H)), was shown to display both genetic and physical interactions with Notch (Fortini *et al*, 1993), and to activate gene expression upon Notch activation by binding to the regulatory DNA of target genes (Bailey and Posakony 1995) However, the significance of the Notch IC-Su(H) interaction remained controversial for many years, and as a result, the vertebrate CSL protein was identified by multiple groups without recognition of its connection to the Notch signalling pathway As a

consequence of this, many names exist to describe the vertebrate CSL protein, including CBF-1 (Epstein Barr Virus (EBV) latency C promoter binding factor, (Henkel *et al.,* 1994)), and RBP-Jε (recombination signal binding protein of the Jε immunoglobulin gene,(Matsunami *et al.,* 1989)). CBF-1 is a transcriptional regulator that binds to the DNA sequence GTGGGAA (Zhou *et al.,* 2000; Kimberly *et al.,* 2003). Initially, CBF-1 was described as a transcriptional repressor *in vitro* (Dou *et al.,* 1994). However, Hsieh and Hayward (1995) demonstrated that, during EBV-mediated cell immortalization, the viral protein EBV nuclear antigen-2 (EBNA-2) subverts the CBF-1 repressor function by binding to it, and converting it to a transcriptional activator. Notch IC was subsequently shown to exert a similar effect on CBF-1 (Hsieh *et al.,* 1996). At present, accumulated data of both *in vivo* (Barolo *et al.,* 2002) and *in vitro* (Zhou *et al.,* 2000) analysis of CBF-1 supports the model in which CBF-1 is a dual function transcription factor, repressing target gene expression in the absence of Notch/EBNA-2 signalling, and activating transcription in their presence.

#### 1.6.5.1.1 CBF-1 Transcriptional Repression

CBF-1-mediated transcriptional repression involves destabilization of the transcription factor IID (TFIID)- transcription factor IIA (TFIIA) interactions (Brulin *et al.,* 2002; Campos *et al.,* 2002), rendering TFIID unable to interact with TFIIA and form a functional complex (Loomes *et al.,* 1999; Kadesch 2000). Repression also involves recruitment of a histone deacetylase (HDAC) co-repressor complex to the promoter (Zhou *et al.,* 2000). Reduction of histone acetylation by HDACs is linked to repressed, transcriptionally inactive chromatin, rendering the chromatin less accessible to the transcription machinery (Kadesch 2000). Kao *et al.,* (1998) has shown that treatment of cells with the HDAC inhibitor, tricostatin A, causes a potentiation of the expression of a CBF-1 target gene in *Xenopus.*

**Figure 17: CBF-1 Co-Repressor Complex**

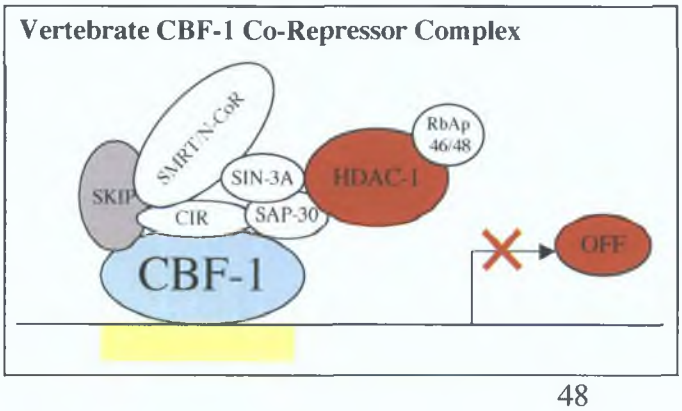



**Figure 17: Schematic diagram of the vertebrate CBF-1 co-repressor complex.** CBF-1 binds to promoter sequence within the nucleus, and also binds a HDAC-containing co-repressor complex resulting in a repression of transcription (Lai 2002).

Several co-repressors make up the HDAC co-repressor complex, which binds to CBF-1 and mediates, at least partially, its transcriptional repressor activity 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 is an adaptor protein which acts as both a tethering point for the CBF-1 co-repressor complex to mediate repression, and as a tethering point for the ankyrin repeat domain of Notch IC during activation (Zhou *et al*, 2000) Therefore, this protein facilitates the switching of the transcriptional regulation role of CBF-1 from that of repression to activation (Lai 2002) SMRT and N-CoR were originally identified as transcriptional repressors for unliganded nuclear hormone receptors (Chen and Evans 1995, Horlein *et al*, 1995) and have both since been shown to act as co-repressors for a variety of other transcription factors, including CBF-1 (Lindner *et al*, 2001, Lai 2002) Both proteins have been shown to bind directly to CBF-1 and antagonize the ability of Notch IC to stimulate gene expression via CBF-1 (Kao *et al*, 1998) Additionally, both SMRT and N-CoR bind directly to SKIP, to other co-repressor proteins designated Sin 3A and SAP 30, and also to HDAC (Lai 2002) Besides acting as bridging proteins in the HDAC co-repressor complex, SMRT and N-CoR have been shown to directly activate the deacetylase activity of the class I enzyme HDAC3 through the SANT DNA binding domain that both proteins possess (Guenther *et al*, 2001, Zhang *et al*, 2001) CIR has also been shown to directly bind to CBF-1 (Goodbourn 1995, Zhou *et al*, 2000), SKIP (Lai 2002) and other members of the co-repressor complex, including HDAC 1/2 and SAP-30 (Kadesch 2000, Oberg *et al*, 2001) SMRT, N-CoR and CIR all fail to bind repression-defective CBF-1 mutants, further highlighting their inclusion and importance in the CBF-1 co-repressor complex (Kadesch 2000, Lai 2002)

#### 1 6 5 1 2 CBF-1 Transcriptional Activation

Activation of CBF-1 by Notch IC involves a two-part mechanism, firstly, the loss of CBF-1 mediated transcriptional repression, and secondly, activation of gene expression mediated through the endogenous Notch IC activation domain (Apelqvist *et al*, 1999, Zhou *et al*, 2000)

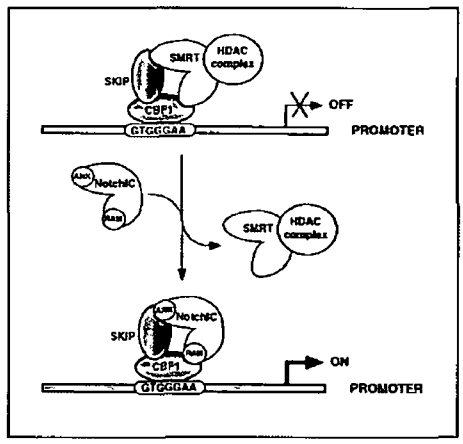



**Figure 18 Model for activation of CBF-1 repressed promoters.** CBF-1 binds promoter sequence within the nucleus SKIP interacts with CBF-1 The SMRT/HDAC co-repressor complex binds to both SKIP and CBF-1 mediating transcriptional repression Notch IC competes with SMRT for contacts on both SKIP and CBF-1 Displacement of the co-repressor complex relieves repression and Notch IC further activates promoter through its transactivational domain (Zhou *et al* 2000)

Evidence supporting the fact that Notch IC acts to displace the co-repressor complex from the CBF-1-SKIP complex has been demonstrated in a number of studies CBF-1 has been shown to bind both SMRT and Notch IC, however, CBF-1 cannot bind these proteins simultaneously (Kadesch 2000, Lindner *et al*, 2001) Additionally, protein-protein interactions assays have demonstrated that SKIP can interact with both SMRT and Notch IC, however, it has been shown that these interactions are mutually exclusive (Zhou *et al*, 2000) SMRT and Notch IC therefore compete for binding to the CBF-1 complex (Kimble *et al*, 1998, Zhou *et al*, 2000), and similar competition has been demonstrated between SMRT and EBNA-2 (Zecchini *et al*, 1999, Zhou *et al*, 2000) Thus, the conversion of CBF-1 from transcriptional repression to activation involves, at least in part, both CBF-1 and SKIP exchanging the SMRT containing co-repressor complex for the Notch IC activation complex (Zhou *et al*, 2000) Notch IC binds to CBF-1 via its RAM23 region, and binds to SKIP via its ankyrin repeat domain Both of these binding sites are necessary for the biological activity of Notch IC Zhou *et al*, (2000) has shown that a mutation in the fourth ankyrin repeat of Notch IC abolished its interaction with SKIP but not CBF-1, and eliminated biological activity in this study Similarly, the ability of Notch IC to block muscle cell differentiation was blocked with the expression of antisense SKIP in these cells

**Figure 19: CBF-1 Co-Activator Complex**

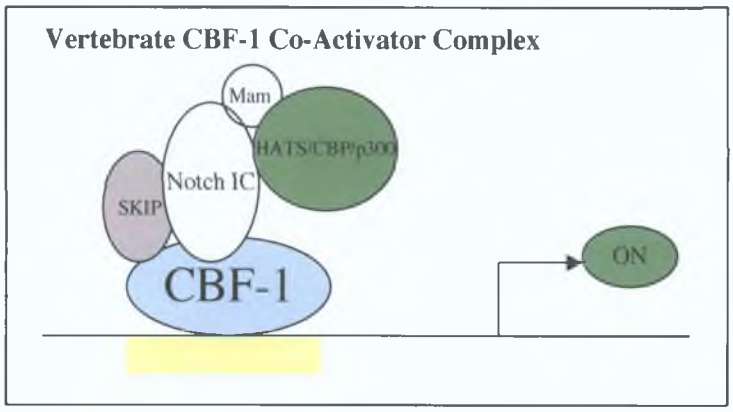



**Figure 19: CBF-1 Co-Activator Complex.** Notch IC recruits a co-activator complex to facilitate transactivation at the CBF-1 promoter (Lai 2002).

Notch IC subsequently activates gene expression through the presence of its endogenous activation domain (Apelqvist *et al.*, 1999; Zhou *et al.*, 2000). In addition, Notch IC recruits a co-activator complex, which includes the nuclear protein Mastermind and histone acetyltransferases (HATS) (Baron 2003). Mastermind was first identified as a modulator of Notch signalling in *Drosophila*, and loss-of-function mutations of *Drosophila* Mastermind generates a similar phenotype to that of loss-of-function Notch (Kojika and Griffin 2001; Iso *et al.*, 2003b). Mastermind is therefore an essential component of the Notch signalling pathway, it does not bind to the DNA directly, but forms a complex with Notch IC and CBF-1, and acts to stabilize this complex (Nakagawa *et al.*, 2000; Kojika and Griffin 2001). Mastermind acts as a positive regulator of Notch signalling as it contains transcriptional activator domains (Baron *et al.*, 2002) and acts to recruit HATS to the co-activator complex (Fryer *et al.*, 2002). These HATS include p300/CBP (Oswald *et al.*, 2001) and PCAF/GCN5 (Kurooka and Honjo 2000) which may act catalytically to produce an open chromatin conformation, thus promoting transcription (Baron 2003). Therefore, repression and activation by CBF-1 proteins are linked to histone hypo- and hyper-acetylation respectively (Lai 2002).

### 1.6.5.2 CBF-1-independent Pathway

The first evidence of the existence of the CBF-1-independent pathway was obtained through analysis of Notch and Su(H) mutant phenotypes in *Drosophila*. The Notch mutant phenotype was seen to be slightly stronger than that of the Su(H) mutant, which suggested that Su(H)-dependent signalling does not mediate all functions of

Notch (Shawber *et al*, 1996, Martinez Arias *et al*, 2002) Further experiments in *Drosophila* highlight the existence and importance of the Su(H)-independent pathway, Notch, for example, has been shown to regulate dorsal closure in *Drosophila*, however, this effect is independent of Su(H), as Su(H) mutant embryos undergo dorsal closure normally (Zecchini *et al*, 1999) Several *in vitro* and *in vivo* experiments also point towards the existence of CBF-1-independent Notch signalling in vertebrates In 1996, Shawber *et al*, provided the first *in vitro* evidence of a Notch-activated CBF-1-independent pathway in vertebrates This study shows that Notch 1 over-expression prevents the differentiation of muscle cells (C2C12) upon serum withdrawal, but does not interact with CBF-1 or up-regulate endogenous Hes-1 expression Additionally, incubation of C2C12 myoblasts with Jagged expressing cells prevented their differentiation into myoblasts, but failed to activate a CBF-1-dependent reporter gene (Bush *et al*, 2001) Endo *et al*, provided the first *in vivo* evidence for the existence of CBF-1-independent Notch signalling This study shows that a Delta 1-activated Notch signal is required for the expression of *Slug*, which is involved in epithelial to mesenchyme cell transition in the developing neural crest during avian ectodermal development However, the expression of *Slug* is not affected by the expression of a dominant negative CBF-1 protein in the ectoderm

### 1 6 5 2 1 Molecular Mechanisms of CBF-1-independent Signalling

A growing repertoire of CBF-1-independent Notch signalling mediators is emerging In some cases the CBF-1-independent signals represent interactions of Notch IC with transcription factors other than members of the CBF-1 family, for example, with Mef 2C in mouse fibroblasts (Wilson-Rawls *et al*, 1999) and LEF1 in human T-cells (Ross and Kadesch 2001) Alternatively, CBF-1-independent signals may involve a direct interaction of Notch IC with other proteins within the cytoplasm that directly alters their function without any change in gene expression Notch signalling, for example, can directly alter the function of the translational regulator Musashi in the cytoplasm of *Drosophila* (Okabe *et al*, 2001)

The most commonly described mediator of CBF-1-independent Notch signalling is deltex The first links between the Notch signalling pathway and deltex were described in *Drosophila* Firstly, over-expression of deltex produces a similar dominant phenotype to that produced with over-expression of Notch IC, in addition, Notch IC has been shown to rescue the deltex negative (dx-) phenotype (Matsuno *et al*, 1995) This

data suggests that deltex acts genetically upstream of Notch, and that deltex acts as a positive regulator of the Notch signalling pathway Deltex encodes a cytoplasmic protein containing three domains, domain I, an SH3 binding domain (domain II) and a zinc-finger domain (domain III)(Matsuno *et al*, 1998), and in *Drosophila*, is expressed ubiquitously throughout development (Busseau *et al*, 1994) Further evidence linking deltex to the Notch signalling pathway are the protein-protein interactions observed between Notch and deltex both in *Drosphila* (Matsuno *et al*, 1995) and humans (Uyttendaele *et al*, 1996, Panin and Irvine 1998) Deltex binds to the conserved ankyrin repeats within Notch via domain I, only the most carboxyl (sixth) ankyrin repeat has been shown to be non-essential for this binding activity (Matsuno *et al*, 1995)

**Figure 20 Model of Physical Interactions between Notch and Deltex**

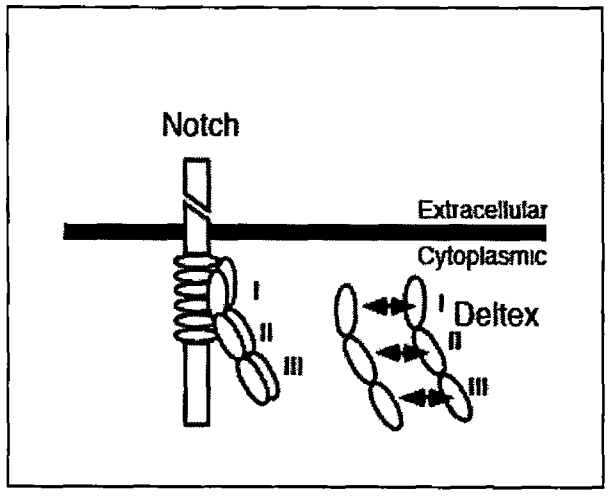



**Figure 20 Model of Physical Interactions between Notch and Deltex** Deltex associates with the first five ankyrin repeats of Notch via its domain I (Matsuno *et al*, 1995)

The function of domain III of deltex, the zinc finger domain is unknown, but it is thought to be involved in protein-protein interactions (Matsuno *et al*, 1995) Both *drosophila* and human deltex have been shown to bind to the SH3-domain containing protein Grb2 via domain II of deltex (Matsuno *et al*, 1998) This study also provides the first link between deltex and Notch-dependent transcriptional events, with deltex exerting a two-fold stimulation of Notch target gene expression in cultured cell assays Whilst transformation experiments demonstrate that only deltex domain I is sufficient to rescue a dx- mutation, they also suggest that domains II and III are also important for deltex function For example, the over-expression of full-length deltex induces dominant phenotypes much more efficiently than over-expression of domain I alone (Matsuno *et al*, 1995)

Notch IC has been shown to antagonize signalling through a JNK MAP kinase pathway (Ordentlich *et al*, 1998, Zecchini *et al*, 1999), and that this effect is mediated through deltex (Ordentlich *et al*, 1998) Through deltex-mediated effects on JNK, Notch signalling has been shown to crosstalk with Ras signalling (Miele and Osborne 1999) Studies in *Drosophila* have identified an antagonist of deltex, Suppressor of Deltex (Fostier *et al*, 1998), however, no mammalian homologue of this gene has, to date, been identified Suppressor of Deltex is an E3 ubiquitin ligase (Cornell *et al*, 1999), rendering it capable of binding to specific target proteins and adding ubiquitin Although its target protein is unknown, Notch is known to be ubiquitinated and degraded through the proteasome pathway (Qiu *et al*, 2000)

Other proteins that are involved in CBF-1-independent Notch signalling include p50-NFεB (Guan *et al*, 1996), the Abl-accessory protein Disabled (Giniger 1998), and the orphan nuclear receptor, nur 77 (Jehn *et al*, 1999) A human Notch 1 construct (delta RAM) has been shown to interact with the inhibitory p50-NFεB homodimers, and that this may be the method of Notch activation of NFεB seen *in vivo* (Guan *et al*, 1996) The *Drosophila abl* gene, and its vertebrate homologue, the protooncogene, *c-abl* encode cytoplasmic tyrosine kinases with a role in Notch signalling in neurons This effect is mediated by the abl accessory protein, disabled, which binds directly to Notch IC, and exerts an effect on axon guidance (Giniger 1998) Additionally, Notch 1 IC has been shown to directly interact with nur 77, resulting in an inhibition of nur 77-dependent transcription (Jehn *et al*, 1999)

The interaction of Notch with these proteins clearly demonstrates that not all of the effects of Notch are mediated through a CBF-1-dependent mechanism, but rather that several other proteins, in addition to the CBF-1 family of transcriptional regulators, can act as putative regulators of Notch signalling Whether some or all of these proteins regulate different functions of the Notch signalling pathway, or whether they represent different mediators used in differing cell types remains to be fully established

### 1 6 6 Effectors of the Notch Signalling Pathway (Notch Target Genes)

Notch IC activates expression of primary target genes of Notch signalling, such as *Hairy/Enhancer of Split (Hes)* and *Hairy Related Transcription Factor (HRT) genes* (Bailey and Posakony 1995, Lecourtois and Schweisguth 1995), which are activated by Notch IC in a CBF-1 dependent manner (Chen *et al*, 1997, Beatus *et al*, 1999, Lee *et al*, 1999b, Ohtsuka *et al*, 1999, Wang *et al*, 2002a) These genes are part of a family

of basic helix-loop-helix (bHLH) type transcriptional repressors that act as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors (Iso *et al*, 2001a, Iso *et al*, 2003b) The Hes family was the first described primary effector of Notch signalling It should be noted that, due to the independent identification of the second family of effectors of Notch signalling by different groups, many different names exist for the HRT family, including HERP (Hes-related repressor protein, (Apelqvist *et al*, 1999, Iso *et al*, 2001b)), CHF (Cardiovascular helix-loop-helix factor, (Chin *et al*, 2000)) and Hey genes (Maier and Gessler 2000)

### 1 6 6 1 bHLH Proteins

bHLH proteins have been implicated in the control of lineage commitment and differentiation in several organs and cell types, such as the heart, nervous system and skeletal muscle cells (Beatus *et al*, 1999) bHLH proteins bind DNA as a dimer, with the basic and HLH domains having distinct functions in this process, the basic domain is a major determinant of DNA binding, while the HLH domains contain hydrophobic residues that allow them to form either homo- or hetero-dimers (Murre *et al*, 1994) These proteins can be grouped into three different classes according to their structural features and biochemical characteristics, class A and B proteins such as Mash1 and Myc respectively are transcriptional activators, whereas class C proteins such as the Hes and HRT families act primarily as transcriptional repressors (Iso *et al*, 2003b)

There are several similarities and differences that render the Hes and HRT families related to, yet distinct from, each other These are evident both in their similar but distinct structures, and their mechanism of transcriptional repression

Consistent with their classification as bHLH proteins, 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*, 2003b) Both families contain another domain, the Orange domain, located carboxy to the bHLH region In addition, 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* 2001b)

Both the Hes and HRT families have been described as transcriptional repressors, with the exception of Hes-6, which antagonizes the function of Hes-1

resulting in de-repression (Bae *et al*, 2000) However, another differing characteristic between these two families is that they achieve transcriptional repression of target genes using different mechanisms

### 1 6 6 2 The Hes Family

Three mechanisms of transcriptional repression have been described as repression mechanisms utilized by the Hes family, active repression, passive repression and repression mediated by the Orange domain

Active repression involves binding of a Hes protein homodimer to an N box (CACNAG) consensus DNA site (Matsuno *et al*, 1995, Chen *et al*, 1997, Panin and Irvine 1998) and recruitment of the co-repressor Transducin-like Enhancer of Split (TLE) in mammals via the C-terminal WRPW motif (Kojika and Griffin 2001, Prakash *et al*, 2002) Chen *et al*, 1999 have shown that the *Drosophila* homologue of TLE, Groucho, can recruit a histone deacetylase Rpd3, an orthologue of mammalian HDAC Reduction of histone acetylation by HDACs is linked to repressed, transcriptionally inactive chromatin, rendering the chromatin less accessible to the transcription machinery (Kadesch 2000), however, whether this mechanism of repression is employed by mammalian TLE remains to be determined Active repression appears to be primary mode of repression employed by the Hes family, however, repression can be achieved in the absence of the C-terminal WRPW domain (Dawson *et al*, 1995, Takke *et al*, 1999)

Passive repression involves protein sequestration in which the Hes protein form a non-function heterodimer with another bHLH protein, such as E47 (Sasai *et al*, 1992, Hirata *et al*, 2000) MyoD and MASH1 are two transcription factors, important in myogenesis and neurogenesis respectively, interact with E47 and activate transcription This is inhibited by Hes-1, probably by disrupting the formation of functional MyoD-E47 and MASH1-E47 complexes

A third mechanism of repression by the Hes family has been shown to involve the Orange domain (Castella *et al*, 2000), either through the direct recruitment of an unidentified co-repressor, or by the stabilization of the TLE co-repressor function, or both

### 1 6 6 3 The HRT Family

In contrast to the Hes family, the primary mechanism of HRT mediated transcriptional repression involves its bHLH domain rather than its C-terminal tetrapeptide motif (Iso *et al*, 2001a) The method of repression employed is similar to the active repression employed by the Hes family, in that, following DNA binding, the HRT protein homodimer recruits a co-repressor complex including HDAC and/or Sin3A/N-Co-R to cause transcriptional repression (Iso *et al*, 2003b)

Therefore, although the Hes and HRT families have similar structures and functions, it is clear that they utilize distinct domains to achieve this transcriptional repression However, it has been suggested that Hes and HRT proteins may co-operate with one another within the cell to achieve transcriptional repression Hes and HRT proteins are co-expressed in many cells and organs, including the embryonic heart, and they have both been shown to bind to the same DNA sequences *in vitro* (Iso *et al*, 2003b) Leinmaster *et al*, (1999) demonstrated that Hes and HRT proteins associate with each other both *in vitro* and in intact cells in the absence of DNA In addition, Hes-HRT heterodimers have been shown to bind more efficiently to target DNA sequences than the respective homodimers (Iso *et al*, 2001b) Therefore, it is suggested that in cells co-expressing Hes and HRT proteins, such heterodimers are likely to form and act in preference to homodimers of the respective proteins (Iso *et al*, 2001b, Iso *et al*, 2003b)

**Figure 21 Model for HES and HRT Cooperation in Notch Signalling**

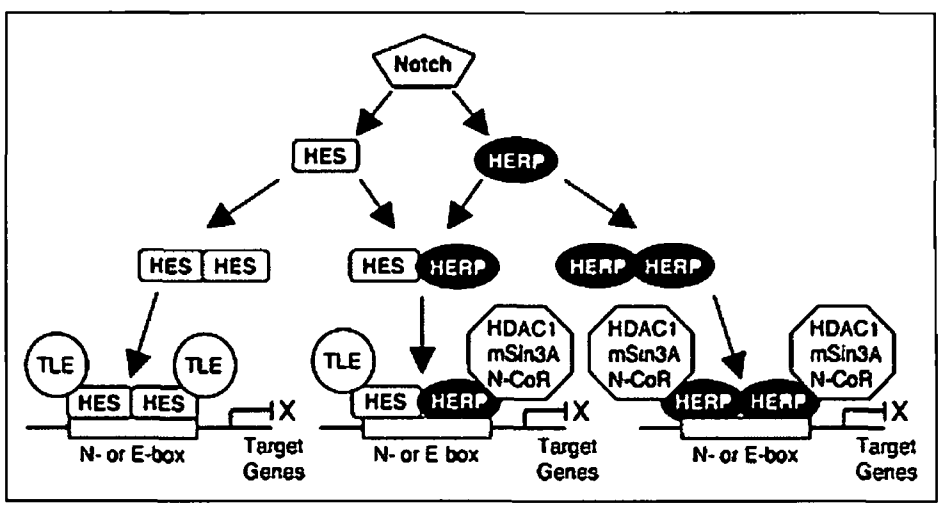



**Figure 21 Model for HES and HRT cooperation in Notch signalling** Refer to text (Iso *et al* 2001b)

Upon Notch stimulation, HES and HRT expression might both be induced In tissues where only HES or HRT is expressed, the respective homodimer binds promoters of target genes The HES homodimers recruit TLE via their WRPW motif, whereas the HRT homodimers recruit the mSin3A-HDAC-N-CoR complex via their bHLH domain In tissues where both HES and HRT are co-expressed, the HES-HRT heterodimers become the predominant complex that binds a specific DNA site newly defined by the basic domains of HES and HRT Because of the higher DNA binding affinity of the heterodimers, a lower concentration of them may be sufficient to achieve repression Repression by HES-HRT heterodimers may be reinforced by their ability to recruit a more diverse set of repressors (Iso *et al*, 2001b)

### 1 6 6 4 Primary Effectors of the Notch Signalling Pathway

Currently, 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) Similarly, 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) In addition, many studies have proven that Notch IC activation of both the Hes and HRT promoters occurs in a CBF-1 dependent manner In cultured mammalian cells, for example, the transactivation of Hes-1 and HRT-2 promoters by Notch IC was reduced in the presence of the CBF-1 mutants, R218H-RPB-Jε and RY227GS (Iso *et al*, 2001a) In addition, 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, CBF-1 is essential for both Hes and HRT expression in response to Notch signalling

Recent studies 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 (de la Pompa *et al*, 1997, Leimeister *et al*, 2000a, Leimeister *et al*, 2000b) In addition, activation of different Notch receptors can result in different levels of transactivation of the target gene Beatus *et al*, (1999) 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 The Hes and HRT families act as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors, for

example, MASH-1 (Ishibashi *et al*, 1994, Chen *et al*, 1997, Hirata *et al*, 2000) and Cbfa-1, a *Drosophila* Runt related protein involved in the regulation of a variety of cell differentiation events (McLarren *et al*, 2000), and thus are involved in the Notch signalling pathway regulation of cell fate decisions In addition, 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) Therefore, Notch signalling in a cell can lead to a decrease in the expression of its ligand

The importance of these 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) In addition, 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 Both HRT and Hes proteins have also been described as being present and active within adult vascular smooth muscle cells (Campos *et al*, 2002), however, their roles remain to be fully elucidated

### 1 6 7 Notch Signalling within the Vasculature

Dysfunctions of the Notch signalling pathway are associated with human pathologies involving cardiovascular abnormalities CADASIL (cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy) and Alagille syndrome are two such disorders (Gridley 2003) These, coupled with the increased expression of Notch signalling pathway genes in both vascular smooth muscle and endothelial cells *in vivo*, highlight both the presence and the importance of the Notch signalling pathway in both the adult and developing cardiovascular system (Lindner *et al*, 2001)

#### 1 6 7 1 CADASIL

The importance of Notch 3 in vascular smooth muscle cells 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 prevalence of this disorder is difficult to estimate, as it is believed that the condition is under- or mis-diagnosed due to its variable mode of presentation (Tournier-Lasserve *et al*, 1993) In support of this theory, of the 300 affected families that have been described worldwide, more than one third of these are found in regions where systematic DNA-based screening and diagnosis are employed (Prakash *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 Ultrastructural analysis of affected arteries have revealed alterations and eventual loss of vascular smooth muscle cells, and the accumulation of granular osmophihc material within the smooth muscle cell basement membrane and the surrounding extracellular matrix (Gridley 2003) In addition, endothelial cells appear to shrink, detach from the basal lamina, and the tight and gap junctions appear to be disrupted (Prakash *et al*, 2002)

All of the mutations associated with CADASIL result in the gain or loss of a cysteine residue in one of the 34 EGF-like repeats of the ExC domain of the Notch 3 protein Most of these mutations are mis-sense mutations and tend to be found within the first five EGF-like repeats (Gridley 2003, Liu *et al*, 2003) The resulting odd number of cysteine residues within the EGF-hke repeats is likely to alter the overall conformation of the Notch 3 protein (Joutel and Tournier-Lasserve 1998) Studies have shown that although this mutation does not affect the cell surface expression or ligand binding ability of the protein (Loomes *et al*, 1999, Gridley 2003), the clearance of the Notch 3 ExC domain from the cell surface is greatly impaired (Luo *et al*, 1997, Gridley 2003) This was first recognized by Joutel *et al*, (1996) upon observing that the ExC domain of the Notch 3 protein accumulates in the cerebral microvasculature of CADASIL patients It has been suggested that the mutant Notch 3 molecules present on

the cell surface compete with non-mutant Notch proteins for ligand binding, thus dominantly inhibiting the normal signalling pathway (Spinner 2000)

### 1 6 7 2 Alagille Syndrome

Alagille Syndrome (AGS) is an autosomal dominant disorder characterized by developmental abnormalities of the heart, liver eye, skeleton and kidneys (Loomes *et al*, 1999) 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) Two groups independently identified Jagged 1 as the defective gene in AGS (Henderson *et al*, 1994, Joutel and Tournier-Lasserve 1998), further highlighting the importance of the Notch signalling pathway in the cardiovascular system

The majority of mutations (90%) identified to date are frame-shift, non-sense and splice mutations, resulting in a truncated protein product (Joutel and Tournier-Lasserve 1998, Iso *et al*, 2002) Mis-sense mutations have also been identified, and have resulted in the defective mtra-cellular transport and processing of the mutant protein (Morrissette *et al*, 2001) However, Jagged 1 mutations have only been found in 70% of AGS patients (Spinner *et al*, 2001), and these patients (90%) typically present with cardiovascular abmormalities in the absence of other characteristic defects (McElhinney *et al*, 2002) This suggests that other genes, perhaps those encoding other components of the Notch signalling pathway, are also responsible for AGS

The two aforementioned disorders highlight the importance of the Notch signalling pathway in the cardiovascular system In addition, a study by Linder *et al*, (2001) demonstrated that component of the Notch signalling pathway were up-regulated in a rat carotid artery model following balloon catheter denudation Therefore, the Notch signalling pathway may also play a role in the cardiovascular response to injury

### 1 6 8 Overlap between the Notch Signalling Pathway and Epstein-Barr Virus

EBV is a human oncogenic herpes virus, which causes lymphoprohferative diseases, such as Burkitt's lymphoma, in immunodeficient individuals (Le Roux *et al*, 1994) The regulation of cell fate decisions (apoptosis, proliferation and differentiation) by EBV is central to the establishment of latent infection and the long-term survival of the infected cell EBV infects primary resting B cells and has been shown to induce unlimited proliferation of virus-infected cells *in vitro* (Hofelmayr *et al*, 2001) In addition, a central component in the overall EBV strategy of infection is the ability of the viral latent proteins to suppress the cellular apoptotic program (Allday 1996) Several viral genes are required for the initiation and maintenance of cell growth, these include genes encoding the nuclear proteins EBNA-1, EBNA-2, EBNA-LP, EBNA-3A and EBNA-3C, and the plasmid membrane protein, Latent Membrane Protein-1 (LMP-1) (Spender *et al*, 1999) EBNA-2 and EBNA-LP are the first viral genes expressed following EBV infection, and are essential for both the initiation and maintenance of immortalization (Hammerschmidt and Sugden 1989)

The overlap between EBV and the Notch signalling pathway is evident as many of the EBV latent proteins, including EBNA-2, EBNA-3A and other viral gene products such as RPMS-I, have been shown to interact with the Notch signalling pathway (Smith *et al*, 2000) In addition, EBV infection and activation of the Notch signalling pathway achieve similar functions phenotypically, by virtue of their ability to modulate cell fate decisions Several studies have highlighted this, including Hofelmayr *et al*, (2001), demonstrating that activated Notch 1 can transiently substitute for EBNA-2 in the maintenance of proliferation of immortalized B cells

#### 1 6 8 1 EBNA-2

EBNA-2 is essential for B-cell immortalization by EBV, and is also necessary for the maintenance of the transformed state of the cell (Hofelmayr *et al*, 1999) EBNA-2 activates a number of cellular and viral genes, including CD21 (B cell differentiation marker), CD23 (B cell activation marker) and the oncogene *c-fgr* In addition, EBNA-2 activates transcription of the viral RNAs coding for the various EBV nuclear and membrane proteins expressed in transformed cells (Kieff 1996) EBNA-2 does not bind to DNA directly, but is recruited to EBNA-2 responsive elements by interacting with the transcription factor, CBF-1, in a manner reminiscent to that of

Notch IC (Schlee *et al.*, 2004). CBF-1 binding sites are present in all of the EBNA-2-regulated promoters identified to date, and this binding site is essential but not sufficient to mediate EBNA-2 responsiveness (Hofelmayr *et al.*, 1999). As with Notch IC, activation of CBF-1 by EBNA-2 involves a two-part mechanism, as shown in figure 22. Firstly, EBNA-2 acts to displace the co-repressor complex from the CBF-1-SKIP complex and, upon binding to this CBF-1 complex, EBNA-2 activates gene transcription through the presence of its endogenous activation domain (Hsieh *et al.*, 1996). Therefore, EBNA-2 can be regarded as a functional homologue of activated Notch, and as such can provide insight into the Notch signalling pathway. In addition, other protein products of EBV can provide tools for manipulating the Notch signalling pathway, as is the case with EBNA-3A and RPMS.

**Figure 22: Model for EBNA-2 Activation of CBF-1 Repressed Promoters**

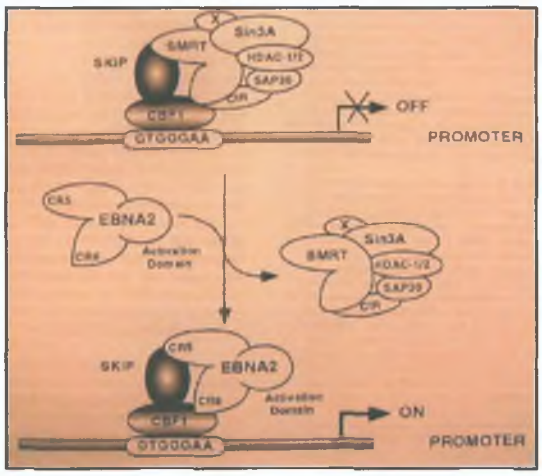



**Figure 22: Model for EBNA-2 activation of CBF-1 repressed promoters.** CBF-1 binds promoter sequence within the nucleus, SKIP interacts with CBF-1. The SMRT/HDAC co-repressor complex binds to both SKIP and CBF-1, mediating transcriptional repression. EBNA-2 competes with SMRT for contacts on both SKIP and CBF-1. Displacement of the co-repressor complex relieves repression, and EBNA-2 further activates promoter through its transactivational domain (Hsieh *et al.*, 1996).

### 1.6.8.2 EBNA-3A

EBNA-3A is essential for EBV transformation of B cells (Cludts and Farrell 1998). Two regions of EBNA-3A have been shown to bind to CBF-1 (Robertson *et al.*, 1995; Robertson *et al.*, 1996; Zhao *et al.*, 1996). EBNA-3A has been shown to repress EBNA-2 mediated transactivation by binding directly and exclusively to CBF-1, thus preventing it binding to its target DNA sequence (Robertson *et al.*, 1995; Robertson *et al.*, 1996). Therefore EBNA-3A negatively modulates transcriptional activity mediated

by EBNA-2, and consequently may also be antagonistic to the Notch signalling pathway

### 1 6 8 3 RPMS-I

The EBV *Bam*HI-A rightward transcripts (BART's) are expressed in all EBV-associated tumours, in addition to latently infected B cells both *in vivo* and *in vitro* One of the EBV BART's contains an open reading frame RPMS-I, encoding the nuclear protein, RPMS-I Smith *et al*, (2000) demonstrated that RPMS-I antagonized both Notch IC and EBNA-2 mediated activation at promoters with CBF-1 binding sites, in addition Zhang *et al*, (2001) have shown that RPMS-I reversed Notch IC-mediated inhibition of differentiation in myoblasts, again highlighting the central role of the Notch signalling pathway in EBV biology RPMS-I interacts with both CBF-1 and components of the CBF-1 co-repressor complex RPMS-I has been shown to bind to the co-repressor component CIR with the highest affinity (Zhang *et al*, 2001) As illustrated in figure 23, RPMS-I acts to stabilize the CBF-1-HDAC co-repressor complex, thus preventing effective displacement of the co-repressor complex by Notch IC or EBNA-2 (Zhang *et al*, 2001)

**Figure 23 Stabilization of CBF-1 mediated repression**

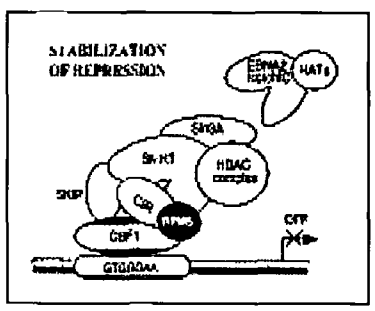



**Figure 23 Stabilization of CBF-1 mediated repression** RPMS-1 mediates repression of CBF-1 through binding to CIR, thus precluding the effective displacement of the co-repressor complex by EBNA-2 and Notch IC (Zhang *et al* 2001)

Thus, several areas of overlap between the Notch and EBNA-2 signalling pathways are evident, and consequently, if both are present and active within a cell, there exists a potential for mutual modification of the activity and function of the pathway

## 1 7 Relevance and Objectives of this Study

Apoptosis is an essential physiological process, important in both vascular development, and maintenance of homeostasis within the adult vasculature Dysregulation of apoptosis, however, is a common response to vascular injury, contributing to the progression and ultimate clinical outcome of vascular disease states, such as hypertension, transplant arteriopathy, and atherosclerosis (Best et al , 1999) Despite its clinical importance, regulation of apoptosis within the adult vasculature is poorly understood Therefore, better knowledge of the control of this process could identify potentially important therapeutic targets, and could have significant therapeutic implications

Numerous studies have implicated the Notch signalling pathway in the regulation of cell fate decisions, including apoptosis, in many cell types (Artavanis-Tsakonas *et al*, 1995, Greenwald 1998, Artavanis-Tsakonas *et al*, 1999) Whilst the presence and function of the Notch signalling pathway was not established in adult VSMC, until recently, several lines of evidence indicated that components of this pathway could be present, and act to regulate apoptosis in adult VSMC The Notch signalling pathway has previously been implicated in several aspects of vascular development, including arterio-venous differentiation, angiogenic processes, and in the regulation of developmental VSMC fate decisions (Artavanis-Tsakonas *et al*, 1999, Gridley 2001, Iso *et al*, 2003a) Moreover, mutations in Notch receptors result in embryonic lethality due to defects in both vasculogenesis and angiogenesis (Xue *et al*, 1999, Singh *et al*, 2000) In addition, the fact that dysregulation of the Notch signalling pathway is involved with human pathologies involving cardiovascular abnormalities, such as CADASIL and AGS, highlights the importance of the Notch signalling pathway in the adult vasculature

Therefore, the principal aims of this study are to establish the presence and activity of the Notch signalling pathway within VSMC, and determine the effect, if any, of this pathway on VSMC apoptosis, induced by both serum-deprivation and exposure to cyclic strain In addition, we aim (i) to determine whether Notch signalling and function within these cells is executed in a CBF-1-dependent or -independent manner, and (ii) to begin to elucidate potential cellular targets of the Notch signalling pathway in the regulation of VSMC apoptosis In addition, *in vivo* models of altered biomechanical force are used as an *in vivo* correlation of the *in vitro* findings of alterations in Notch signalling pathway expression and apoptosis due to changes in cyclic strain

This study aims to provide increased understanding into the regulation of

apoptosis in VSMC, both under physiological and pathological conditions, and additionally aims to direct future research, with the ultimate aim of the eventual development of diagnostic, prognostic and therapeutic tools in an attempt to decrease mortality due to cardiovascular disease

# Chapter 2

# Materials & Methods

## 2 1 Introduction

All general purpose chemicals and reagents used in experimental work were of analytical grade, and were purchased from the companies indicated in section 2 2 All cell culture plastic-ware was purchased from Sarstedt (Drinagh, Wexford, Ireland)

## 2 2 Materials

### 2 2 1 Biological Materials

#### 2 2 1 1 Antibodies

The anti-Notch 1 and -Notch 3 were purchased from Upstate Cell Signalling Solutions (Milton Keynes, UK) The anti- HRT-1, -HRT-2, -HRT-3, -Hes-1, -Hes-5, -Jagged and an additional -Notch 3 antibody were purchased from Santa Cruz Biotechnology (CA, USA) The HRP conjugated anti-mouse, anti-goat and anti-rabbit antibodies were purchased from Amersham Biosciences (Buckinghamshire, UK) The Alexa-fluor conjugated secondary antibodies were purchased from Molecular Probes (Leiden, The Netherlands)

## 2 2 1 2 Expression and Reporter Constructs

**Table 3 Plasmids used in this study**



| Plasmid | Gift from | Description |
|---|---|---|
| pCMV-ED1-HA<br>pCMV-ED4<br>pHACS1 | Dr Bettina Kempkes,<br>GSF-Institute of Clinical<br>Molecular Biology, Neuherberg,<br>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,<br>Karolinska Institute,<br>Stockholm,<br>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<br>pGa98-1-6 | Dr Bettina Kempkes,<br>GSF-Institute of Clinical<br>Molecular Biology, Neuherberg,<br>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<br>pGL3-mHRT1<br>pGL3-mHRT3 | Prof Eric Olson,<br>University of Texas,<br>Southwestern Medical Centre,<br>Dallas,<br>Texas,<br>USA | 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<br>pTK Hes-5-luc | Dr Urban Lendahl,<br>Karolinska Institute,<br>Stockholm,<br>Sweden | Hes-1 and –5 promoter fragments were inserted into pTK-luc vector |
| pcDNA3RPMS-I | Dr Paul J Farrell,<br>Ludwig Institute for Cancer Research,<br>Imperial College School of Medicine,<br>London,<br>U K | Contains the full length RPMS-I cDNA ORF cloned into the pcDNA3HA vector (Invitrogen) (Smith, 2000) |

| | |
|---|---|
| pCMX-R218H-RBP-Jκ | Prof Diane Hayward,<br>Johns Hopkins School of Medicine,<br>Baltimore,<br>Maryland 21231,<br>USA |
| pSG5<br>pSG5EBNA2 | Lindsey Spender,<br>Ludwig Institute for Cancer Research,<br>Imperial College School of Medicine,<br>London,<br>U K |
| pCMV<br>pCMV-EBNA3A | Dr Celine Gelinas,<br>University of Medicine and Dentistry of New Jersey,<br>New Jersey,<br>USA |
| p-1374/+81 Bfl-1 Luc | Dr Brendan D'Souza,<br>Laboratory for Molecular and Cellular Biology,<br>Dublin City University,<br>Dublin,<br>Ireland |
| pPGKpuro | Dr Peter Laird,<br>University of Southern California,<br>Kerk School of Medicine,<br>Los Angeles,<br>California,<br>USA |
| 3 x enh κB-luc<br>pSG5 IκB | Prof Martin Rowe,<br>University of Wales,<br>Cardiff,<br>U K |

pCMX-R218H-RBP-Jκ is a dominant-negative CBF-1 contstruct, with an arginine to histidine mutation at position 218



PSG5EBNA2 (pPDL151) expresses the wild type B95-8 EBNA2 gene which has been cloned into pSG5 (Stratagene)

Contains the EBNA3A cDNA inserted into the pCMV vector (Stratagene) (le Roux, 1994)

Consists of the BamH1-Xbal fragment of –1374/+81 Bfl-1 CAT inserted into the BamH1-HinDIII portion of the pGL2 basic vector which contains the luciferase reporter (D'Souza, 2000)

Puromycin plasmid (Tucker *et al* , 1996)

3 x enh κB-luc contains three κB elements upstream of a minimal conalbumin promoter driving the expression of the firefly luciferase gene *(Floettmann and Rowe 1997)* pSG5 IκB contains IκB cloned into the pSG5 vector

**Table 4 Generic name assigned to plasmids used in this study**

| Plasmid (Generic name) | Generic name assigned to plasmids used in this study |
|---|---|
| pCMV-ED1-HA | Notch 1 IC |
| pCMV-ED4 | mut Notch 1 IC |
| pCMX-Notch 3 IC-HA | Notch 3 IC |
| pGa98-1-6 | CBF-1-luc |
| pGa50-7 | CBF-1-luc Con |
| pcDNA3RPMS-I (RPMS-I) | RPMS-I |
| pCMX-R218H-RBP-Jκ | R218H |
| pSG5EBNA2 | EBNA-2 |
| pCMV-EBNA3A | EBNA-3A |
| 3 x enh κB-luc | NFκB |
| pSG5 IκB | IκB |

### 2 2 1 3 Commercial Kits and Oligonucleotides

| | |
|---|---|
| Oligonucleotides (see section 2 3 8 3 ) | MWG Biotech |
| BCA Protein Assay Kit | Pierce |
| High Sensitivity Mgalactosidase Assay Kit | Stratagene |
| Luciferase Reporter Reagents | Promega |
| Wizard$^{R}$ *Plus* Midipreps DNA purification kit | Promega |
| Super Signal West Pico Chemiluminescent Reagent | Pierce |

### 2 2 2 Chemical Materials

AGB Scientific (Dublin, Ireland)

Whatmann Chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)

ECL Hybond nitrocellulose membrane

ECL Hyperfilm

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

BioRad (Alpha Technologies, Dublin)

Bio-Dot SF Micro filtration apparatus

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DMEM

dNTP's

DEPC-treated water

Trizol® reagent

Calbiochem (Bad Soden, Germany)

Hygromycin

Gibco (Dun Laoghaire, Ireland)

G418

Oxoid (Hampshire, U K )

Tryptone

Skim Milk Powder

Yeast Extract

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Promega (UK)

Taq DNA Polymerase

MLV-RT

RNase H

Oligo dT

Sigma Chemical Company (Poole, Dorset, England)

Mglycerophosphate

Mmercaptoethanol

Acetic Acid

Acetone

Agarose

Ammonium Persulphate

Acrylamide/bis-Acrylamide

Bovine Serum Albumin

Brefeldin A

Brightline Haemocytometer

Bromophenol blue

Calcium Chloride

CHAPSO

Chloroform

DMEM

DMSO

DTT

EDTA

EGTA

Ethidium Bromide

Methanol

Mineral oil (molecular grade)

Penicillin-Streptomycin (100x)

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

p-Nitroaniline

RPMI-1640

Sodium Acetate

Sodium Chloride

Sodium Doecly Sulphate

Sodium Hydroxide

Sodium Nitrite

Sodium Orthovanadate

Sodium Phosphate

Sodium Pyrophosphate

Sulphuric Acid

Tetracycline

TEMED

Foetal Calf Serum
Glycerol
Glycine
Hanks Balanced Salt Solution
Hydrochloric acid
Isopropanol
Lauryl Sulphate
Leupeptin
Monensin
N-Acetyl-Asp-Glu-Val-Asp-pNitroanilide
Tris Acetate
Tris Base
Tris Chloride
Triton X-100
Trypsin-EDTA solution (10x)
Tween 20

Qiagen (West Sussex, U K )
SYBR Green® PCR Kit

## 2 3 Methods

### 2 3 1 Cell Culture

#### 2 3 1 1 Introduction

All cell culture procedures were performed using aseptic technique in a sterile environment created by an Aura 2000 m a c laminar airflow cabinet, Bioair Instruments Cells were visualised using an Oylmpus CK30 phase contrast microscope

#### 2 3 1 2 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 passed using a trypsinisation method This method 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 3 1 3 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 3 1 2

### 2 3 1 4 Cell Counting

Cell counts were carried out using a Sigma bright line haemocytometer In brief, a drop of cell suspension was used to fill the haemocytometer counting chamber Following visualization under 10X magnification, the number of cells were counted in each of the four outer quadrants of the haemocytometer The average of these four counts was equal to the number of cells x $10^4$/ml of cell suspension

### 2 3 1 5 Cell Storage and Recovery

For longterm storage of cells, freshly trypsinised and pelleted cells, as described in section 2 3 1 2, were re-suspended in freezing down media (RPMI-1640, 20% FCS, 1% P/S and 10% DMSO) and transferred to sterile cryovials Typically cells from a 175 $cm^2$ tissue culture flask were re-suspended in 3 ml freezing down media, and stored in 1 ml aliquots The cryovials were then placed in a Nalgene cryofreezing container, and placed in the -80°C freezer overnight This allows for gradual freezing of the cells at a rate of -1 °C/min Cells were then transferred to the Thermoylen locater jr cryostorage system for longterm storage in liquid nitrogen, the levels of which were monitored regularly

Recovery of cells from liquid nitrogen storage involves gradual thawing of a 1 ml aliquot, and the immediate addition of the aliquot to 9 ml of RPMI growth medium when thawing occurs Cells were then centrifuged at 3,500 rpm for 5 min, resuspended in 1 ml of RPMI growth medium, and added to 14 ml of medium in a 75 $cm^2$ tissue culture flask Media was changed the following day, and cells were routinely sub-cultured twice before being used in an experiment to ensure complete recovery

### 2 3 1.6 Exposure of RVSMC to Cyclic Strain

RVSMC were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of $1x10^5$ cells/well Bioflex® plates contain a pronectin-coated silicon membrane bottom which enables precise deformation of cultured cells by microprocessor controlled vacuum (Banes *et al*, 1995) When cells had reached approximately 80-90% confluency, a Flexercell® Tension Plus™ FX-4000T™ system (Flexcell International Corporation, Hillsborough, NC, USA) was subsequently employed to apply cyclic strain, typically 10% cyclic strain, 60 cycles/mm, 24 h was applied unless otherwise stated Control cells remained unstrained Following strain, the cells were washed twice in 1x PBS, and either protein or RNA samples isolated as described in sections 2 3 2 or 2 3 8 1 respectively Alternatively, cells were assayed for apoptosis using Acridine Orange/Ethidium Bromide or Annexin V/Propidium Iodide dual stains as described in sections 2 3 9 2 and 2 3 9 3 respectively

### 2 3 1 7 Culture and Maintenance of HeLa Cells

A HeLa-derived cell line, HtTA-jag 10 cells, expressing human Jagged 1 under tetracycline control was also used in this study, and were obtained as a kind gift from Prof Celine Gelinas, Centre for Advanced Biotechnology and Medicine, NJ, USA Cells were maintained in DMEM, supplemented with 10% FCS, 1% P/S, hygromycin (225 U/ml), G418 (125 μg/ml) and tetracycline (2 μg/ml), and routinely sub-cultured To induce jagged expression, cells were cultured onto 6-well plates at a density of 1 x $10^5$ cells/well Following overnight recovery, cells were maintained in media in the absence of tetracycline for a period of 48 h To facilitate jagged expression, cells were washed three times in growth media (- tetracycline) at 0 and 24 h following initiation of jagged induction

## 2 3 2 Preparation of Whole Cell Lysates

The preparation of whole cell lysates involved the trypsinsation and centrifugation of cells, as described in section 2 3 1 2 The cell pellet was then re-suspended in an appropriate volume of 1x Lysis Buffer (20 mM Tris, 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2 5 mM sodium pyrophosphate, 1 mM M glycerophosphate, 1 mM sodium orthovanadate and 1 ug/ml leupeptin), typically cells from a 175 $cm^2$ tissue culture flask were re-suspended in 300 – 500 μl 1x Lysis Buffer

The lysates were then subjected to freeze-thawing three times, and stored at –80°C prior to use

### 2 3 3 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 3 4 DNA Manipulations

#### 2 3 4 1 Preparation of Competent Cells

Competent JM109 cells were used during this study for transformation of DNA A modified Rhubidium chloride ($RbCl_2$) method was employed to prepare competent cells This method gives better transformation efficiencies than the Calcium chloride ($CaCl_2$) procedure for most strains This procedure is an adaptation of one described by (Hanahan 1983) An *E coli* strain was streaked from a glycerol stock onto 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 The resulting culture (2 5 ml) was then used to inoculate 250 ml of LB broth, supplemented with 20 mM $MgSO_4$, and incubated in a 1 L flask at 37°C until the O D of the culture at 640 nm was between 0 4 and 0 8 (approximately 4-5 h) The cells were then transferred to two sterile 250 ml centrifuge tubes and pelletted by centrifugation at 4,500 x g, 4°C for 5 min The resulting pellets were re-suspended in 0 4 original volume ice-cold TFB1 (30 mM Potassium Acetate, 10 mM $CaCl_2$, 50 mM

$MgCl_2$, 100 mM RbCl, 15% glycerol, pH 5 8 and filter sterilize 100 ml for 250 ml culture – 50 ml per centrifuge tube) and the two pellets combined Cells were kept on ice for all subsequent steps, and pipettes, tubes and flasks were chilled The re-suspended cells were then incubated on ice at 4°C for 5 min, and pelleted by centrifugation at 4,500 x g, 4°C for 5 mm Cells were then gently re-suspended in 1/25 of the original volume of ice-cold TFB2 (100 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH 6 5 and filter sterilize for 250 ml culure use 10 ml) Cells were then incubated on ice for 1 h, ahquoted at 100 µl per tube for storage at -80°C Prior to storage the ahquoted cells were snap frozen in a dry ice/isopropanol bath JM 109 compenent cells prepared using this method are stable for 1 year

**2 3 4 2 Transformation of DNA into JM 109**

Frozen JM 109 competant cells were removed from –80°C environment and placed on ice for 5 min, or until just thawed The cells were mixed gently, by flicking the tube, and 100 µl of cells per transformation were transferred to pre-chilled culture tubes Plasmid DNA (10 ng) was added to the 100 µl of JM 109 cells, ensuring even addition of DNA to the cells by trituration and by gently flicking the tube several times The tube was immediately placed on ice for 30 mm, after which time the cells were heat-shocked for 45-50 sec at exactly 42°C, without shaking The tube was then immediately placed on ice for 2 min After this time, 900 µl of SOC medium (0 5% Yeast Extract, 2 0% Tryptone, 10 mM NaCl, 2 5 mM KCl, 10 mM $MgCl_2$, 20 mM$MgSO_4$, 20 mM glucose, pH 7 0) was added to the transformation reaction tube and incubated for 60 mm at 37°C, shaking at approximately 225 rpm The tube was then centrifuged at 3000 rpm for 1 min, 900 µl of the supernatant was removed, and the pellet resuspended in the remaining 100 µl The resuspended cells (20 µl and 80 µl) were plated out, with the appropriate controls, on LB plates (1 0% Tryptone, 0 5% Yeast Extract, 0 5% NaCl, 1 5% Agar) containing the appropriate selective medium (LB + 125 µg/ml ampicillin) and incubated at 37°C for 12-16 h If the cells are transformed they become ampicillan resistant, thus only the transformed cells will yield colonies After this incubation period, a single cell colony is picked under aseptic conditions, and introduced into a starter culture as described in section 2 3 4 4 for eventual plasmid preparation

### 2 3 4 3 Glycerol Stock Preparation

Following the transformation procedure (section 2 3 6 2), glycerol stocks of the plasmid of interest were made by aseptically removing 800 µl of the starter culture and mixing it with 200 µl glycerol in a sterile eppendorf The glycerol stock was then stored at -80°C

### 2 3 4 4 Preparation of Plasmid DNA

All procedures were carried out under aseptic conditions A glycerol stock of the plasmid of bacteria of interest was streaked out onto LB ampicillin agar (1 0% tryptone, 0 5% yeast extract, 0 5% NaCl, 1 5% Agar, 125 µg/ml ampicillm) and incubated overnight at 37°C An isolated colony from this plate was used to inoculate a 3 ml starter culture of LB broth (1 0% tryptone, 0 5% yeast extract, 1 0% NaCl) with the appropriate antibiotic (ampicillm 100 µg/ml), which was incubated at 37°C with gentle agitation at approximately 225 rpm 200 µl of the starter culture was then used to inoculate 25 ml of LB broth (secondary culture) with the appropriate antibiotic in a 250 ml sterile flask The secondary culture was then incubated overnight at 37°C with gentle agitation at approximately 225 rpm, with the appropriate controls The O D of the culture should read 1-1 5 at 600 nm The secondary culture was then transferred to a sterile centrifuge tube and centrifuged, using a T533 rotor in an ALC PK 131R centrifuge, at 6,000 x g for 15 min at 4°C The supernatant was then removed and the pellet was dried by inverting the tube on tissue paper and allowing the remaining supernatant to drain off

Plasmids were prepared using the Wizard$^{R}$ *Plus* Midipreps DNA purification kit (Promega) according to the manufacturers instructions Briefly, the cell pellet prepared was completely re-suspended in 3 ml of Cell Resuspension Solution, complete re-suspension being critical for optimal yields Cell Lysis Solution (3 ml) was then added, and the mixture inverted four times, after which time 3 ml of Neutralization Solution was added and again mixed by inversion The mixture was centrifuged at 14,000 x g for 15 min at 4°C, and the supernatant then decanted to a new centrifuge tube (DNA solution) Ten ml of DNA purification resin was added to the DNA solution, and swirled for 30 sec The resin/DNA mixture was then transferred to a midicolumn that was attached to a vacuum port A vacuum of at least 15 inches of Hg was applied to pull the sample through the column, after which time the vacuum was disconnected Column wash solution (15 ml) was added to column and vacuum reapplied, this washing procedure was repeated twice The column was allowed dry for 30 sec after

the second wash. The midicolumn reservoir was then removed, placed in a micro-centrifuge tube and centrifuged at 10,000 x g for 2 min. The reservoir was then placed in a new microcentrifuge tube, 300 µl of preheated (65-70°C) nuclease-free water was added to the reservoir, incubated for 1 min, and the reservoir was then centrifuged at 10,000 x g for 20 sec. The reservoir was then discarded and eluate was centrifuged at 10,000 x g for 5 min. The DNA containing supernatant was then transferred to a new microcentrifuge tube and stored at –20°C in 1x Tris EDTA (TE) buffer (10 mM TrisCl, 1 mM EDTA, made up in DNase/RNase free water, pH 7.4), for example add 30 µl of 10x TE buffer to 300 µl of eluted DNA.

#### 2.3.4.5 DNA Quantitation and Storage

To determine the amount of DNA in sample obtained from the plasmid midi kit, the sample was diluted 1:100 in sterile TE (1x) buffer and spectrophotometric analysis carried out using the Shimadzu UV-160A dual spectrophotometer, blanked with TE. The sample was measured, using 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.

### 2.3.5 Transient Transfections

#### 2.3.5.1 LipofectAMINE ™ (LFA) Mediated Transfection

Transient transfection was carried out in RVSMC using LipofectAMINE™ (Invitrogen), according to the manufacturers instructions. For transfection in a 6-well plate, cells were seeded at a density of 1.0 x $10^5$ cells/well, and transfected at 70% confluency 2 days later. Per well of a 6-well plate (10 $cm^2$), the cells were transfected with a luciferase reporter construct, and various expression constructs, with the addition of a total of 2.0 µg DNA/well. The pCMV-LacZ expression construct (0.5 µg/well) was co-transfected for normalization. Briefly, the appropriate volumes of DNA, mixed before use, were added to 500 µl of DMEM/well not containing serum or antibiotics as these can decrease transfection efficiency. Per well of a 6 well plate, 4 µl of LFA was made up to 100 µl in DMEM, mixed and added to the DNA/DMEM

mixture, and incubated for 30 min at room temperature to allow DNA-liposome complexes to form During this incubation period, each well to be transfected was washed twice with HBSS, and 400 µl DMEM was added to each well Following completion of the incubation period, 600 µl DNA/DMEM/LFA mixture was placed on each well, and incubated for 3 h at 37°C in a humidified atmosphere Following incubation, transfection medium was aspirated from well and replaced with RPMI-1640 growth medium, and the cells were allowed to recover for 18-24 h following transfection Each transfection on 6-well plates was performed in triplicate For transfections in flasks of a larger surface area, the amount of DNA and reagents were calculated up in the same proportion as was used in transfecting each well of a 6-well plate which is 2 µg DNA/10 $cm^2$

**2.3 5 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 mm, 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 40 µl 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, with is measured by the luminometer (Labsystems Luminoskan)

**2 3.5 3 β-Galactosidase Assay**

A β-Galactosidase assay (High Sensitivity β-Galactosidase Assay, Stratagene) was carried out according to the manufacturers instructions to normalise for differing transfection efficiencies β-Galactosidase, which is expressed due to transfection with pCMV-LacZ, catalyzes the hydrolysis of chlorophenol red-β-D-galactopyranoside (CRPG) into galactose and the chromophore chlorophenol red, yielding a dark red solution, which can be quantified using a spectrophotometer at 570 nm In brief, per sample, 20 µl of cell lysate was analysed in triplicate in a 96 well

plate 130 µl of 1x CRPG substrate was added per well and the time recorded The plate was then covered and incubated at 37°C for 30 min – 72 h, until the sample turned a dark red The incubation time should be recorded and the reactions terminated by the addition of 80 µl Stop solution (0 5 M $Na_2CO_3$) to each well The absorbance was then analysed at 570 nm using a microplate spectrophotometer A blank was set up for each experiment by substituting the 20 µl of sample for 20 µl of lysis buffer

#### 2 3 5 4 Calculations

Luciferase activity was corrected for both MGalactosidase activity and differing protein concentrations, and expressed as fold activation over the empty vector

#### 2 3 5 5 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 100 ug streptomycin, and 0 8 µg/ml puromycin for 48 h

### 2 3 6 Dot Blot Immunoassay

The dot blot immunoassay technique was used to optimize both primary and secondary antibody dilutions prior to western blot analysis A Bio-Dot SF Microfiltration apparatus (170-6542) from Bio-Rad was used for all dot blots The dot blot apparatus was used to immobilize protein from whole cell lysates directly onto a nitrocellulose membrane The dot blot procedure was carried out as follows, the dot blot apparatus was cleaned and dried prior to use, and subsequently assembled as per manufacturers instructions (Bio Rad) In brief, this involved the soaking of three sheets of Whatman chromotography paper in phosphate buffered saline (PBS) Nitrocellulose membrane (PALL BioTrace) was pre-soaked in distilled water, and also soaked in PBS The dot blot apparatus was then assembled with the filter paper and membrane as per instruction manual The vacuum was then applied to the apparatus, and the four corner screws of the apparatus tightened in a diagonal crossing pattern under vacuum to ensure no cross well contamination The membrane was then re-hydrated with 100 µl PBS per well The flow valve was then adjusted so that the vacuum chamber was open to the atmosphere A volume of 200 µl protein solution containing 10 µg RVSMC total

protein lysate was then added to each well, and the samples were allowed to filter through the membrane by gentle vacuum Each well was then washed with 200 µl PBS, and the wash solution allowed filter through membrane by gentle vacuum After the wells were completely drained, the membrane was removed from the apparatus The membrane was then blocked in a 5% skim milk solultion in PBS-Tween20 (PBS-T20) (10 mM Tris, 100 mM NaCl, 0 1% Tween-20), for 2 h at room temperature, whilst being rocked gently on an orbital shaker The membrane was then incubated in primary and secondary antibodies, and developed as described in sections 2 3 5 5-2 3 5 7 In this procedure, however, the blocked membrane was cut and incubated in varying primary or secondary antibody concentrations Following development the blots were analyzed, and the optimum concentration for the antibody of interest was determined prior to use in Western Blot analysis

### 2 3 7 SDS-PAGE and Western Blot Analysis

#### 2 3 7 1 Preparation of SDS-PAGE Gel

An Atto AE-6450 Dual Mini Slab Kit was used for all protein electrophoresis carried out during this study Prior to preparation of the SDS-PAGE gel, all gel cast parts were cleaned with ethanol and assembled as per manufacturers instructions (AHO, Tokyo, Japan) Stock solutions of 5% (stacking gel) and the 7 5/12% (resolving gel, depending on antibody used) un-polymerized bis-acrylamide were made up and stored at 2-8°C for no longer than 3 months As an example, 500 ml of 7 5% stacking gel solution was made up as follows,

| | |
|---|---|
| Resolving Gel Buffer (1 5 M Tris/HCL, pH 8 8) | -125 ml |
| Acrlyamide/Bis Acrlyamide (40% solution, 29 1 ratio) | -93 5 ml |
| 10% w/v Sodium Dodecyl Sulphate | -5 ml |
| Ultra-pure water | -276 5 ml |

An aliquot of resolving gel solution (10 ml per gel) was mixed with 50 µl of freshly prepared 10% ammonium persulphate, and 15 µl TEMED This solution was then immediately poured in to gel cast, overlaid with a film of ethanol to remove any air-bubbles, and allowed to polymerize undisturbed for 5 to 15 min When gel polymerized, the ethanol was poured off, and traces of ethanol washed away with stacking gel buffer The stacking gel (5 ml per gel) was then prepared in the same manner as the resolving gel This was then poured onto the resolving gel, the 12-well

comb put in place, and the gel allowed to polymerize for 5 to 15 min The electrophoresis chamber was then half filled with reservoir buffer (25 mM Tris, 192 mM Glycine, 0 1% Sodium Doecyl Sulphate (SDS), 2-8°C) When the gel had set, the comb, clips and gaskets were removed, and the gel cast placed in to the chamber and secured in place, care was taken to avoid any airlocks as gel was placed in chamber The chamber was then filled with reservoir buffer, and the wells flushed out to remove any un-polymerized bis-acrlyamide

### 2 3 7 2 Preparation and Addition of Samples

Ten µg of whole protein lysate was added per lane used in the experiment Samples were prepared as follows, a volume of the total protein lysate giving a concentration of 10 µg protein was mixed 1 4 with 5 µl of 4x Sample Solubilisation Buffer (SSB) (4 g SDS, 20 ml glycerol, 2 ml Mmarcaptoethanol, 0 04 g Bromophenol blue, 24 ml 0 25M TrisCl, made up to 50 ml, pH 6 8), and made up to a total volume of 20 µl with lysis buffer per sample required The sample was made up in a screw-cap eppendorf, boiled at 100°C for 3 min, and immediately placed on ice prior to loading on to gel The 20 µl sample were then slowly loaded into required well, and 2 µl Rainbow molecular weight marker (Amersham) was also loaded into first lane The loading arrangement was recorded

### 2 3 7 3 Electrophoresis

The proteins and markers were then electrophoretically separated at 200 V, 90 mA and 150 W until the dye front had migrated to the end of the gel

### 2 3 7 4 Semi-Dry Transfer, Ponceau S Staining, and Blocking of Membrane

An Atto AE-6675 semi-dry blotting apparatus was used for all transfer procedures during this study The transfer procedure was carried out as follows, when the gel had finished running, the gel was removed from the gel cast, and the stacking gel cut away The resolving gel was soaked in semi-dry transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol) for 5 min Two stacks of 15 sheets of Whatman chromotography were cut to the size of the resolving gel (9x6 $cm^2$), and were also soaked in semi-dry transfer buffer for 5 min In addition, a piece of nitrocellulose membrane (PALL BioTrace) was cut to the size of the resolving gel, pre-soaked in ultra pure water for 5 min, and subsequently soaked in semi-dry transfer buffer for 5 mm

The transfer stack was then assembled as follows, 1 stack of Whatman chromotography paper was placed on the semi-dry transfer apparatus, and over-laid with the nitrocellulose membrane, resolving gel, and the second stack of Whatman chromatography paper A pen was then rolled over the entire stack to expel any air-bubbles that may have been trapped The apparatus was then closed, and run at 90 V, 500 mA and 150 W for 45 min

Upon completion of the semi-dry transfer, the nitrocellulose membrane was removed and stained in Ponceau S solution for 5 min The membrane was then rinsed briefly in ultra pure water to remove any background staining, placed between two clear acetates, and the image scanned using an Epson perfection 1200S scanner The image was saved and subsequently used to assess equality of protein loading onto gel, and quality of protein transfer onto nitrocellulose membrane The membrane was then de-stained using distilled water, until all ponceau stain was removed

The membrane was then blocked in a 5% skim milk solultion in PBS-T20, for 2 h at room temperature The membrane was rocked gently on an orbital shaker whilst blocking

### 2 3 7 5 Incubation with Primary and Secondary Antibodies

Following blocking, the membrane was washed vigorously with PBS-T20, for two min, twice The membrane was then incubated with the primary antibody of interest diluted in 2 5% skim milk in PBS-T20 Both the primary and secondary antibody dilutions were determined by dot blot immunoassay, as described in section 2 3 4, or according to manufacturers recommendations The membranes were typically incubated in the primary antibody overnight at 2-8°C, and then 1 h at room temperature, whilst being gently rocked on an orbital shaker

Following incubation with the primary antibody, the membrane was rinsed twice, and washed vigorously for 2 x 15 min in PBS-T20 The membrane was then incubated in the appropriate secondary antibody diluted in PBS-T20, for 2 h at room temperature, whilst being gently rocked on an orbital shaker Prior to development of membrane, the membrane was again washed vigorously for 2 x 15 min in PBS-T20

**Table 5 Incubation conditions for antibodies used in Western Blot Analysis**

| 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-2 (Santa Cruz) | 1 500 | HRP-Conjugated Anti-rabbit IgG (Amersham Biosciences) | 1 2000 |

### 2 3 7 6 Detection and Development of Blot

Supersignal west chemiluminescent substrate (Pierce) was the detection reagent used in developing all blots during this study Briefly, the substrate was prepared according to manufacturers instructions, and allowed to equilibrate to room temperature in a dark environment Following the last wash, any excess fluid was removed from membrane by a gentle capillary action, and the membrane was placed on cling-film, protein side-up The detection reagent was then placed on the membrane for 5 min, ensuring that the whole membrane was covered After this incubation period, any excess detection reagent was removed by a gentle capillary action, and the membrane was placed on a fresh piece of cling-film, protein side down, and covered, ensuring any air bubbles were removed Development of the blot was carried out in a dark room, where the covered membrane was placed protein side up in an x-ray film cassette A piece of Amersham Hyperfilm autoradiography film was then placed on the membrane for the desired amount of time, usually between 10 sec and 5 mm depending on the strength of the chemiluminescent signal The film was then removed and developed in an Amersham hyperprocessor automatic developer

### 2 3 7 7 Densitometric Analysis of Blot

Bands on a developed film were photographed using a Kodak DC290 digital camera The image generated was then analysed using Kodak 1D (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 Microsoft Excel™

## 2 3 8 Polymerase Cham Reaction (PCR)

### 2 3 8 1 Preparation of Total RNA

Total RNA was isolated from RVSMC's using Trizol® reagent according to the method of Chomczynski and Sacchi (1987) The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is a modification on the isolation method developed by Chomcznski and Sacchi 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 minutes 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 30-50 µl of RNase free water All total RNA preparations were stored at -80 °C

### 2 3 8 2 Quantification of Total RNA in Samples

To determine the amount of total RNA in samples obtained in section 2 3 8 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 3 8 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°C

Notch 1 Primer Set (designed from rat sequence) Fragment size 550 bp
Forward primer 5'-GAG TCA CCC CAT GGC TAC-3'
Reverse primer 5'-GTG GCT GCA CCT GCT GGG-3'

Notch 3 Primer Set (designed from rat sequence) Fragment size 390 bp
Forward primer 5'-GAC CGT GTG GCC TCT TTC TAT TGT-3'
Reverse primer 5'-GCA GCT GAA GCC ATT GAC TCT ATC CT-3'

HRT-1 Primer Set (designed from rat sequence) Fragment size 290 bp
Forward primer 5'-GAA GCG CCG ACG AGA CCG AAT CAA-3'
Reverse primer 5'-CAG GGC GTG CGC GTC AAA ATA ACC-3'

HRT-2 Primer Set (designed from rat sequence) Fragment size 330 bp
Forward primer 5'-CAT CAG AGT CAA CGC CAT GT-3'
Reverse primer 5'-GAC ACT GAT AAC GGT GGG CT-3'

HRT-3 Primer Set (designed from rat sequence) Fragment size 340 bp
Forward primer 5'-GTG GCA CAG GGT TCT TTG AT-3'
Reverse primer 5'-GCT GAG ATA GGG TAA GGG GG-3'

Hes-1 Primer Set (designed from rat sequence) Fragment size 280 bp
Forward primer 5'-TCA ACA CGA CAC CGG ACA AAC-3'
Reverse primer 5'-GGT ACT TCC CCA ACA CGC TCG-3'

Hes-5 Primer Set (designed from rat sequence) Fragment size 380 bp
Forward primer 5'-GCT CAG TCC CAA GGA GAA AA-3'
Reverse primer 5'-GTC GGG GTC TCC TTG ACA G-3'

Jagged Primer Set (designed from rat sequence) Fragment size 147 bp
Forward primer 5'-AAC AGA ACA CAG GGA TTG CC-3'
Reverse primer 5'-AGG TTT TGT TGC CAT TCT GG-3'

GAPDH Primer Set (designed from rat sequence) Fragment size 186 bp
Forward primer 5'-TGC TGA GTA TGT CGT GGA GT-3'
Reverse primer 5'-GCA TTG CTG ACA ACT TTG AG-3'

Bad Primer Set (designed from rat sequence) Fragment size 175 bp
Forward primer 5'-TGT TCC AGA TCC CAG AGT TT-3'
Reverse primer 3'-TGA TGA CTG TTA TTG GCT GC-3'

Bax Primer Set (designed from rat sequence) Fragment size 207 bp
Forward primer 5'-TTG CCC TCT TCT ACT TTG CT-3'
Reverse primer 5'-CAA AGA TGG TCA CTG TCT GC-3'

Bcl-2 Primer Set (designed from rat sequence) Fragment size 256 bp
Forward primer 5'-CCA GAA TCA AGT GTT CGT CA-3'
Reverse primer 5'-AGG TAC CAA TAG CAC TTC GC-3'

Bcl-$x_L$ Primer Set (designed from rat sequence) Fragment size 170 bp
Forward primer 5'-AAT GAC CAC CTA GAG CCT TG-3'
Reverse primer 5'-AGT GAG CCC AGC AGA ACT AC-3'

### 2 3 8 4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

#### 2 3 8 4 1 Reverse-Transcriptase

All total RNA samples were prepared by the Trizol® method as previously described in section 2 3 8 1 RNA preparations were then quantified by absorbance spectroscopy as described in section 2 3 8 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 minutes 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 minutes 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 minutes 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 3.8 4.2 Polymerase Chain Reaction (PCR)

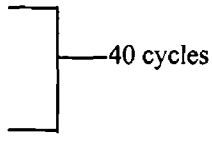
PCR was carried out using Promega Taq DNA Polymerase A PCR mix was made up for each sample to be amplified The mix was made up as follows

| | |
|---|---|
| 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 was 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

57°C for the apoptosis primers For the purpose of this protocol the program was as follows

Denaturing Phase 94°C – 4 minutes
Annealing Phase 55°C/57°C – 2 minutes
Elongation Phase 72°C – 3 minutes
4°C – Hold




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

### 2.3.8 5 Agarose gel electrophoresis

All DNA gel electrophoresis was carried out using a GibcoBRL Horizon 20 25 Gel Electrophoresis Apparatus Before use the gel box was cleaned with ethanol and the gel cast was set up as described in the manufacturers instruction manual A 2 5% agarose gel stock was made up by dissolving 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 mm 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 gel was then disposed of in the appropriate EtBr waste container

### 2 3 8 6 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 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 10 µM | 1 0 µl |
| Reverse primer 10 µM | 1 0 µl |

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

| | | |
|---|---|---|
| Denaturing Phase | 95°C - 20 s | |
| Annealing Phase | 55°C - 30s (Notch primers) | 55 cycles |
| | 57°C - 45 s (Apoptosis primers) | |
| Elongation Phase | 72°C - 30 s | |

### 2 3.9 Acid Phosphatase Assay

The Acid Phosphatase (AP) cell viability assay was carried out to determine the concentration of Brefeldin A and Monensin with which to treat the RVSMC's Brefeldin A and Monensin were used as pharmacological inhibitors of the Notch signalling pathway, and a concentration which rendered the cells 70% viable post-24 h treatment was determined by AP cell viability assay In brief, cells were plated onto 6-well plates at 1 5 x $10^5$ and treated 2 days later or when 80% confluent The wells were washed twice with 1x PBS, and 100 µl of freshly prepared phosphatase substrate (10 mM p-nitrophenolphosphate, 0 1 M sodium acetate, 0 1% Triton-X-100, pH 5 5) was added per well Plates were then wrapped in aluminium foil and incubated in the dark at 37°C for 2 h The enzyme reaction was stopped after the appropriate incubation time with the addition of 50 µl of 1 0 M NaOH The samples were then analysed at 405 nm using a microplate spectrophotometer

### 2 3 10 Apoptosis Assays

#### 2 3.10 1 Caspase Assay

A colourimetric caspase 3 assay was carried out on cell lysates (prepared as described in section 2 3 2) to determine the level of caspase 3 activation in the sample, and therefore the level of apoptosis Briefly, 10 µl caspase 3 substrate (2 mM

Ac-DEVD-pNA containing 10% DMSO in 20 mM HEPES, 0 1% CHAPSO, 5 mM DTT and 2 mM EDTA) was added to 50 µl cell lysate, and diluted in assay buffer (20 mM HEPES, 0 1% CHAPS, 5 mM DTT and 2 mM EDTA) to a final volume of 100 µl Samples were incubated for 90 min and the absorbance measured at 405 nm using a Tecan Spectra plate reader Appropriate negative controls and blanks were included A pNA standard curve (0-200 µg/µl) allowed for the specific activity of caspase 3 to be calculated for each sample

**2 3 10 2 Acridine Orange/Ethidium Bromide Cell Staining**

The staining of RVSMC's with the Acridme 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 3 10 3Annexin V/Propidium Iodide Cell Staining**

Staining of RVSMC's with Annexin V (AV, Roche)/Propidium Iodide (PI, Sigma) dual stain allows for the simultaneous detection of viable, apoptotic and necrotic cells In the early stages of apoptosis, changes occur at the cell surface, one such change is the translocation of phosphatidlyserine from the inner part of the plasma membrane to the outer layer, by which phosphatidlyserine becomes exposed at the external surface of the cell Annexin V is a $Ca^{2+}$-dependent phospholipid-binding protein with high affinity for phosphatidlyserine (Schmid *et al*, 1992) However, as

necrotic cells also expose phosphatidlyserine due to the loss of membrane integrity, the simultaneous application of a DNA stain, which is used for dye exclusion tests is necessary to distinguish apoptotic from necrotic cells Propidium Iodide (50 μg/ml) was the DNA stain used in this case The Annexin V-Fluos labeling solution was prepared according to manufacters instructions Per 1 ml of labeling solution, 20 μl of AV reagent was pre-diluted in 1 ml of incubation buffer (10 mM Hepes/NaOH, 140 mM NaCl, 5mM $CaCl_2$, pH 7 4), and 20 μl PI (50 μg/ml) was subsequently added Briefly, media was removed and cells were washed twice in 1x PBS, 150 μl of labeling solution was added per well of a 6-well plate and incubated for 10 min at room temperature, before being aspirated off cells The cells were then analyzed immediately using an Olympus DP-50 fluorescent microscope (excitation 450-500 nm, emission 515-565 nm) With this assay, viable cells (AV negative, PI negative) do not fluoresce, apoptotic cells (AV positive, PI negative) appear green, and necrotic cells (AV positive, PI positive) appear green and orange/red

### 2 3 11 *In Vivo* Studies

#### 2 3 11 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 3 11 2 Portal Vein Ligation

This procedure involved the ligation of the portal vein 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 momtered 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 3 12 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

# The Notch Signalling Pathway in Adult VSMC

## 3.1 Introduction

The initial aim of this study was to identify the presence of Notch signalling pathway components in adult VSMC, and to determine the ability of this pathway to be activated in a CBF-1-dependent manner in these cells. Pathway activation was analyzed through transient over expression of the active form of the Notch receptor (Notch IC), serum stimulation, and exposure of the cells to the endogenous ligand, Jagged. In addition, we analyzed the ability of the EBV nuclear protein, EBNA-2, to mimic activation of the Notch signalling pathway in VSMC.

Several studies have reported the importance of the Notch signalling pathway in the development of many tissues and organs in the body (Artavanis-Tsakonas *et al.*, 1999; Miele and Osborne 1999). Formation of the vascular system is one of the earliest and most important events during embryogenesis in mammals. During the early stages of vascular development, the *de novo* formation of blood vessels occurs from mesodermally-derived endothelial cell precursors, angioblasts. Angioblasts differentiate and subsequently assemble into primitive blood vessels, termed the primary vascular plexus, in a process known as vasculogenesis. The primary vascular plexus is subsequently modified by angiogenesis to generate the small and large vessels of the mature vascular system. During angiogenesis, the endothelial cell layer of large vessels is covered by multiple layers of SMC, providing structural support and stability for the vessel wall. A number of signalling 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. Mutation of many components of the Notch signalling pathway 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 respectively (Han *et al.*, 2000; Singh *et al.*, 2000). In addition, mice with mutations in both Delta-like 1 and Notch 2 show embryonic haemorrhage, possibly resulting from poor development of vascular structures (Kojika and Griffin 2001). Mutations in Notch target genes also highlight the importance of the Notch signalling pathway in vascular development. Zebrafish embryos harbouring a mutation in Gridlock, a HRT-2 orthologue, show impairment of vascular formation in the form of aortic coarctation (Zhong *et al.*, 2000). In contrast, HRT-2 null mice do not present with aortic coarctation, suggesting a level of redundancy between mammalian Notch

target genes, however, they present with massive post-natal cardiac hypertrophy, and a resulting high rate of lethality in the first 10 days of life (Gessler *et al*, 2002) The importance of the CBF-1-dependent pathway of Notch signalling in vascular development is supported by the fact that mutations in the presenilin 1 gene results in embryonic blood vessel haemorrhage (Shen *et al*, 1997, Wong *et al*, 1997) In addition, Notch 4 gain-of-function mutations result in embryonic lethality due to abnormal vessel structure and patterning of the vascular network (Uyttendaele *et al*, 2001) The fact that both gain-of-function and loss-of-function mutations result in altered vascular phenotype suggests that the appropriate levels of Notch signalling pathway activation are critical in vascular formation

Whilst the role of the Notch signalling pathway in development is reasonably well established, there is increasing recognition of the importance of the Notch signalling pathway in adult cells Throughout life multi-cellular organisms produce and replenish mature functional cells from immature non-functional cells One of the well recognized functions of the Notch signalling pathway is to delay the differentiation of immature cells The Notch signalling pathway has been documented in both physiological and pathological roles in mammals, for example, T cell development and maturation, and cancer development respectively (Kojika and Griffin 2001) The "synthetic" adult SMC phenotype are not terminally differentiated, and are similar both in morphology and gene expression to foetal or embryonic SMC (Gittenberger-de Groot *et al*, 1999) Therefore, it is conceivable that components of the Notch signalling pathway could be present and play an important functional role in adult VSMC Whilst several studies reveal the presence of some of the components of the Notch signalling pathway in VSMC (Leimeister *et al*, 2000, Lindner *et al*, 2001, Campos *et al*, 2002), this study aims to provide a more comprehensive analysis of the presence, activation, and function of components of the Notch signalling pathway, both *in vivo* and *in vitro*

The human disorders CADASIL and AGS provide additional evidence of the importance of the Notch signalling pathway in the adult vasculature These disorders involve dysregulation of components of the Notch signalling pathway, and resulting cardiovascular abnormalities CADASIL, which is caused by mutations in Notch 3, is characterized by degeneration of SMC, primarily in cerebral arteries, but also in systemic arteries and some veins CADASIL patients present with an abnormal accumulation of Notch 3 in VSMC, impaired Jagged 1/Notch 3 binding and defective CBF-1-dependent Notch 3 signalling (Brulin *et al*, 2002, Prakash *et al*, 2002, Joutel *et al*, 2004) AGS also highlights the importance of the Notch signalling pathway, and in

particular Jagged 1, in the development and maintenance of the cardiovascular system AGS patients typically present with congenital heart defects, heart murmurs and/or peripheral pulmonary stenosis (Joutel and Tournier-Lasserve 1998, Loomes *et al*, 1999)

The importance of Jagged as a Notch ligand is further highlighted as Jagged has been shown to bind and activate all four Notch receptors (Shimizu *et al*, 1999) The presence of Jagged has been shown in VSMC, and Jagged is reported to influence cell matrix and cell-cell interactions in VSMC (Lindner *et al*, 2001) However, it remains to be established which Notch receptor Jagged activates in VSMC, and whether it activates the Notch receptor in a CBF-1-dependent or -independent pathway Several studies have revealed the presence of Notch 1, Notch 2 and Notch 3 in VSMC, both *in vivo* and *in vitro* (Leimeister *et al*, 2000, Lindner *et al*, 2001, Campos *et al*, 2002) However, it is only Notch 3 expression that is SMC specific (Joutel *et al*, 2000), which could be indicative of a significant role for this receptor in VSMC To date, vascular expression of Notch 4 has not been documented in VSMC, which is reported to be endothelial cell specific (Uyttendaele *et al*, 1996) Therefore, whilst significant advances have been made in recent years in detailing the regulation, expression and role of the Notch signalling pathway in the adult vasculature, the pathway remains poorly characterized

This study further characterizes the Notch signalling pathway in adult VSMC The effect of Notch 1 and Notch 3 activation on VSMC was performed through transient transfection of the respective IC portion, which is the active form of the receptor (Weinmaster 1998) Activation of the CBF-1-dependent pathway and Notch target gene activation was analyzed using reporter plasmids, with luciferase constructs upstream of the promoter of interest, as detailed in Chapter 2, table 3 The CBF-1 plamid, pGA98-1-6, contains an enhancer consisting of 12 CBF-1 binding sites linked to the κ-globin promoter driving a luciferase gene pGA50-7 is the corresponding control vector lacking the CBF-1 enhancer (Minoguchi *et al*, 1997) The reporter plasmids for the Notch target genes contain promoter fragments of the relevant target gene inserted into a basic luciferase vector The activity of both endogenous and transfected Notch was inhibited using documented pharmacological and molecular inhibitors of the Notch signalling pathway The pharmacological inhibitors, Brefeldin A and Monensin have been shown to inhibit Notch 1 IC activity in fibroblasts (Schroeter *et al*, 1998) Whilst neither are specific inhibitors of the Notch signalling pathway, Brefeldin A and Monensin are chemically distinct inhibitors of protein

translocation to the golgi apparatus, thus inhibiting Notch IC translocation to the nucleus In addition to the pharmacological inhibitors, specific inhibitors of the Notch signalling pathway were utilized in this study, these include pCMV-ED4, RPMS-I, R218H-RBP-Jκ and EBNA-3A pCMV-ED4 is a mutant form of Notch 1 IC, generated without the RAM domain Mutant Notch 1 IC can compete with endogenous and transfected Notch IC for both CBF-1 binding at the ANK domain, and for co-activator binding RPMS-I has previously been shown to prevent Notch IC-mediated activation at promoters with CBF-1 binding sites by stabilizing the CBF-1/HDAC co-repressor complex This prevents effective displacement of the co-repressor complex by Notch IC (Smith *et al*, 2000) The R218H-RBP-Jκ plasmid is a mutant CBF-1 plasmid that competes with CBF-1 for Notch IC binding, but cannot bind effectively to DNA (Sakai *et al*, 1998) EBNA-2 has been shown to signal in a CBF-1-dependent manner in B cells, in a manner reminiscent to that of the Notch signalling pathway (Hsieh *et al*, 1996, Zhang *et al*, 2001) EBNA-3A has been shown to modulate EBNA-2-mediated CBF-1 activity (Robertson *et al*, 1995, Robertson *et al*, 1996), therefore the effect of EBNA-3A as a Notch signalling pathway inhibitor was also investigated The activity of EBNA-2 in VSMC has not previously been documented, and was also investigated

## 3 2 Results

### 3 2 1 Components of the Notch Signalling Pathway are present in RVSMC

Analysis of RVSMC has revealed the presence of components of the Notch signalling pathway in adult vascular smooth muscle cells (Figures 3 1 – 3 8) The Notch receptors, Notch 1 (Figure 3 1) and Notch 3 (Figure 3 2) are present in RVSMC The antibodies used in this study are directed against the active IC portion of the Notch receptors Therefore, these antibodies detect both the cleaved IC portion of the Notch receptor, in addition to the full-length receptor This is clearly illustrated in Figure 3 2D, using the Notch 3 receptor as an example The detection of Notch 3 IC with antibodies from two different commercial sources (Figure 3 2A - Upstate Cell Signaling Solutions and Figure 3 2D Santa Cruz Biotechnology), coupled with the competing away of Notch 3 expression with an appropriate blocking peptide confirms the rigour of the antibodies used As expression of the active IC form of Notch is of interest in this study, expression changes in Notch 1 IC and Notch 3 IC will hereafter be presented The specificity of the antibodies used in detecting Notch 1 IC and Notch 3 IC was further confirmed by over-expression of Notch 1 IC or Notch 3 IC in RVSMC using HA-tagged plasmids The molecular weight of the tagged Notch IC detected with an anti-HA antibody corresponded to that of Notch 1 IC (Figure 3 2E) and Notch 3 IC respectively Expression of the Notch ligand, Jagged, was also evident in RVSMC (Figure 3 3) Additionally, the presence of the Notch target genes, HRT-1 (Figure 3 4), HRT-2 (Figure 3 5), HRT-3 (Figure 3 6), Hes-1 (Figure 3 7) and Hes-5 (Figure 3 8) was also established The presence of protein was confirmed by western blot and immunocytochemical analysis, and the presence of mRNA was verified through semi-quantitative PCR analysis

Immunocytochemical analysis revealed the sub-cellular localization of the components of the Notch signalling pathway Figure 3 1C illustrates that Notch 1 is predominately located in the nuclei of RVSMC, however, it is present both in the cell cytoplasm and on the plasma membrane Nuclear Notch 1 does not appear to be present in the nucleoli of the cell, and the distribution of Notch 1 in the cytoplasm appears to be clustered around the nucleus Membrane-tethered Notch 1 appears to constitute a small proportion of the overall cellular distribution of Notch 1 Similarly, Notch 3 appears to be primarily located in the nuclei of RVSMC (Figure 3 2C), however, in this case, a discrete localization of the receptor around the nuclear envelope is also in evidence

Notch 3 is also present in the cytoplasm of the cell, however, nuclear clustering as seen with Notch 1 is not apparent The presence of the Notch ligand, Jagged, has also clearly been shown in RVSMC (Figure 3 3C) Similar to the Notch receptors, the sub-cellular localization of Jagged is predominately cytoplasmic and nuclear

Immunocytochemical analysis also revealed the presence of Notch target genes of the HRT and Hes families in RVSMC HRT-1 (Figure 3 4C), HRT-2 (Figure 3 5C) and HRT-3 (Figure 3 6C) all exhibit a strong nuclear localization pattern within the cell, as is evident with Notch receptors and ligands The HRT family members examined however, appear to be absent from the nucleoli of the cells Cytoplasmic HRT-1 appears to exhibit nuclear clustering, which does not appear to be the case with cytoplasmic HRT-2 and HRT-3 Both Hes-1 (Figure 3 7C) and Hes-5 (Figure 3 8C) exhibit significant nuclear localization within RVSMC Hes-1 appears to be absent from the nucleoli of the cells, and distinct clusters or areas of high concentration of Hes-1 are also in evidence within the nucleus Both Hes-1 and Hes-5 are present within the cytoplasm, Hes-1 appears to be evenly distributed throughout the cytoplasm whilst Hes-5 exhibits a low level of nuclear clustering In the case of all of the components of the Notch signalling pathway examined, the cytoplasmic proportions appear to be contained in discrete vesicles

In all cases the appropriate primary and secondary controls were performed in parallel with each experiment

**Figure 3.1 The Presence of Notch 1 in Adult RVSMC**

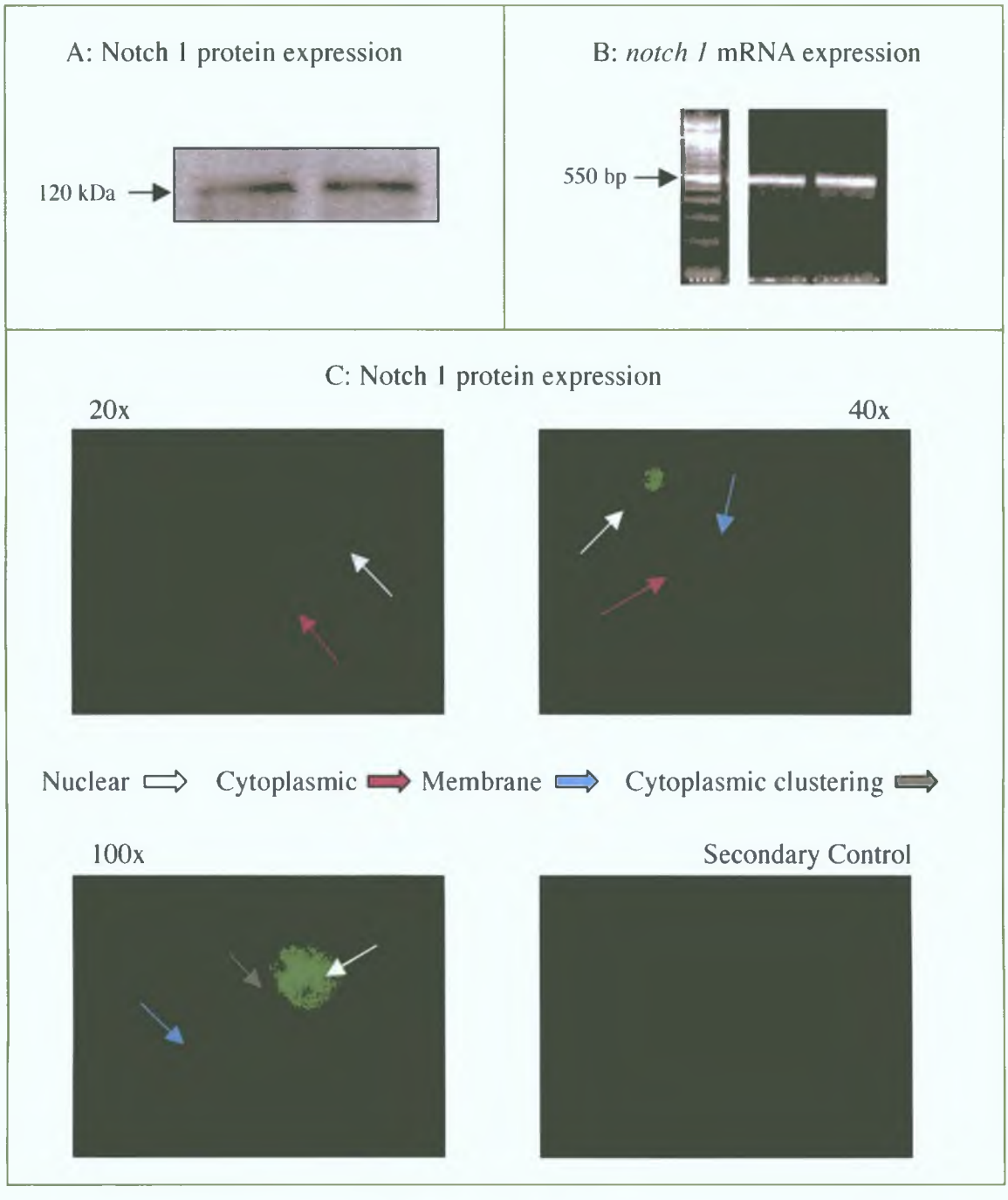



**Figure 3.1 The presence of Notch 1 in adult VSMC**
A & B) Notch 1 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing Notch 1 expression at 20x, 40x, and 100x magnifications, and a secondary control. Arrows indicate sub-cellular localization of Notch 1. n=3.

**Figure 3.2 The Presence of Notch 3 in Adult RVSMC**

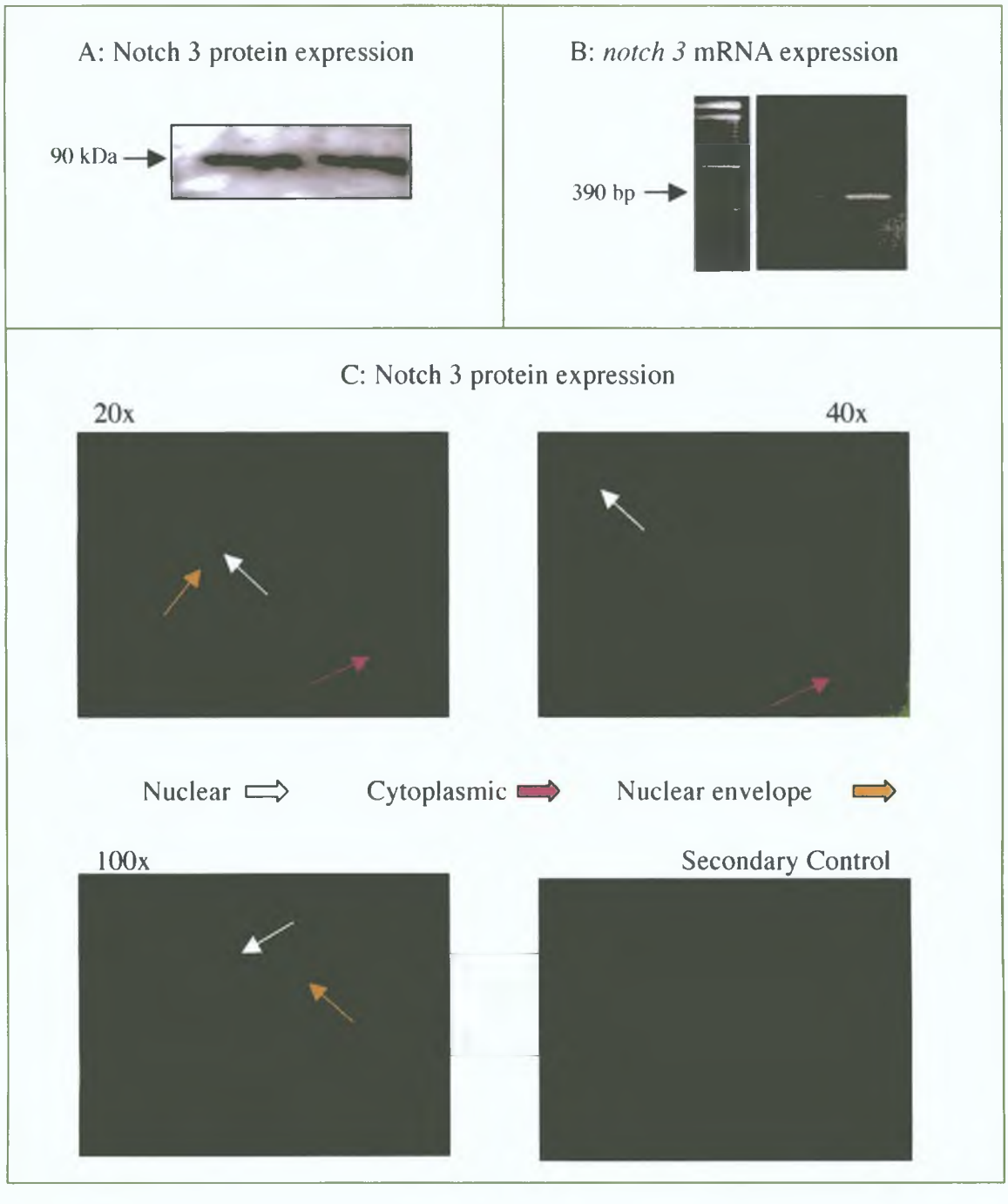


**Figure 3.2 continued: The Presence of Notch 3 in Adult RVSMC**

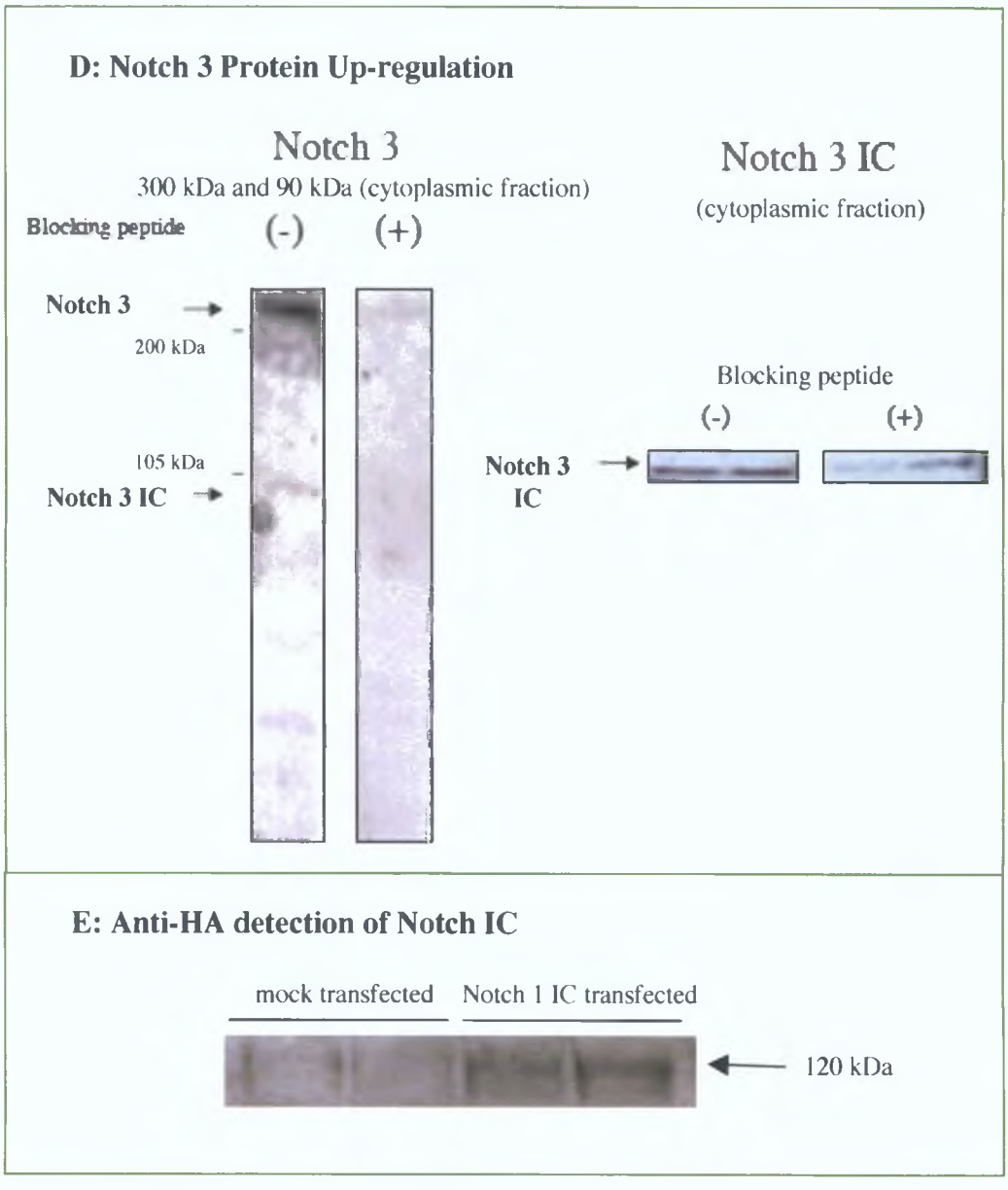



**Figure 3.2 The presence of Notch 3 in adult VSMC.** A & B) Notch 3 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing Notch 3 expression at 20x, 40x, and 100x magnifications, and a secondary control. Arrows indicate sub-cellular localization of Notch 3. D) Additional analysis of Notch 3 expression in VSMC showing full length un-cleaved Notch 3 (300 kDa) and Notch 3 IC expression. Co-expression with the Notch 3 blocking peptide proves Notch 3 detection. E) Detection of Notch 1 IC using an anti-HA antibody. n=3.

**Figure 3.3 The Presence of Jagged in Adult RVSMC**

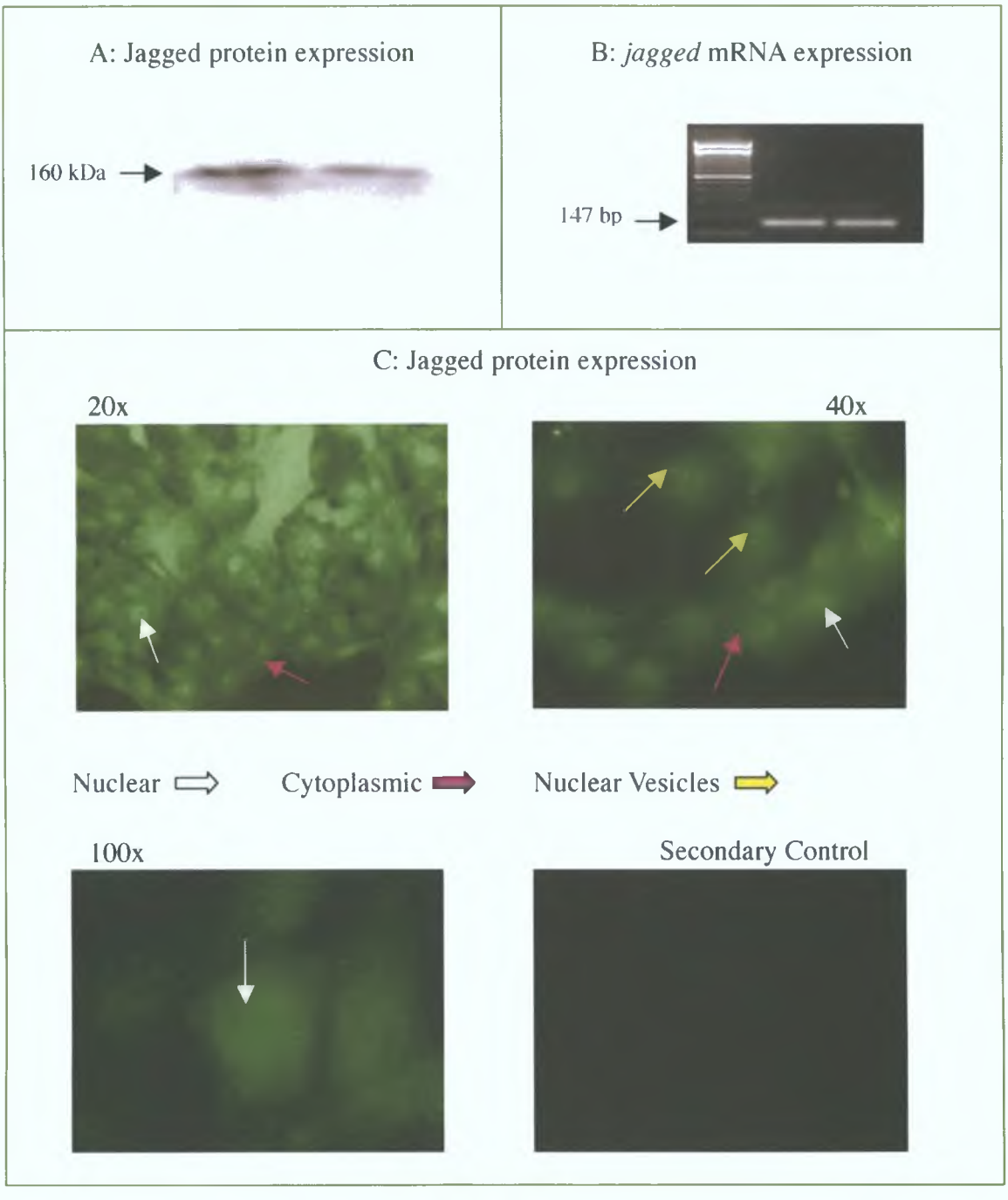



**Figure 3.3 The presence of Jagged in adult VSMC**
A & B) Jagged protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing Jagged expression at 20x, 40x, and 100x magnifications, and a secondary control. Arrows indicate the sub-cellular localization of Jagged in VSMC. n=3.

**Figure 3.4 The Presence of HRT-1 in Adult RVSMC**

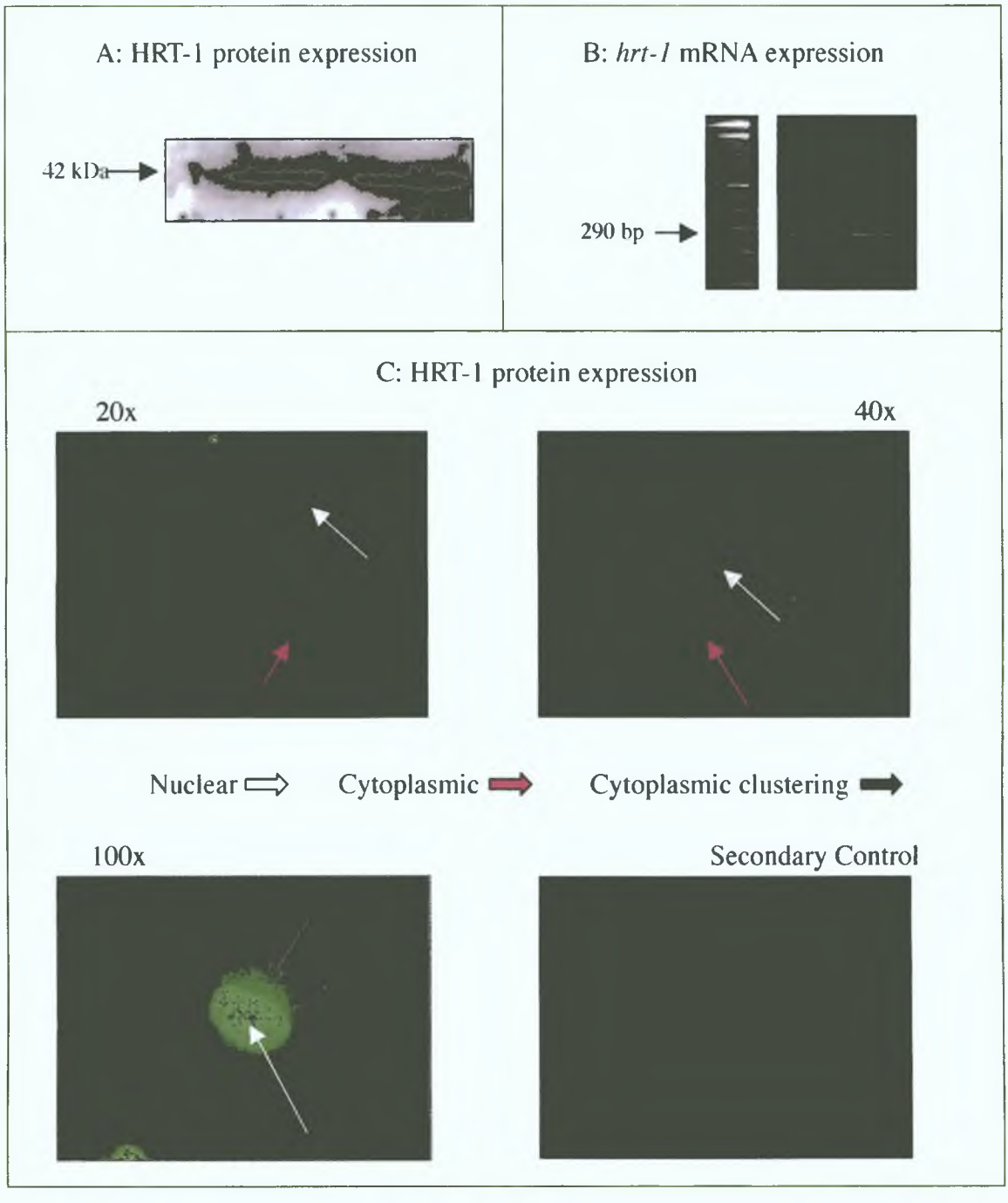



**Figure 3.4 The presence of HRT-1 in adult VSMC**
A & B) HRT-1 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing HRT-1 expression at 20x, 40x, and 100x magnifications, and a secondary control. Arrows indicate sub-cellular localization of HRT-1. n=3.

**Figure 3.5 The Presence of HRT-2 in Adult RVSMC**

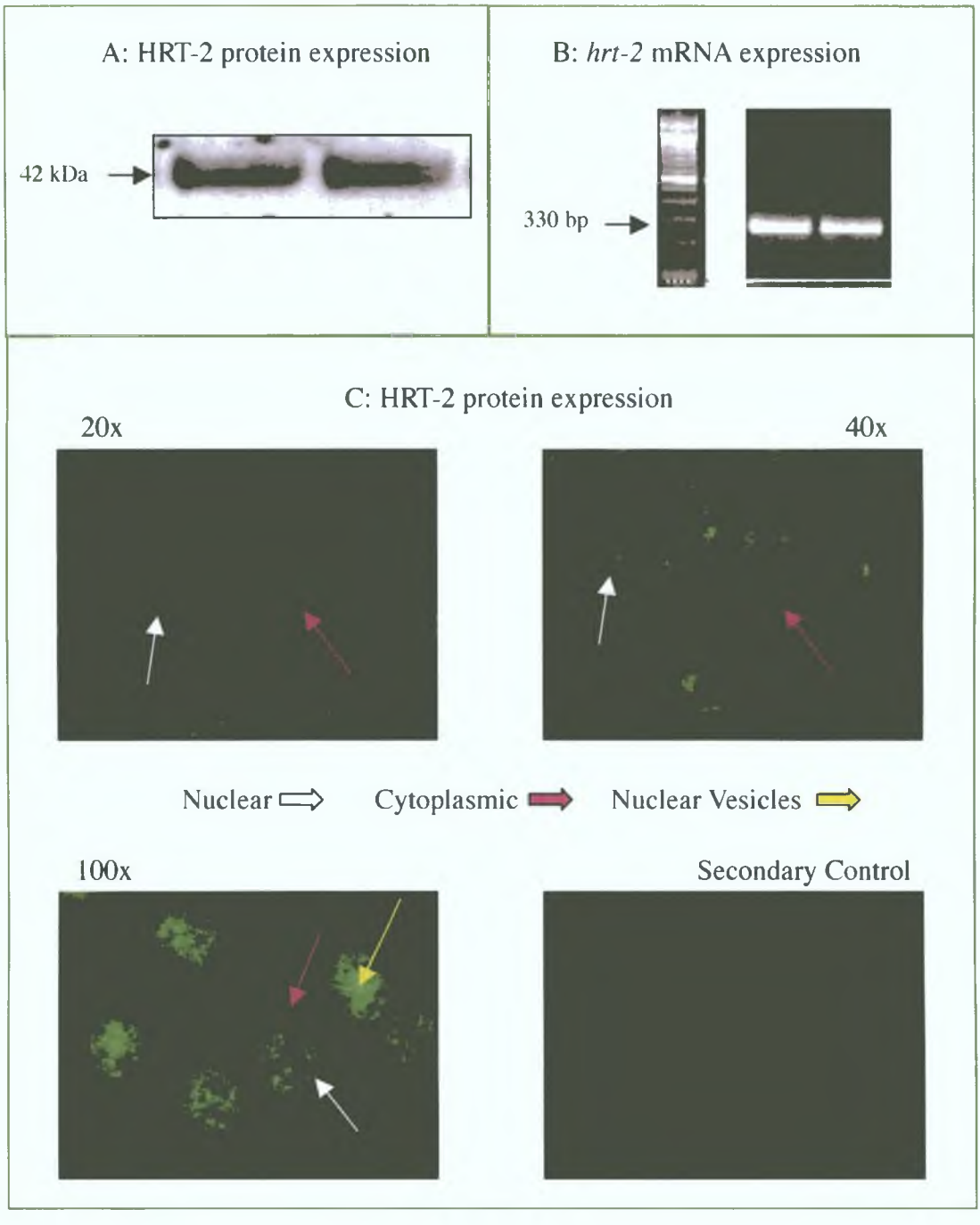



**Figure 3.5 The presence of HRT-2 in adult VSMC**
A & B) HRT-2 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing HRT-2 expression at 20x, 40x, and 100x magnifications. n=3.

**Figure 3.6 The Presence of HRT-3 in Adult RVSMC**

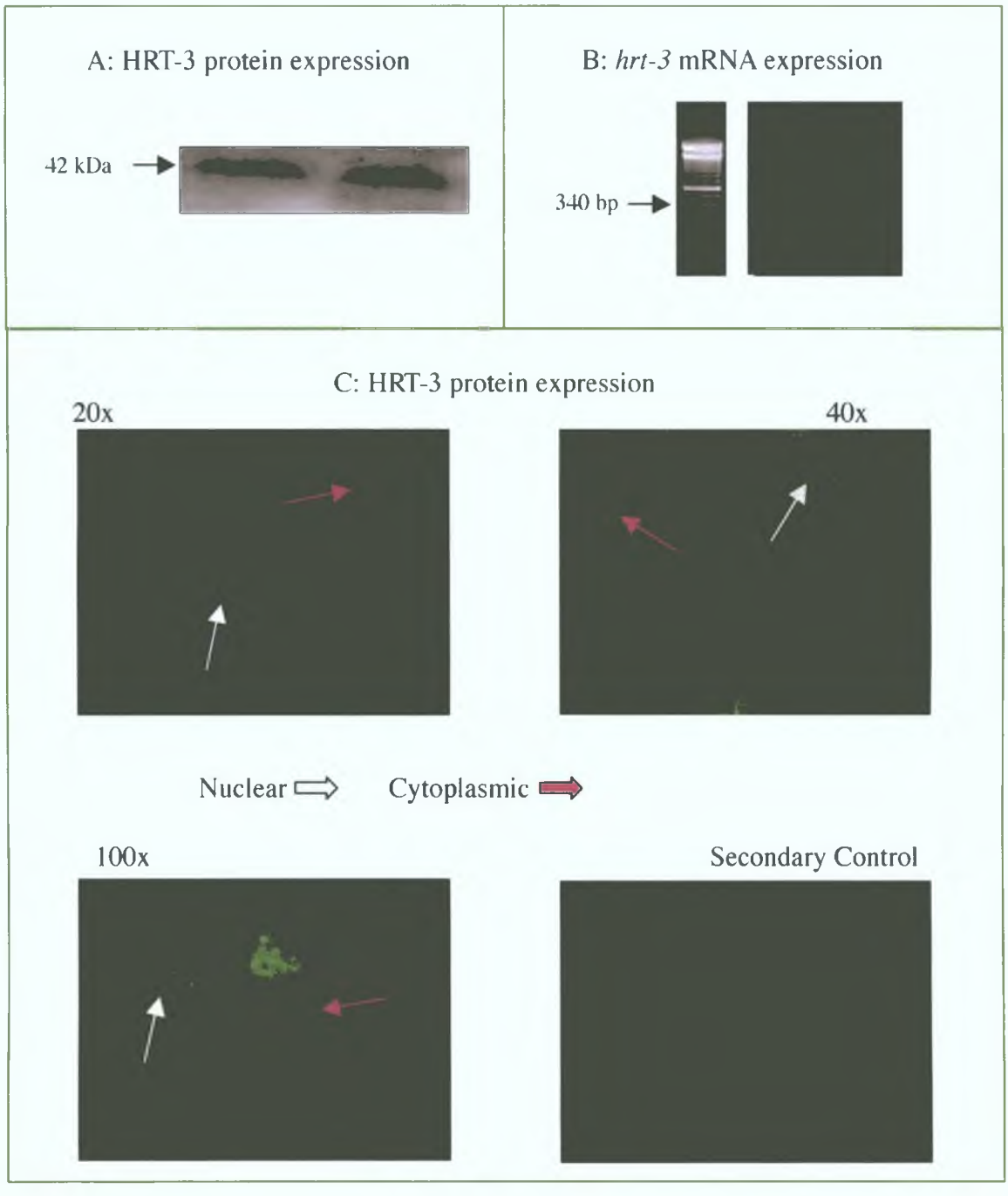



**Figure 3.6 The presence of HRT-3 in adult VSMC**
A & B) HRT-3 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing HRT-3 expression at 20x, 40x, and 100x magnifications. n=3.

**Figure 3.7 The Presence of Hes-1 in Adult RVSMC**

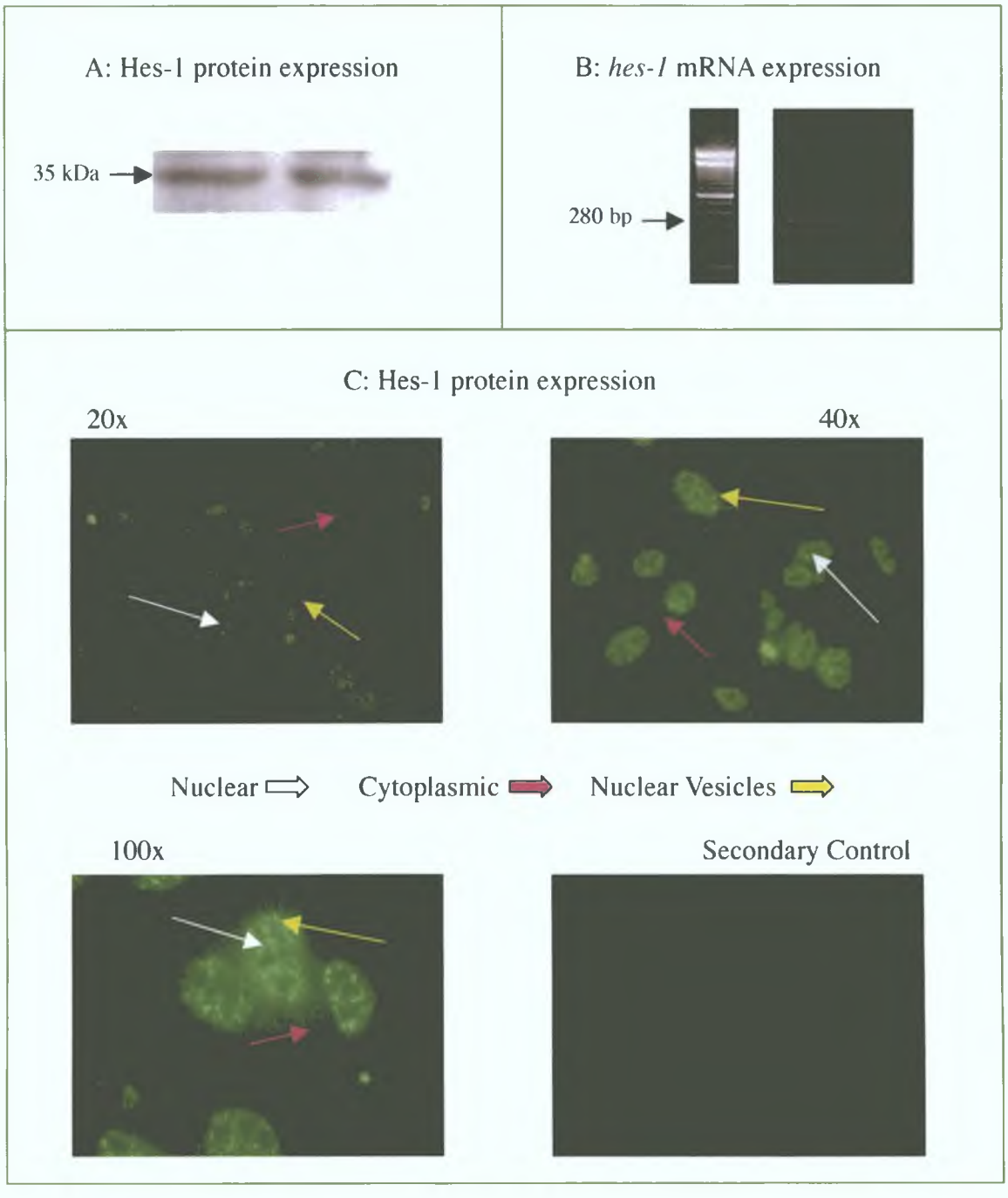



**Figure 3.7 The presence of Hes-1 in adult VSMC**
A & B) Hes-1 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing Hes-1 expression at 20x, 40x, and 100x magnifications. n=3.

**Figure 3.8 The Presence of Hes-5 in Adult RVSMC**

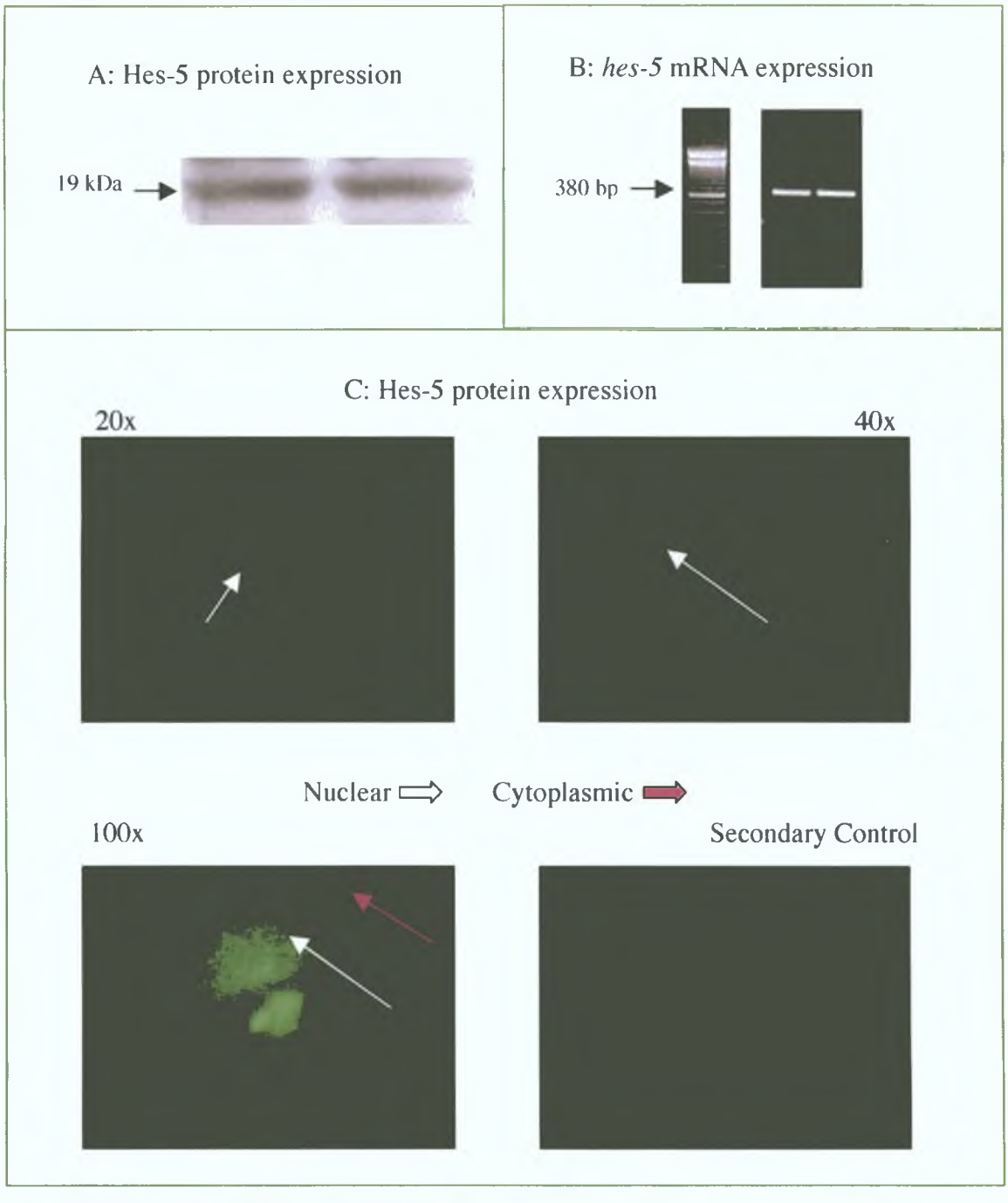



**Figure 3.8 The presence of Hes-5 in adult VSMC**
A & B) Hes-5 protein (A) and mRNA (B) is expressed in RVSMC, as determined by western blot analysis and semi-quantitative PCR respectively. C) Immunocytochemistry showing Hes-5 expression at 20x, 40x, and 100x magnifications. n=3.

### 3 2 2 Activation of the Notch Signalling Pathway in RVSMC

This study has successfully detected components of the Notch signalling pathway in cycling RVSMC In addition, we investigated whether the Notch signalling pathway could be activated in these cells This was examined through the transient transfection of RVSMC with the active (IC) portion of either the Notch 1 or Notch 3 receptor, co-transfected with either a CBF-1- or Notch target gene-luciferase-tagged reporter plasmid Activation of the reporter plasmid results in generation of the firefly luciferase enzyme, which catalyzes the conversion of D-luciferin to oxyluciferin, with the concomitant production of a photon of light, which is measured by a luminometer In addition, CBF-1-dependent activation of the Notch signalling pathway was confirmed through analysis of Notch target gene mRNA levels in VSMC following over-expression of the active form of the Notch 1 and Notch 3 receptors

Up-regulation of Notch 1 IC and Notch 3 IC through transient transfection was verified at the protein and mRNA level (Figure 3 9) Over-expression of the active forms of both Notch 1 and Notch 3 clearly activates both CBF-1-dependent promoter activity (Figure 3 10) and Notch target gene promoter activity (Figure 3 11) in RVSMC

The co-transfection of Notch 1 IC and the CBF-1 luciferase-tagged promoter (CBF-1-luc) resulted in a significant fold increase (19 29±3 09) in luciferase activity over control cells lacking transfected Notch 1 IC (Figure 3 10A) Co-transfection of Notch 1 IC and CBF-1-luc with inhibitors of the Notch signalling pathway resulted in an attenuation of the Notch 1 IC-induced increase in luciferase activity Mutant Notch 1 IC (pCMV-ED4) co-transfection, for example, resulted in a 71 0±2 0% decrease in luciferase activity as compared to Notch 1 IC transactivation of CBF-1-luc, whereas RPMS-I, and EBNA-3A co-transfection resulted in 71 38±4 1% and 87 46±4 48% decreases in luciferase activity respectively Notch 1 IC and CBF-1-luc transfected cells were also exposed to the pharmacological inhibitors Brefeldin A and Monensin, which resulted in a 57 86±4 56% and 75 12±2 85% decrease in luciferase activity respectively, as compared to Notch 1 IC transactivation of CBF-1-luc The attenuation of the Notch 1 IC-induced increase in luciferase activity was shown to be statistically significant as compared to Notch 1 IC transactivation of CBF-1-luc for all of the co-transfected inhibitors, and for the pharmacological inhibitors, Brefeldin A and Monensin

Co-transfection of Notch 3 IC and CBF-1-luc resulted in a significant fold

increase (15 72±3 80) in luciferase activity over control cells lacking transfected Notch 3 IC (Figure 3 10B) Similarly, co-transfection with inhibitors of the Notch signalling pathway resulted in an attenuated fold increase in luciferase activity with respect to that due to Notch 3 IC alone Mutant Notch 1 IC co-transfection, resulted in a 52 29±19 1% decrease in luciferase activity as compared to Notch 3 IC transactivation of CBF-1-luc, whereas RPMS-I, and R218H-RBP-Jκ co-transfection resulted in 79 95±8 27% and 69 78±3 75% decrease in luciferase activity respectively Exposure to the pharmacological inhibitors Brefeldin A and Monensin resulted 78 75±3 75% and 80 98±4 77% decrease in luciferase activity respectively as compared to Notch 3 IC transactivation of CBF-1-luc, with a significant attenuation being shown for RPMS-I, Brefeldin A and Monensin

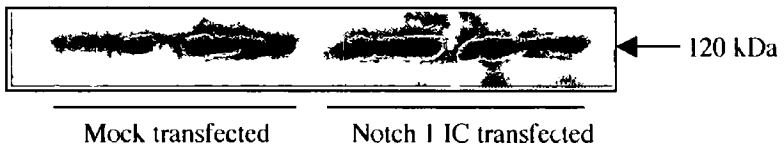
Over-expression of Notch 1 IC and Notch 3 IC in RVSMC also resulted in the transactivation of Notch target gene luciferase-tagged promoters (Figure 3 11) Co-transfection of both Notch 1 IC and Notch 3 IC resulted in a significant fold increase (3 25±1 04 and 8 16±1 73 respectively) in *hes-5* luciferase activity over control cells lacking transfected Notch Notch 1 IC and Notch 3 IC, however, was found to have a lesser effect on *hes-1* promoter activity, resulting in 1 54±0 32 and 1 3±0 17 fold increase in luciferase activity respectively The effect of Notch 1 IC and Notch 3 IC on the *hrt-1* luciferase-tagged promoter was also investigated Notch 1 IC resulted in a 19 51±4 17 fold increase in luciferase activity over control cells lacking transfected Notch, whereas Notch 3 IC over-expression resulted in a 4 96±1 22 fold increase (Figure 3 11C), however, both receptors caused a significant increase with respect to control As was evident with the CBF-1 luciferase-tagged promoter, co-transfection of the Notch 1 mutant with Notch 1 IC resulted in a significant attenuation (77 25±7 89%) of the Notch 1 IC-induced increase in *hrt-1* luciferase activity (Figure 3 11D)

The effect of Notch 1 IC and Notch 3 IC over expression on the Notch signalling pathway was confirmed using quantitative real time-PCR analysis The effect of Notch 1 IC (Figure 3 12A) and Notch 3 IC (Figure 3 12B) on Notch target gene mRNA expression levels was investigated Notch 1 IC over expression resulted in a 2 98±0 81 fold increase in *hrt-1* mRNA expression levels over mock transfected control cells (data not shown, assigned a value of 1), an increase in *hrt-2* (1 51±0 52) and *hes-5* (2 19±0 05) was also observed (Figure 3 12A) Co-transfection of Notch 1 IC with the Notch signalling pathway inhibitor, RPMS-I, significantly attenuated the Notch 1-mediated increase in *hrt-1*, *hrt-2* and *hes-5* mRNA expression by 71 48±0 33%,

79 48±3 31%, and 90 42±1 14% respectively (Figure 3 12A) Similarly, Notch 3 IC over expression resulted in increased mRNA expression levels of Notch target genes over mock transfected control cells, exhibiting a 3 55±0 5, 1 62±0 41 and 1 52±0 39 fold increase for *hrt-1*, *hrt-2* and *hes-5* respectively (Figure 3 12B) Co-transfection of Notch 3 IC with the Notch signalling pathway inhibitor, RPMS-I, significantly attenuated the Notch 3-mediated increase in *hrt-1*, *hrt-2* and *hes-5* mRNA expression by 80 0±0 28%, 53 1±4 3%, and 53 95±3 2% respectively (Figure 3 12B)

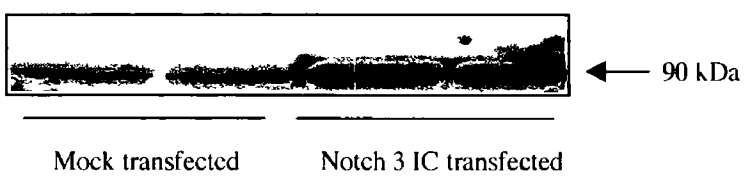
**Figure 3 9 Up-regulation of Notch 1 IC and Notch 3 IC in transfected VSMC**

**A Notch 1 Protein Up-regulation**




**B Notch 1 mRNA Up-regulation**


**C Notch 3 Protein Up-regulation**




**D Notch 3 mRNA Up-regulation**


**Figure 3 9 Up-regulation of Notch 1 IC and Notch 3 IC in transfected VSMC** Up-regulation of Notch 1 protein (A) and mRNA (B) expression following transfection with Notch 1 IC Up-regulation of Notch 3 protein (C) and mRNA (D) expression following transfection with Notch 3 IC M=mock transfected, T=Notch 1 IC/Notch 3 IC transfected

**Figure 3 10 Activation of the Notch Signalling Pathway in RVSMC**

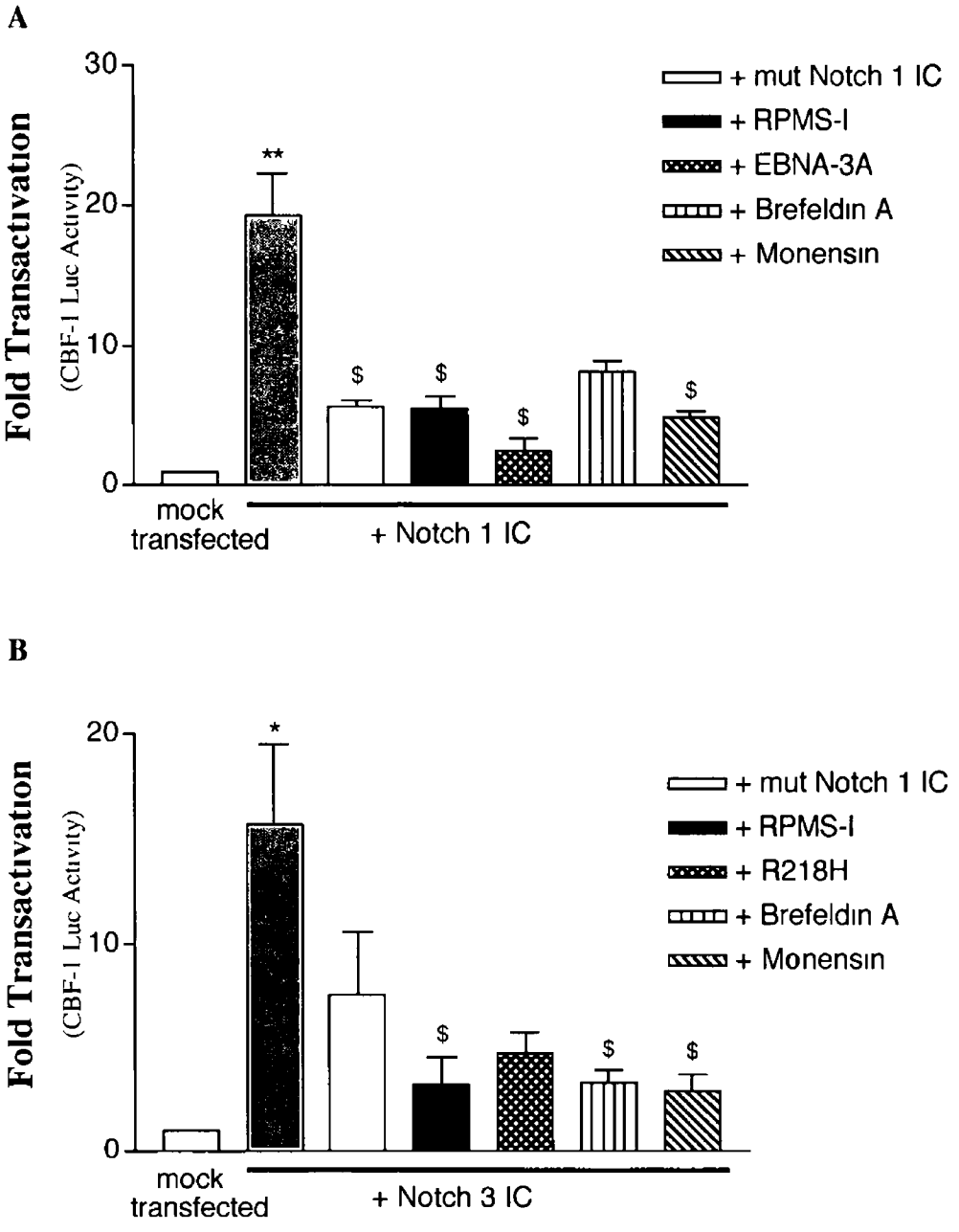
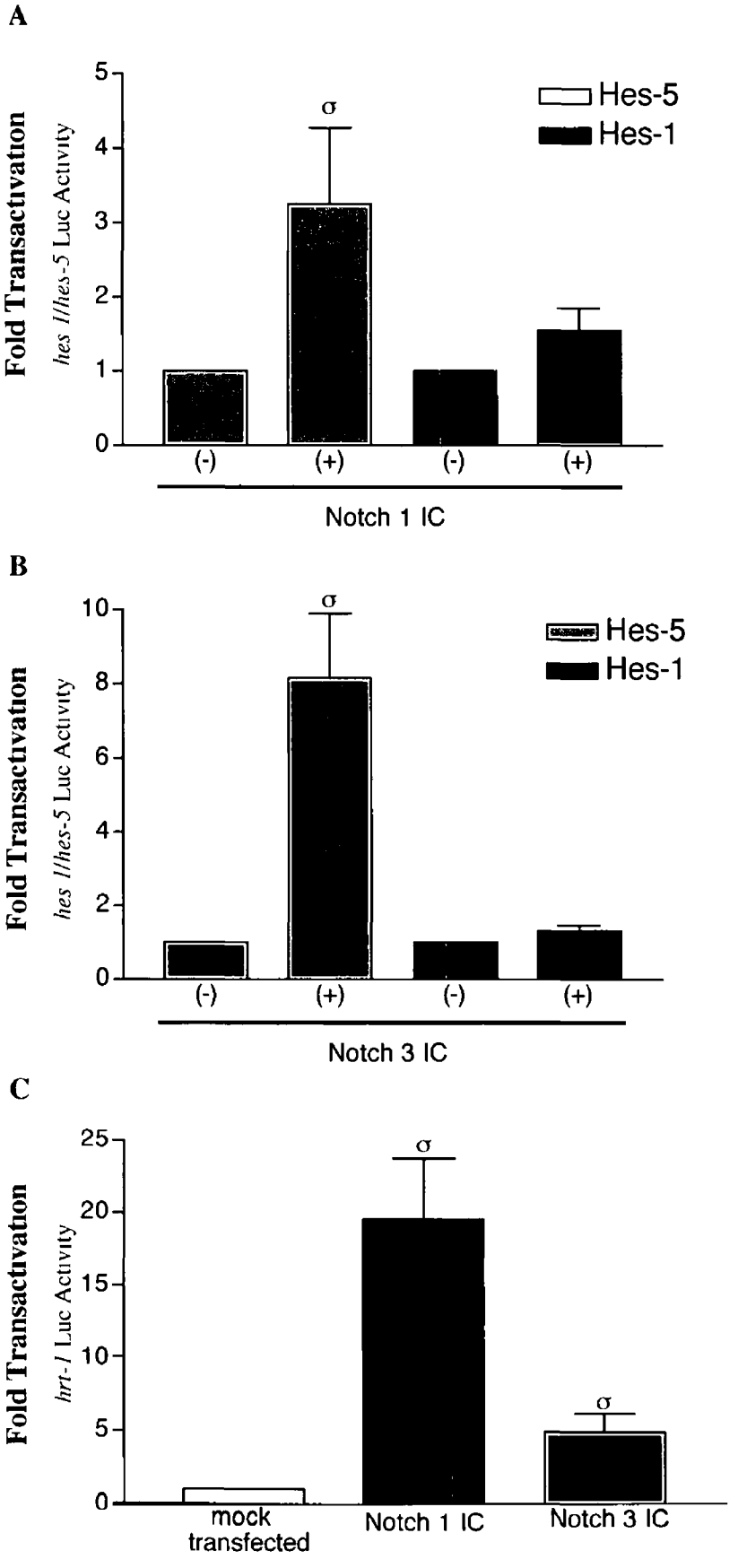



**Figure 3 10 Notch 1 and Notch 3 activate CBF-1 activity in adult VSMC** RVSMC were transiently transfected with the CBF-1 luciferase-tagged reporter plasmid, and co-transfected with either Notch 1 IC (A) or Notch 3 IC (B) Additionally, cells were co-transfected with inhibitors of the Notch signalling pathway, mut Notch 1 IC, RPMS-I, R218H, or EBNA-3A, and treated with the pharmacological inhibitors Brefeldin A (0 1μg/ml, 24 h) and Monensin (0 25 μg/ml, 24 h) Luciferase assays were normalized to α-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) * $p<0.05$ ** $p<0.005$ (rank test) as compared to mock transfected control, $ $p<0.05$ as compared to Notch transfected cells (student's t-test)

**Figure 3.11 Activation of Notch Target Genes in RVSMC**



D

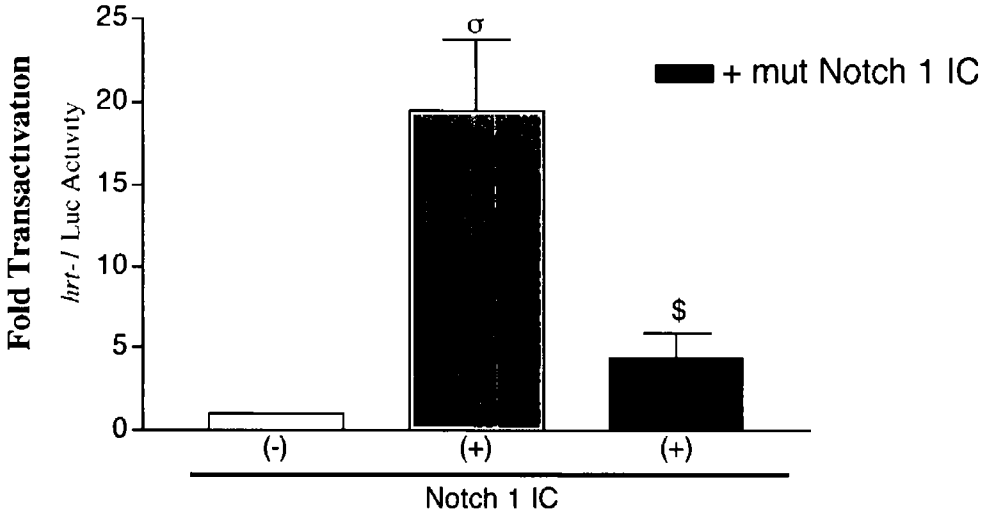



**Figure 3 11 Over-expression of Notch 1 and Notch 3 activates notch target genes in RVSMC** A & B) RVSMC were transiently transfected with the Hes-5 and Hes-1 reporter plasmids and co-transfected with either Notch 1-IC (A) or Notch 3-IC (B) Luciferase assays were normalized to σ-galactosidase activities and protein levels, n=3, and expressed as fold increase over control (= the value obtained with Hes-5 or Hes-1 reporter plasmid transfected cells arbitrarily assigned a value of 1) C) RVSMC were transiently transfected with the HR1-1 reporter plasmid and co-transfected with either Notch 1 IC or Notch 3 IC D) 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 σ-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 12 Notch Target Gene mRNA Levels in RVSMC**

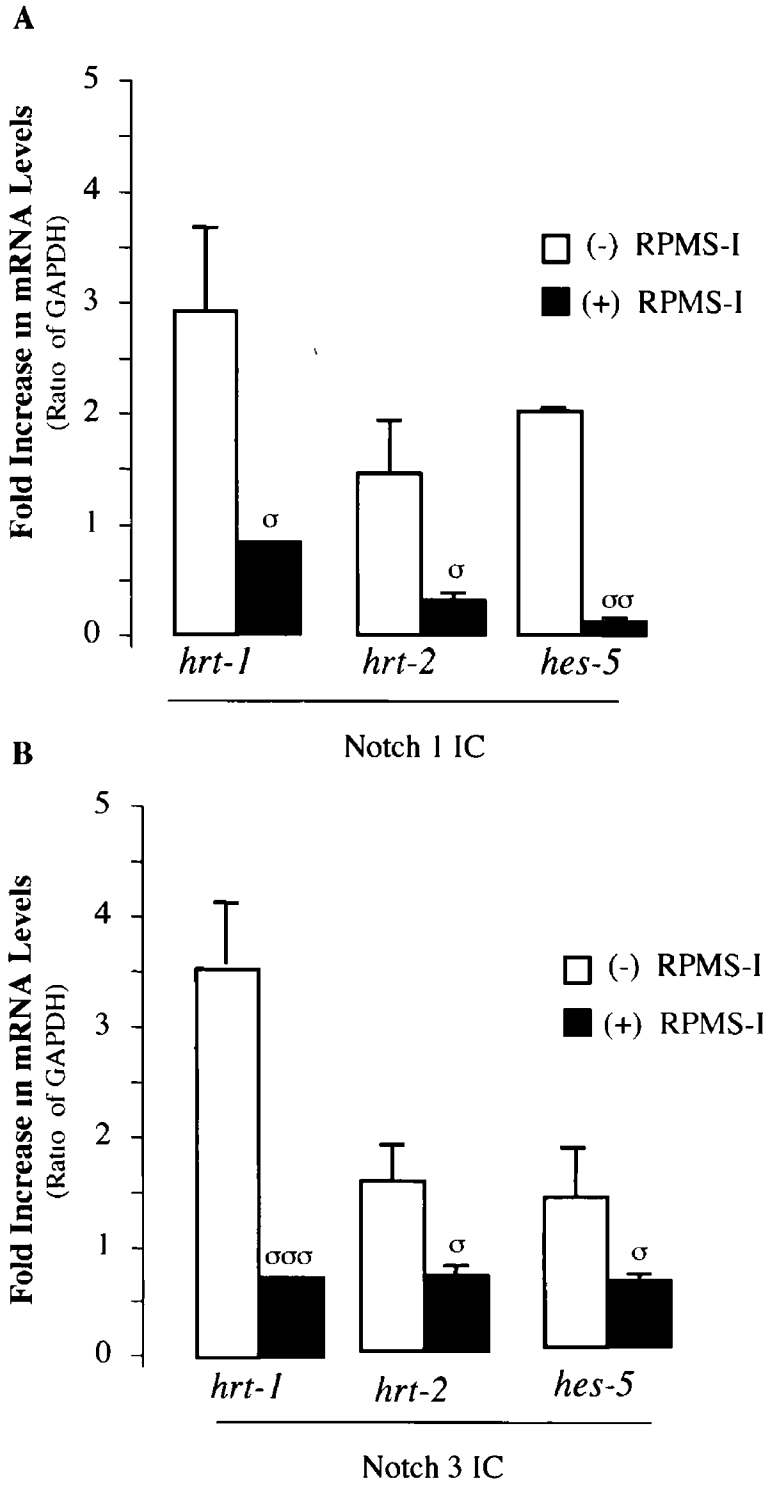



**Figure 3 12 Notch 1 and Notch 3 up-regulate Notch Target Gene mRNA expression, this is inhibited by the Notch inhibitor, RPMS-I** RVSMC were transiently transfected with Notch 1 IC (A) and Notch 3 IC (B) in the presence and absence of the Notch inhibitor, RPMS-1 mRNA levels were measured using quantitative RT-PCR, and expressed as fold increase over control (= the levels of target gene present in untransfected cells (not shown)), n=3 $^{\sigma}$ $p<0.05$, $^{\sigma\sigma}$ $p<0.005$, $^{\sigma\sigma\sigma}$ $p<0.005$ as compared to mock transfected control (student's t test)

### 3 2 3 Serum-dependent Activation of the Notch Signalling Pathway in RVSMC

The Notch signalling pathway in RVSMC can be activated through over expression of either Notch 1 IC or Notch 3 IC in these cells It was also of interest to examine whether the Notch signalling pathway could be activated in RVSMC by less artificial means, therefore the effect of serum stimulation the Notch signalling pathway in RVSMC was studied This was achieved through the transient transfection of RVSMC with a luciferase-tagged reporter plasmid, followed by serum deprivation for 48 h, and serum stimulation over a period of 24 h The results obtained clearly demonstrate that serum activates the Notch signalling pathway in RVSMC (Figure 3 13 and 3 14) Serum stimulated CBF-1 promoter activity over a period of 24 h (Figure 3 13 A and B), with a significant fold increase over the 0 h timepoint control (arbitrarily assigned a value of 1) evident at 8 h post-serum addition (26 1±7 9) This fold increase in luciferase activity was also present at 10 h post-serum addition (13 9±4 2), with the 24 h timepoint almost returning to control level (1 5±0 2) The effect of serum stimulation on the CBF-1-luc control vector, pGA50-7, was also investigated (Figure 3 13A) pGA50-7 exhibited an increase in luciferase activity over its 0 h control at 8 h (8 1±2 3) and 10 h (3 9±0 3) However, the fold increase in CBF-1 luciferase activity was significantly increased over its control vector at the 8 h and 10 h timepoints The effect of co transfection of mutant Notch 1 IC, and the pharmacological inhibitors, Brefeldin A and Monensin, on serum-stimulated increases in CBF-1 luciferase activity was investigated (Figure 3 13B) 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 a 81 61±7 85% decrease in luciferase activity respectively, and cells co-transfected with mutant Notch 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% due to Brefeldin A, Monensin and mutant Notch 1 IC respectively An additional experiment was performed to examine the effect of co-transfecting the Notch inhibitors, RPMS-I or R218H-RBP-Jκ, on serum-stimulated CBF-1 luciferase activitation In this case, the fold increase in luciferase activity was examined at 4 h, 8 h, and 24 h post-serum

addition (Figure 3 13C) The 4 h timepoint post-serum addition resulted in a 17 9±5 4 fold increase in CBF-1 luciferase activity, whereas the 8 h and 24 h timepoints exhibited a significant increase of 26 6±2 3 and 11 0±0 32 respectively Co-transfection of RPMS-I resulted in an attenuation of the serum-induced increase in luciferase activity 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 respectively R218H-RBP-Jκ co-transfection also resulted in an attenuation of the serum-stimulated increase in CBF-1 luciferase activity 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 timepoint (Figure 3 13C)

Serum stimulation also resulted in the transactivation of Notch target gene luciferase-tagged promoters (Figure 3 14) Serum stimulation resulted in an increase in *hes-5* luciferase activity over a period of 24 h, with significant increases of 2 9±0 6 and 6 4±1 0 evident at 4 h and 8 h post-serum addition respectively, and an increase of 3 2±1 2 evident at 24 h post-serum addition (Figure 3 14A) In contrast, serum stimulation did not significantly increase *hes-1* luciferase activity, with 1 2±0 1, 1 5±0 2 and 1 3±0 1 fold increases in luciferase activity evident at 4 h, 8 h and 24 h post-serum addition respectively The effect of serum stimulation on *hrt-1* promoter activity was also investigated over a period of 24 h (Figure 3 14B) 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-I, resulted in an attenuated fold increase in luciferase activity due to serum stimulation (Figure 3 14B) 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 timepoint

As serum stimulation activates CBF-1-dependent Notch signalling, the effect of serum on Notch receptor and target gene expression was investigated The effect of serum on the Notch signalling pathway was confirmed using western blot analysis and both semi-quantitative PCR and quantitative real time-PCR analysis Serum stimulation significantly increases expression of the activated portion of the Notch 1 receptor, Notch 1 IC, by 1 4±0 1 fold over the non-serum stimulated control cells (Figure 3 15A) Analysis of mRNA expression levels of Notch 1 and Notch 3 at 8 h post-serum

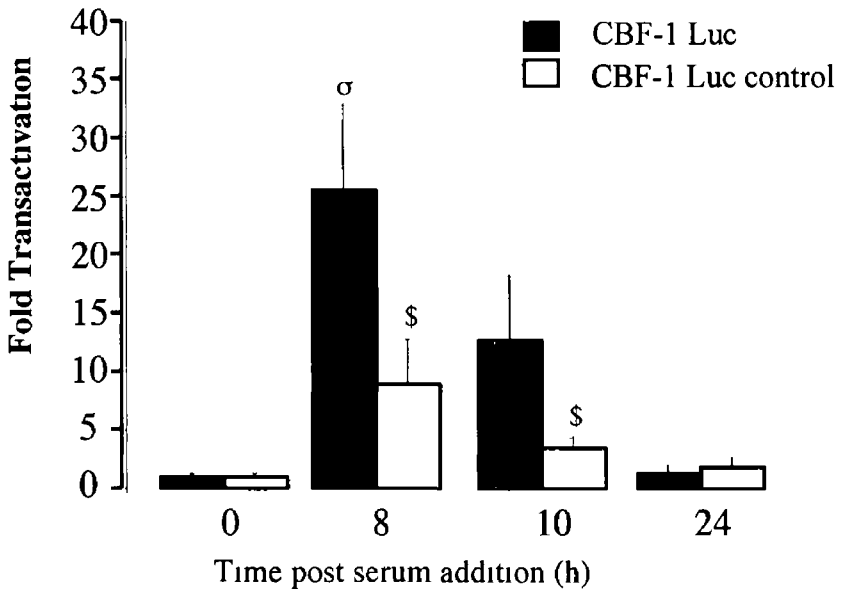
stimulation (Figure 3 15B) revealed that serum causes a significant fold increase in *notch 1* (1 78± 27) and *notch 3* (1 81±0 1) mRNA levels over the non-serum stimulated 0 h control

The effect of serum stimulation on *hrt-1* (Figure 3 16A) and *hrt-2* (Figure 3 16B) mRNA expression levels was investigated over a period of 24 h Semi-quantitative PCR revealed a temporal increase in both *hrt-1* and *hrt-2* mRNA expression levels, which were maximal at 4 - 10 h post serum addition Similarly, serum stimulation for 8 h resulted in significant increases in Notch target gene mRNA as compared to un-stimulated control cells (Figure 3 16C) 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

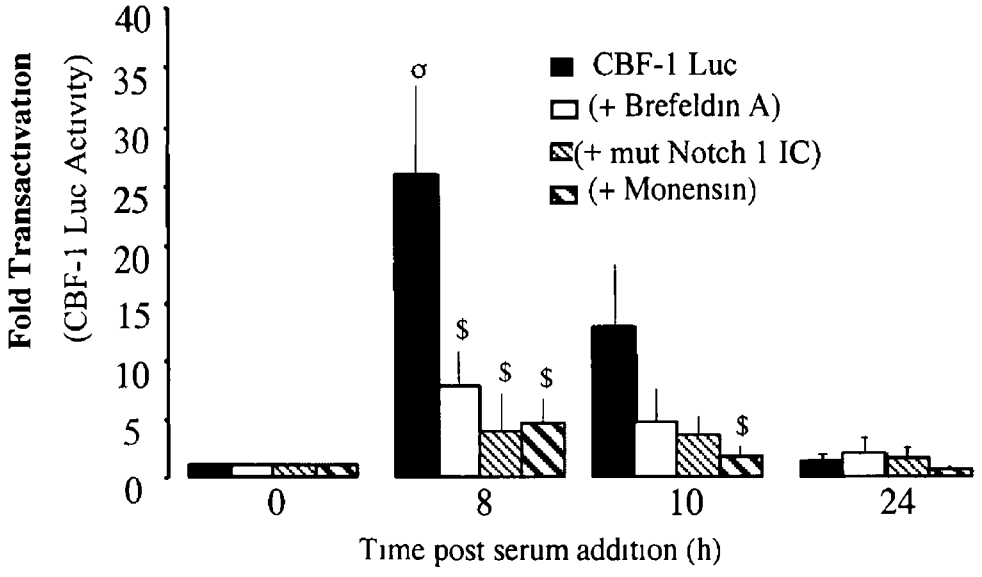
To further verify the involvement of the CBF-1 dependent Notch signalling pathway in serum-stimulated increases in Notch target gene mRNA expression, cells were mock-transfected, or transfected with the CBF-1-dependent Notch signalling pathway inhibitor, RPMS-I (Figure 3 17) Quiesced cells were serum 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 due to the presence of RPMS-I by 58 0±1 0% and 65 0±5 0% respecitvely Similarly, *hrt-1* and *hes-5* mRNA expression levels were also decreased by 58 0±12 0% and 67 0±13 0% respectively

**Figure 3 13 Serum Stimulation of the Notch Signalling Pathway in RVSMC**

A




B



C

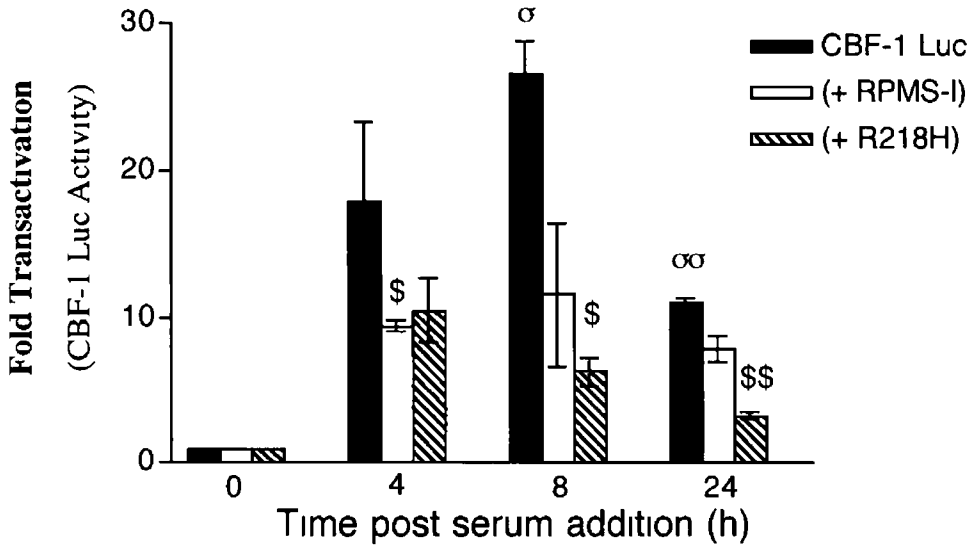



**Figure 3 13 Serum stimulates CBF-1 activity in adult VSMC in a time-dependent manner, which can be inhibited by inhibitors of the Notch signaling pathway** A) RVSMC were transiently transfected with the CBF-1 reporter plasmid, pGA98-1-6, or the CBF-1 mutant, pGA50-7 Following overnight recovery from transfection, cells were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h B) RVSMC were transiently transfected with the CBF-1 reporter plasmid, pGA98-1-6, and co-transfected with the Notch 1 mutant plasmid, mut Notch 1 IC 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 C) 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 Luciferase assays were normalized to σ-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) $^{\sigma}$ $p<0$ 05, $^{\sigma\sigma}$ $p<0$ 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 14 Serum Stimulation of Notch Target Genes in RVSMC**

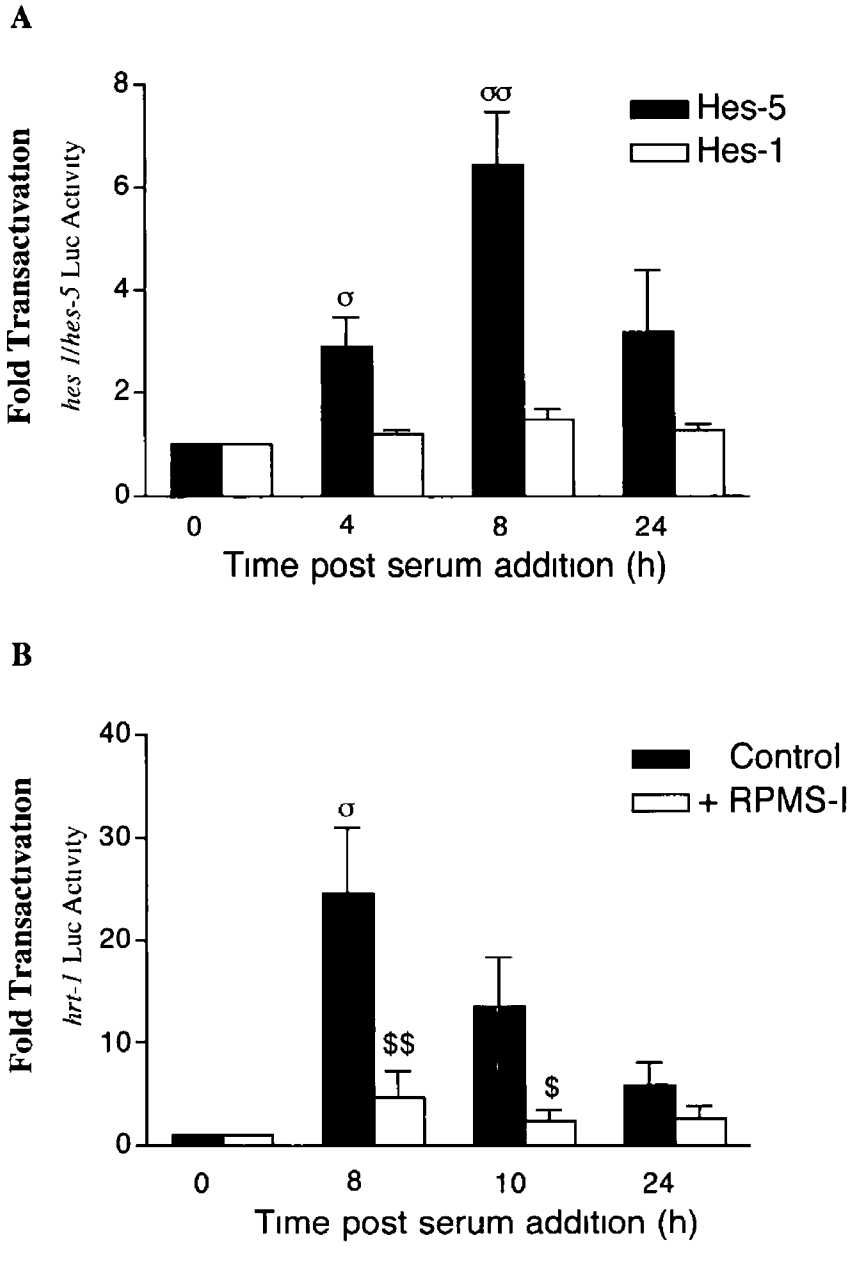
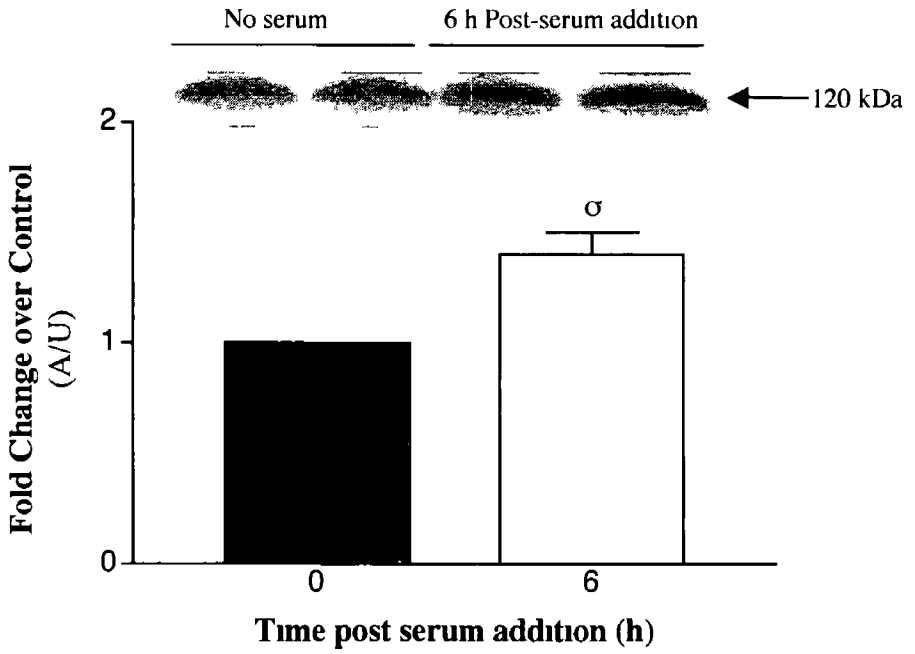



**Figure 3 14 Serum stimulates Notch target gene activity in adult VSMC in a time-dependent manner, which can be inhibited by inhibitors of the Notch signaling pathway** A) RVSMC were transiently transfected with the *hes- 5* or *hes 1* reporter plasmid B) RVSMC were transiently transfected with the HRT-1 reporter plasmid, and co-transfected with the Notch inhibitor RPMS-I In both cases, 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 σ-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) $^{\sigma}$ $p<0\,05$, $^{\sigma\sigma}$ $p<0\,005$ as compared to 0 h serum control, $^{\$\$}$ $p<0\,005$ as compared to serum control at that timepoint (student's t test)

**Figure 3 15 Serum Stimulation of Notch Receptor Expression in RVSMC**

**A Notch 1 Protein Expression**




**B Notch Receptor mRNA Expression**

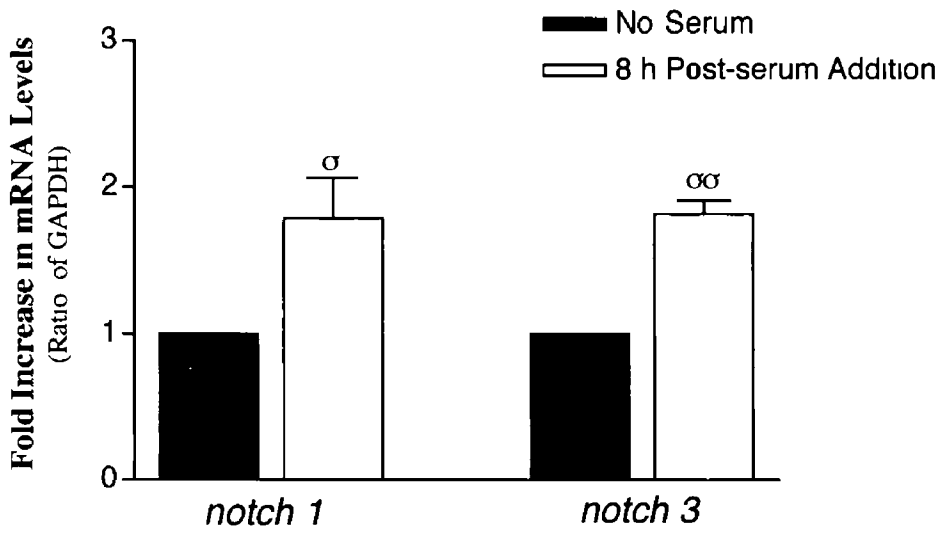



**Figure 3 15 Serum increases Notch protein expression in RVSMC** A) RVSMC were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h as indicated Samples were taken at 6 h post-serum addition in serum containing and non-serum containing samples, and subsequently assayed using for Notch 1 protein expression Samples are expressed as fold increase over non-serum stimulated control (arbitrarily assigned a value of 1) B) 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 fold increase over control (= the value obtained with serum depleted mRNA levels at 8 h arbitrarily assigned a value of 1), n=3 $^{\sigma}p<0.05$, $^{\sigma\sigma}p<0.005$ as compared to 0 h serum control (student's t test)

**Figure 3 16 Serum Stimulation of Notch Target Gene mRNA Expression in RVSMC**

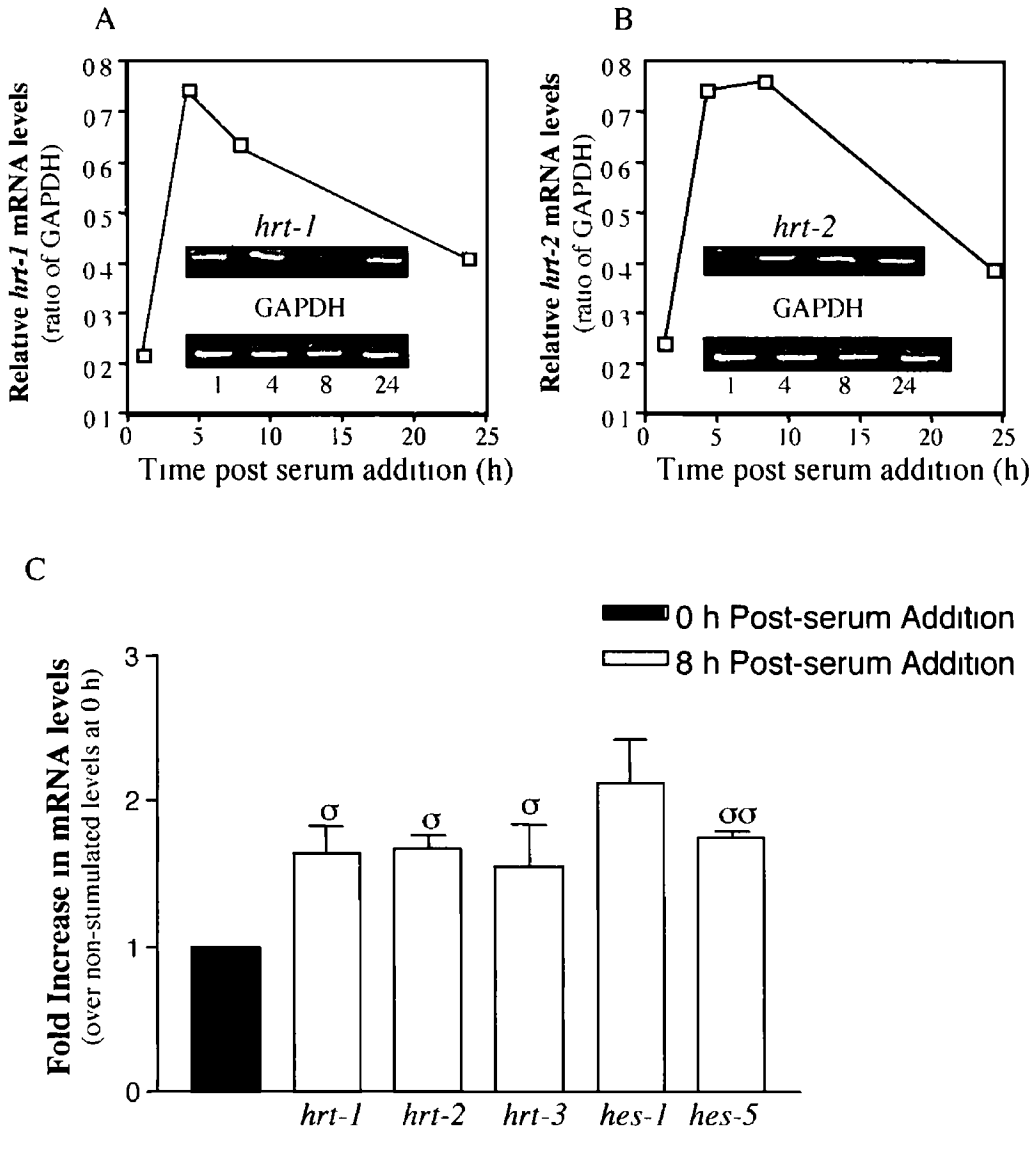



**Figure 3 16 Serum stimulates Notch target gene activity in adult VSMC in a time-dependent manner** A & B) RVSMC were quiesced for 48 h, and stimulated with media containing 10% FBS at 0 h Samples were taken over a period of 0–24 h, and subsequently assayed using semi-quantitative PCR C) RVSMC were 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 $^{\sigma}$ $p<0.05$, $^{\sigma\sigma}$ $p<0.005$ as compared to 0 h serum control (student s t test)

**Figure 3 17 Inhibition of Serum Stimulated Notch Target Gene mRNA Expression in RVSMC**

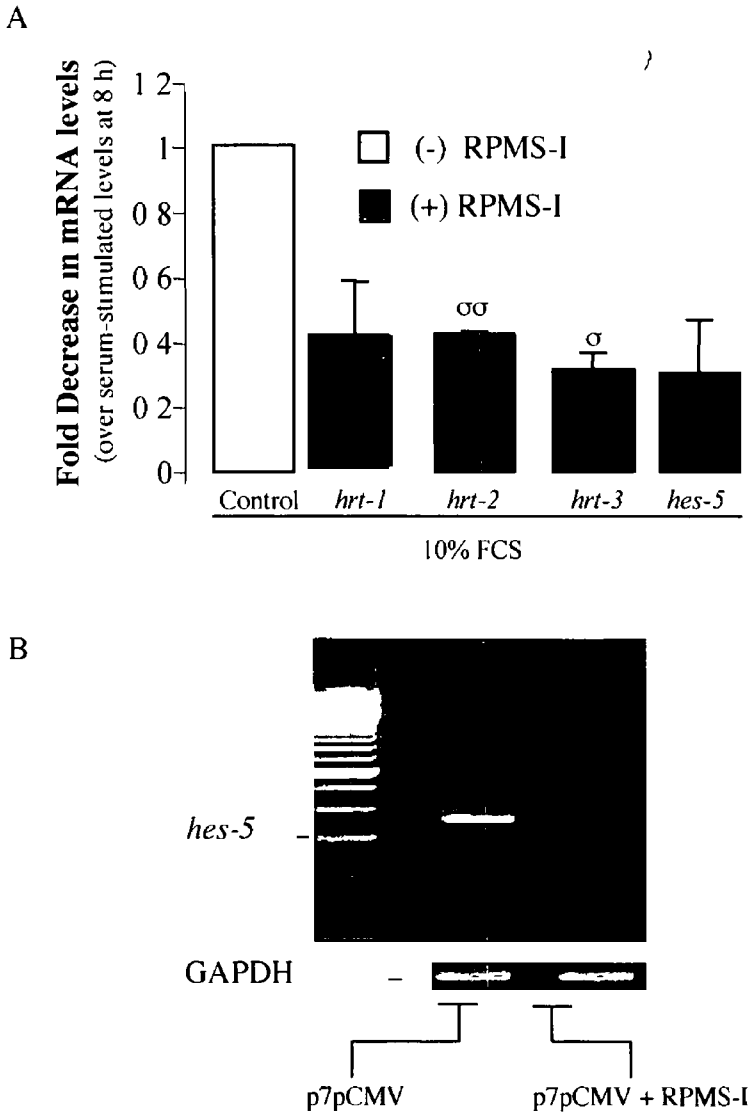



**Figure 3 17 Inhibition of serum stimulated Notch target gene mRNA expression in RVSMC** A) RVSMC were transfected with and empty vector (-RPMS-I) or with the a Notch signalling pathway inhibitor (+RPMS-I) Following recovery overnight cells were quiesced for 48 h, and stimulated with media containing 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 example of semi-quantitative PCR gel for the Notch target gene, *hes-5*, and GAPDH in both the presence and absence of RPMS-I $^{\sigma}$ $p<0.05$, $^{\sigma\sigma}$ $p<0.005$ as compared to mock transfected control (student's t test)

### 3 2 4 Endogenous ligand activation of the Notch Signalling Pathway in RVSMC

This study has demonstrated the activation of the Notch signalling pathway through over expression of Notch 1 IC and Notch 3 IC, and through serum stimulation of quiesced cells In addition, we investigated the effect of exposure of RVSMC transfected with a CBF-1 luciferase-tagged promoter to the endogenous ligand, Jagged, on the Notch signalling pathway (Figure 3 18) Jagged expression was induced in HeLa cells through removal of tetracycline from the growth media for 48 h, and Jagged induction was confirmed by western blot analysis *(S Loughran personal communication)* Exposure of RVSMC to Jagged expressing HeLa cells resulted in a significant 2 51±0 53 fold increase in CBF-1 luciferase activity over control (RVSMC exposed to non-Jagged expressing HeLa cells, arbitrarily assigned a value of 1)

### 3 2 5 EBNA-2 Activates the Notch Signalling Pathway in RVSMC

The overlap between EBV and the Notch signalling pathway is evident as many of the EBV latent proteins, including EBNA-2, EBNA-3A and other viral gene products such as RPMS-I, have been shown to interact with the Notch signalling pathway (Zhang *et al*, 2001) We have previously shown that RPMS-I and EBNA-3A attenuate a Notch IC mediated-, and also serum stimulated- (in the case of RPMS-I), increase in Notch signaling pathway activity in RVSMC This study investigated whether EBNA-2 could mimic the activation of the Notch signalling pathway in RVSMC The co-transfection of EBNA-2 and the CBF-1 luciferase-tagged promoter resulted in a significant fold increase (12 3±3 21) in luciferase activity over control cells lacking transfected EBNA-2, arbitrarily assigned a value of 1 (Figure 3 19) Co-transfection with the viral gene products EBNA-3A, RPMS-I and the CBF-1 mutant R218H-RBP-Jκ, resulted in an attenuation of the EBNA-2 induced fold increase in luciferase activity by 88 54±4 87%, 78 54±11 95% and 56 1±27 72% respectively This attenuation was significant in the case of EBNA-3A and RPMS-I Exposure of the EBNA-2 and CBF-1-luc co-transfected cells to the pharmacological inhibitors, Brefeldin A and Monensin, resulted in a very slight attenuation of the EBNA-2 induced fold increase in luciferase activity, by 7 32±39 83% and 17 89±24 87% respectively

**Figure 3 18 Stimulation of the Notch Signalling Pathway in RVSMC due to exposure to the Notch Ligand, Jagged**

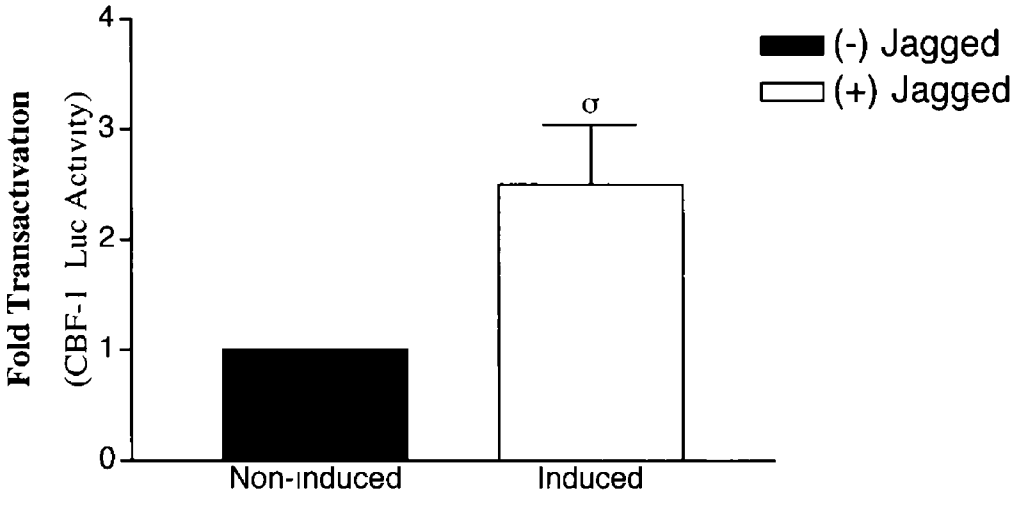



**Figure 3 18 Stimulation of the Notch Signalling Pathway in RVSMC due to exposure to the Notch Ligand, Jagged** HeLa cells were plated onto 6-well plates and Jagged expression was induced as indicated through the removal of tetracycline from the growth media over a period of 48 h In parallel, RVSMC were transiently transfected with the CBF-1 luciferase-tagged reporter plasmid, and co-transfected with the puromycin resistance plasmid, pGK-puro Following overnight recovery, transfected cells were selected out with exposure of the cells to puromycin containing media (0 8 μg/ml) for 48 h RVSMC were seeded onto the non-induced and induced HeLa cells at equal densities Samples were isolated after 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 non-induced HeLa cells co-cultured with the CBF-1 reporter plasmid, pGA98-1-6, expressing RVSMC, arbitrarily assigned a value of 1) $^{\sigma}p<0\,05$, as compared to mock transfected control (student s t test)

**Figure 3 19 Stimulation of the Notch Signalling Pathway in RVSMC with the EBV protein, EBNA-2**

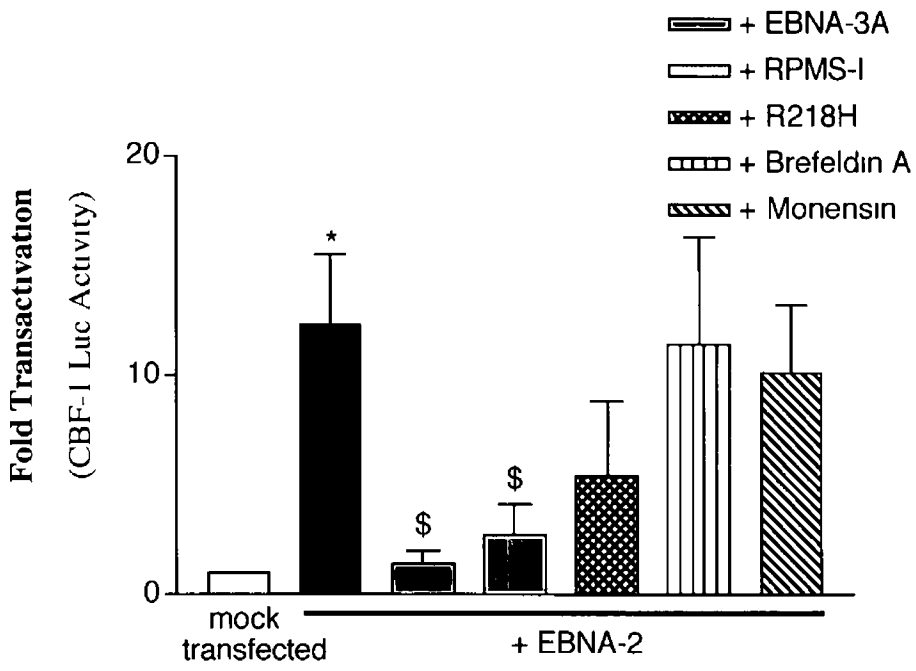



**Fig 3 19 EBNA-2 activates CBF-1 activity in adult VSMC** RVSMC were transiently transfected with the CBF-1 luciferase-tagged reporter plasmid, pGA98-1-6, and co-transfected with the EBV nuclear antigen, EBNA-2 plasmid Additionally, cells were co-transfected with inhibitors of the Notch signalling pathway EBNA-3A, RPMS-I, or R218H, and treated with the pharmacological inhibitors Brefeldin A (0 1μg/ml, 24 h) and Monensin (0 25 μg/ml, 24 h) Luciferase assays were normalized to σ-galactosidase activities and protein levels, n=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, $^{\$}$ $p<0.05$ as compared to EBNA-2 over-expressing cells (student s t test)

## 3 3 Discussion

This study comprehensively documents the presence of many components of the Notch signalling pathway in RVSMC This is largely in agreement with several recent studies (Campos *et al*, 2002, Iso *et al*, 2002), and provides additional evidence of the presence of the Notch signalling pathway in VSMC Campos *et al*, for example, documented the presence of Jagged 1, Notch 3, HRT-1, HRT-2 and HRT-3 protein and mRNA expression in RVSMC *in vitro* Contrary to the present study, however, Campos *et al*, could detect Notch 1 mRNA but not protein expression, and could not detect any evidence of Hes-1 or Hes-5 in RVSMC, the presence of which is unequivocally proven in this study The Notch receptor antibodies used in this study are directed against the IC portion of the Notch receptor and, as such, detect both the IC and full-length forms of the Notch receptor This study focused on the presence and the over-expression of the IC portion of the Notch receptor, as this is the active form of the receptor involved in CBF-1-dependent signalling (Weinmaster 1998) In addition, several studies have revealed the predominance of the IC portion of the Notch receptor, both in vascular cells (Campos *et al*, 2002, Wang *et al*, 2002a, Wang *et al*, 2002b) and other cell types (Fehon *et al*, 1991, Aster *et al*, 1994) This study also investigated the sub-cellular localization of the components of the Notch signalling pathway using immunocytochemical analysis The components of the Notch signalling pathway appear to localize predominately in the nucleus, which is in agreement with several other reports, which document the predominant sub-cellular localization of Notch IC in the nucleus (Fortini *et al*, 1993, Lieber *et al*, 1993, Kopan *et al*, 1996)

This study presents the novel finding that both Notch 1 IC and Notch 3 IC signal via a CBF-1-dependent pathway in adult RVSMC This method of signalling is likely to be conserved from development, as CBF-1-dependent signalling of Notch 1 and Notch 3 has recently been observed in rat embryonic VSMC (Campos *et al*, 2002) Over-expression of Notch 1 IC and Notch 3 IC resulted in increased CBF-1-dependent promoter activity, and Notch target gene mRNA levels In most cell types Notch 1 IC expression results in increased CBF-1-dependent activity, however, the role of Notch 3 IC appears to be cell-type specific In 1999, Beatus *et al*, suggested that Notch 3 IC acts as an antagonist of the CBF-1-dependent Notch signalling pathway, as Notch 3 IC expression inhibited the Notch 1 IC CBF-1-dependent increase in Hes-1 activity Our study, and others, establishes Notch 3 as an agonist of the CBF-1-dependent pathway,

and in addition, Wang *et al*, have shown that co-expression of Notch 1 and Notch 3 IC in VSMC results in a potentiation of the Notch 1 IC response (Wang *et al*, 2002a) The CBF-1-dependent nature of Notch IC signalling in VSMC was further confirmed with the attenuation of the Notch IC-mediated response on CBF-1 promoter activity and Notch target gene mRNA levels due to co-transfection with CBF-1-specific inhibitors Intracellular Notch IC transport is also likely to be an important factor in Notch activity in VSMC, as pharmacological inhibitors of protein transport inhibited the observed effects of Notch IC, a phenomenon that is also reported in fibroblasts (Schroeter *et al*, 1998) Notch 3 IC and Notch 1 IC activates CBF-1 promoter activity to a similar extent in adult VSMC This contradicts the finding in embryonic VSMC, that the activation of Notch 3 IC on the CBF-1 promoter is considerably more potent (2 5 fold) than that of Notch 1 IC (Campos *et al*, 2002) The reason for this discrepancy is not clear, but perhaps points to a more important role for Notch 3 signalling developmentally, or indeed, a more significant role for Notch 1 in adult VSMC

Further analysis of the effect of Notch 1 IC and Notch 3 IC over-expression revealed that both receptors activate *hrt-1* and *hes-5*, but not *hes-1*, promoter activity in adult VSMC This is achieved through CBF-1-dependent signalling This establishes both HRT-1 and Hes-5, but not Hes-1, as a direct downstream target gene of Notch 1 and Notch 3 in adult RVSMC This is in agreement with several studies that have documented the HRT-1, -2 and -3 as direct downstream targets of all Notch receptors in myoblasts and fibroblasts (Maier and Gessler 2000, Nakagawa *et al*, 2000, Iso *et al*, 2002) In addition, Wang *et al*, have shown that both HRT-1 and HRT-2 are direct downstream targets of Notch 3 IC in adult VSMC (Wang *et al*, 2002a) Whilst Notch 3 IC evokes a similar level of activation at both the *hes-5* and *hrt-1* promoters, the effect of Notch 1 on the *hrt-1* promoter is considerably greater than that on the *hes-5* promoter This could indicate a significant role for Notch 1-mediated activation of HRT-1 in adult VSMC This is consistent with the preferential expression of HRT-1 in the cardiovascular system (Chin *et al*, 2000), furthermore, Notch 3 IC significantly increases *hrt-1* over *hrt-2* promoter activity in VSMC (Wang *et al*, 2002a) Consistent with this, we have documented that both Notch 1 IC and Notch 3 IC cause a greater increase in *hrt-1* as opposed to *hrt-2* mRNA levels The activation of Notch target genes is likely to be cell context specific, as although Notch 1 and Notch 3 IC over-expression did not activate *hes-1* promoter activity in VSMC, Hes-1 has been shown to be a direct downstream target of Notch 1 in endothelial cells (Liu *et al*, 2003) In addition, Notch 1 has also been reported to increase Hes-1 expression in T lymphoma

cell lines (Lee *et al*, 1999), however, expression of Hes-5, HRT-1, HRT-2 and HRT-3, but not Hes-1, was greatly diminished in Notch 1 deficient mice in various tissues (de la Pompa *et al*, 1997, Leimeister *et al*, 1999, Leimeister *et al*, 2000) Hes-1 and Hes-5 have been shown to functionally compensate each other, for example, in Notch-mediated neuronal degeneration (Ohtsuka *et al*, 1999), therefore whether Hes-1 activity in VSMC could be induced by disruption of Hes-5 remains to be investigated

Further evaluation of Notch signalling pathway activation *in vitro* revealed the novel finding that serum temporally promotes Notch signalling in VSMC in a CBF-1-dependent manner Interestingly, although *hes-1* promoter activity was not activated as a result of serum addition, an increase in *hes-1* mRNA expression was observed The reason for this discrepancy is not clear, put points to the fact that Hes-1 could potentially play a role in modulating the effects of the Notch signalling pathway in adult VSMC

This study has, however, clearly shown that serum stimulation results in an increase in Notch receptor protein and mRNA expression, in addition to Notch target gene mRNA expression Using a robust antibody directed against the IC portion of the Notch receptor, we demonstrate that serum significantly increases the active IC component of the Notch 1 receptor We have previously shown that Notch IC over-expression increases activation of the artificial CBF-1 luciferase-tagged promoter, and also the natural CBF-1 promoter within VSMC, as evidenced by Notch target gene promoter activation and up-regulation in a CBF-1-dependent manner Therefore, we propose that the serum-mediated up-regulation of Notch signalling pathway activity is due to a serum-regulated increase in the IC portion of the Notch receptor within VSMC The explanation of the serum-induced activation of the Notch signalling pathway is not immediately apparent, however, several studies have revealed that serum mitogens influence Notch receptor expression and Notch signalling in several cell types VEGF, for example, has recently been shown to induce Notch 1 and Delta-like 4 expression in arterial endothelial cells via the PI3K/Akt pathway (Liu *et al*, 2003) Additionally, serum components have been shown to stimulate Jagged expression via NFκB activation in non-vascular cells (Artavanis-Tsakonas *et al*, 1999, Nickoloff *et al*, 2002) Serum mitogens are also reported to result in decreased Notch signalling pathway expression Angiotensin II and PDGF cause a time-dependent decrease in Notch 3, Jagged 1, HRT-1 and HRT-2, mediated, at least in part, by ERK-dependent signalling mechanisms (Campos *et al*, 2002, Wang *et al*, 2002a) Notwithstanding, our study has clearly demonstrated that serum activates the Notch signalling pathway in

RVSMC, and that this is achieved in a CBF-1-dependent manner

This study also demonstrates that the expression of the endogenous ligand, Jagged, induces CBF-1-dependent activation of the Notch signalling pathway in VSMC Whilst the level of CBF-1 activation is markedly lower than that induced by either Notch IC over-expression or serum stimulation, the mode of activation is the same Many authors claim that Notch IC over-expression produces differing results compared with endogenous ligand activation (Iso *et al*, 2002), however, others claim that constitutively activated Notch produces a phenotype identical to that produced by ligand activation (Uyttendaele *et al*, 2000) We observed similar levels of CBF-1 activation in Notch IC- and serum-stimulated cells, indicating that either the level of activation produced by Notch IC over-expression is physiological, or that the level of Notch signalling following serum stimlation of quiesced cells might represent a response similar to that seen in vascular insult or injury

It is widely accepted that EBNA-2 activates the Notch signalling pathway in many cell types (Hsieh *et al*, 1996), and this study adds VSMC to that list Increased understanding of the activities of EBNA-2 may increase understanding of EBV-related pathologies, in addition, it is possible that EBNA-2, or other EBV components, could be used to manipulate the level of Notch pathway activation in VSMC

## 3 4 Conclusion

This study demonstrates the presence of many components of the Notch signalling pathway in RVSMC, and illustrates that Notch 1 IC, Notch 3 IC, EBNA-2 and serum stimulation result in a CBF-1-dependent activation of the Notch signalling pathway in these cells In conjunction with this, we have clearly shown that HRT-1 and Hes-5, but not Hes-1, are among the primary target genes of the Notch signalling pathway in RVSMC

# Chapter 4

# Results

# The Notch Signalling Pathway and VSMC Apoptosis

## 4 1 Introduction

Components of the Notch signalling pathway are present in both embryonic and adult VSMC, therefore it is conceivable that the functional role of this pathway in development is also conserved in adult cells However, the functional role of the Notch signalling pathway in adult VSMC remains poorly characterized Therefore, this study attempts to further elucidate the role of Notch signalling in adult VSMC As the Notch signalling pathway has previously been implicated in the regulation of apoptosis in both the developing vasculature and other non-vascular adult cells, this study investigated the role of this pathway m regulating VSMC apoptosis In addition, we investigate whether Notch regulation of apoptosis occurs in a CBF-1-dependent manner, and attempt to determine some of the mechanisms whereby the Notch signalling pathway acts to regulate apoptosis This was achieved through investigating Notch interaction with members of the Bcl-2 and NFκB families

In recent years numerous studies have identified the Notch signalling pathway as an important factor in controlling cell fate decisions in many tissues (Artavanis-Tsakonas *et al*, 1995, Greenwald 1998, Artavanis-Tsakonas *et al*, 1999) This role is conserved from *Drosophila* to humans, and many examples of Notch signalling pathway control of cell fate decisions abound Whilst accumulated data from *Drosophila, C Elegans* and vertebrates suggest that the Notch signalling pathway plays a fundamental role in the differentiaton of uncommitted cells, it is now emerging that Notch has multiple developmental roles, including the regulation of other developmental cell fate decisions Notch has been shown to restrict neural differentiation in *Drosophila* (Parks *et al*, 1997), and similarly represses both neurogenesis and myogenesis in vertebrates (Lai 2004) Aberrant regulation of the Notch signalling pathway during development can result in embryonic lethality Studies using *notch 1* knockout mice documented that the mice did not survive beyond the second stage of gestation, and the embryos exhibited widespread apoptotic cell death (Swiatek *et al*, 1994, Conlon *et al*, 1995) Recent studies have implicated the Notch signalling pathway in multiple aspects of vascular development, including control of VSMC fate decisions, angiogeneic processes, and arterial-venous differentiation (Artavanis-Tsakonas *et al*, 1999, Gridley 2001, Iso *et al*, 2003) Dysregulation of the Notch signalling pathway in embryogenesis can result in embryonic lethality due to defects in vasculogenesis (Xue *et al*, 1999) and angiogenesis (Singh *et al*, 2000), postulated to be as a result of aberrant regulation of cell fate decisions in the developing

decisions in the developing vasculature Although the role of the Notch signalling pathway in vasculogenesis and organogenesis is well established, the role of this pathway in the adult vasculature remains to be fully defined

A growing body of evidence also implicates the Notch signalling pathway in the control of cell fate decisions in adult cells Notch has been widely implicated in the control of cell fate decisions in haematopoiesis (Bigas *et al*, 1998, Jehn *et al*, 1999), and is increasingly recognized as an important arbiter of cell fate in other tissues and organs Notch, for example, inhibits murine pancreatic cell (Apelqvist *et al*, 1999) and myoblast differentiation (Bush *et al*, 2001) Notch has also been shown to implicate other cell fate decisions in mature organisms Increased Notch receptor and ligand expression in carotid arteries decreases cellular migration through regulation of cell-cell and cell-matrix interactions (Lindner *et al*, 2001) In addition, Notch signalling pathway activation results in increased proliferation in bone marrow stem cells and promyelotic leukaemia cells (Carlesso *et al*, 1999) As many signalling pathways involved in the control of proliferation also regulate apoptosis, it is not surprising that the Notch signalling pathway has also been shown to regulate cellular apoptosis Whilst Notch is generally considered to exert an anti-apoptotic effect, some reports indicate that Notch can promote apoptosis, suggesting that the effect of Notch is cell type specific Notch 1 inhibits glucocorticoid-induced and T-cell receptor-mediated apoptosis in thymocytes (Deftos *et al*, 1998, Kaneta *et al*, 2000), and Notch 4 has recently been shown to inhibit endothelial cell apoptosis (MacKenzie *et al*, 2004) In contrast, Notch 1 activation has been reported to promote apoptosis in chicken B lymphocytes and human monocytes (Morimura *et al*, 2000, Ohishi *et al*, 2000) Notch regulation of cell fate decisions occurs both in a CBF-1-dependent and -independent manner Notch regulation of cell fate determination of marginal zone B cells is CBF-1-dependent (Tanigaki *et al*, 2002), whereas inhibition of murine muscle cell differentiation occurs in a CBF-1-independent manner (Shawber *et al*, 1996) In addition, inhibition of apoptosis in endothelial cells is mediated via both CBF-1-dependent and -independent pathways (MacKenzie *et al*, 2004)

Dysregulation of cell fate decisions is associated with a wide variety of diseases, including cancer, neurodegeneration and heart disease As Notch is intimately involved in the regulation of cell fate decisions it was postulated and proven that aberrant regulation of the Notch signalling pathway contributes to tumourigenesis This was first demonstrated in a recurrent translocation that is associated with many types of T-cell acute lymphoblastic leukaemias, resulting in a constitutively active form of Notch 1 in

the cells (Ellisen *et al*, 1991) Increased expression of Notch receptors and ligands has since been identified in several cancers, including epithelial malignancies, colon adenocarcinomas and lung squamous carcinomas (Zagouras *et al*, 1995, Daniel *et al*, 1997) Expression of the Notch ligands, Jagged 1 and Delta 1 are markedly increased in cervical carcinomas, with a concomitant increase in Notch 1 and Notch 2 receptor expression (Gray *et al*, 1999)

Dysregulation of cellular proliferation and apoptosis are evident in multiple forms of vascular disease, including hypertension, transplant arteriopathy and atherosclerosis (Best *et al*, 1999) Clinically, the most common and important manifestation of excessive and progressive apoptosis is the loss of tissue from myocardial infarction and strokes In both instances, although there is a loss of tissue from ischemic necrosis, the larger loss of tissue results from the extension of the original vascular insult by apoptosis (Misao *et al*, 1996) Therefore, a better understanding of the regulation of these processes in vascular cells could identify important therapeutic targets, and have potentially important clinical implications Dysregulation of the Notch signalling pathway underlies a number of adult disorders with associated vascular pathologies, such as CADASIL and AGS (Loomes *et al*, 1999, Brulin *et al*, 2002) Therefore, it is conceivable that the Notch signalling pathway plays a role in the regulation of cell fate decisions within the vasculature Recent reports have provided initial evidence that this is the case Mac Kenzie *et al*, (2004) has shown that Notch 4 inhibits EC apoptosis triggered by lipopolysaccharide In addition, Wang *et al*, (2002b) demonstrate that Notch 3 inhibits Fas-mediated apoptosis in VSMC through of induction of c-FLIP

Apoptosis is a normal physiological process required for the maintenance of homoestasis in all tissues and organs of the body, including the vasculature Therefore, this study initially investigated the effect of manipulation of the Notch signalling pathway on VSMC apoptosis in cycling cells Serum deprivation is a well-documented trigger for apoptosis in many cells types, including vascular cells (Yao and Cooper 1995, Kono *et al*, 2002) The effect of manipulation of the Notch signalling pathway was also investigated on serum deprivation-induced apoptosis in VSMC Mac Kenzie *et al*, (2004) have documented that Notch 4 inhibits EC apoptosis in both a CBF-1-dependent and -independent manner, in addition, Wang *et al*, revealed that Notch 3 protects VSMC from apoptosis in a CBF-1-independent manner Therefore, this study investigated whether Notch signalling pathway activation in VSMC could affect apoptosis in a CBF-1-dependent manner This was achieved through transfection of

CBF-1 specific inhibitors, including mutant Notch 1 IC, RPMS-I, and R218H-RBPJκ

The process of apoptosis is tightly regulated through a number of gene products that promote or inhibit cell death at different stages of apoptosis The most extensively studied, and perhaps the most important, are the Bcl-2 family (Gupta 2003) The net influence of the Bcl-2 family on apoptosis appears to be the ratio between the pro- and anti-apoptotic molecules in a cell at any given time A number of signalling pathways, such as the MAPK and NFκB pathways, can influence the relative concentration of Bcl-2 family of proteins in mammalian cells (Gupta 2003), this study investigates whether the Notch signalling pathway should be added to this list EBNA-2, which mimics and subverts the Notch signalling pathway, has been shown to up-regulate *bfl-1* mRNA levels in a Burkitts lymphoma (BL) cell line, and regulate *bfl-1* promoter activity in a CBF-1-dependent manner *(P Pegman personal communication)* Therefore, the effect of Notch over-expression on *bfl-1* promoter activity in VSMC was examined In addition, Notch 1 has been shown to physically interact with the NFκB signalling pathway in T cells (Guan *et al*, 1996) Notch exhibits an IκB-like activity, physically interacting with the p50 NFκB subunit, sequestering it in the cytoplasm, thus inhibiting NFκB activity It is currently not documented whether Notch IC, or indeed EBNA-2, exerts the same effect on NFκB activity in VSMC This study, therefore, examined the effect of Notch 1 IC, Notch 3 IC and EBNA-2 on NFκB activity in VSMC using a luciferase-tagged promoter upstream of NFκB binding sites, and an IκB control plasmid It is conceivable, and indeed likely, that the Notch signalling pathway could be part of a complex multi-signalling network that exerts an influence over VSMC fate decisions, including apoptosis This, however, remains largely undefined An increased understanding into the regulation of apoptosis in the vasculature, in particular in VSMC, has the potential to translate into future therapeutic benefits

## 4 2 Results

### 4 2 1 Inhibition of Endogenous Notch-mediated CBF-1-dependent Signalling in RVSMC Results in Increased Apoptosis

To determine the effects of the Notch signalling pathway on RVSMC apoptosis *in vitro*, the effect of inhibition of endogenous Notch signalling on RVSMC apoptosis was initially investigated This was achieved either through transfection of cycling RVSMC with inhibitors of the Notch signalling pathway, RPMS-I, R218H-RBP-Jα, or mutant Notch 1 IC, or treatment of cells with the pharmacological inhibitors Brefeldin A or Monensin Cells were co-transfected with the puromycin resistance plasmid, and exposed to puromycin-containing media, to ensure a high level of plasmid expression

Apoptosis was measured using histochemical analysis and caspase 3 activity assays, as described in section 2 3 10 Briefly, the annexin V/propidium iodide and the acridine orange/ethidium bromide dual stains, which allow simultaneous detection of viable, apoptotic and necrotic cells, were used in this study Using the annexin V/propidium iodide dual stain, viable cells (AV negative, PI negative) do not fluoresce, apoptotic cells (AV positive, PI negative) appear green, and necrotic cells (AV positive, PI positive) appear orange/red The acridine orange/ethidium bromide dual stain causes viable cells to appear to have a bright green nucleus with intact structure, while early 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 The reliability of all assays was confirmed using a number of ubiquitous activators of apoptosis, such as hydrogen peroxide and serum deprivation *(data not shown)*

Transfection of RVSMC with RPMS-I and mutant Notch 1 IC, and treatment of the cells with Brefeldin A all resulted in significantly increased apoptosis, as measured by the annexin V/propidium iodide dual stain Inhibition of Notch with RPMS-I, mutant Notch 1 IC or Brefeldin A resulted in significantly increased apoptotic (annexin V positive/green) cells with respect to mock transfected control, which exhibited predominately viable (annexin V negative/propidium iodide negative) cells (Figure 4 1) Representative histochemical analysis are shown in Figure 4 1A, and cumulative data on the percentage of apoptotic (annexin V positive/propidium iodide negative cells) are also presented (Figure 4 1B) Mock transfected cycling RVSMC exhibited a low level of apoptosis (4 3±2 72%), whereas inhibition of endogenous CBF-1-dependent Notch

signalling with RPMS-I, mutant Notch 1 IC and Brefeldin A resulted in an increase in the percentage of apoptotic cells to 16 18±1 4%, 16 5±1 5% and 14 5±0 5% respectively This increase in apoptosis in RVSMC due to endogenous Notch inhibition was further confirmed using the acridine orange/ethidium bromide dual stain Transfection of cycling RVSMC with the Notch inhibitor RPMS-I resulted in a significant increase in apoptotic cells, as compared to mock transfected control cells (Figure 4 2A) Cumulative data on the percentage of apoptotic cells again revealed a low level of apoptosis in control cells (5 3±0 3%), which is significantly increased due to Notch inhibition to 31 0±8 32% (Figure 4 2B) In addition, analysis of caspase 3 activity in RVSMC revealed an increase in the activity of this marker of apoptosis due to endogenous CBF-1-dependent Notch inhibition Transfection with the Notch signalling inhibitor, RPMS-I, resulted in a significant fold increase in caspase 3 activity (1 44±0 07) with respect to mock transfected control cells (Figure 4 3A) Furthermore, transfection of cells with mutant Notch 1 IC, R218H-RBP-Jα, or treated with the pharmacological inhibitor Brefeldin A resulted in a fold increase of caspase 3 activity over control to 1 59±0 07, 1 55±0 05 and 1 2±0 15 respectively, with significant increases being observed due to mutant Notch 1 IC- and R218H-RBP-Jα̃nduced inhibition of the Notch signalling pathway (Figure 4 3B)

The effect of endogenous CBF-1-dependent Notch pathway inhibition on the *bcl-2* family of apoptotic genes was also investigated (Figure 4 4) RPMS-I transfection resulted in an increase in the pro-apoptotic *bad* and *bax* gene expression to 1 52±0 24 and 1 3±0 3 over mock transfected control cells respectively A significant fold increase in the anti-apoptotic *bcl-2* gene expression was also seen in RPMS-I transfected cells to 1 83±0 33 over the mock transfected control In contrast, a decrease of 27 0±10% in anti-apoptotic *bcl-x*$_L$ mRNA expression was evident in RPMS-I transfected cells (to 0 73±0 1) as compared to mock transfected control cells (Figure 4 4A) To further investigate whether these genes were under the control of the Notch signalling pathway, or whether the change in gene expression observed following CBF-1-dependent Notch inhibition was compensatory, we over-expressed Notch 3 IC in the cells and examined *bcl-2* family gene expression Notch 3 over-expression increased *bad* expression to 1 35±0 45 over mock transfected control cells, and a significantly deceased *bax* expression by 34 0±10 0% (to 0 66±0 1) as compared to mock transfected cells In addition, an increase in both *bcl-2*, and *bcl-x*$_L$ expression to 1 5±0 29 and 1 6±0 21 over mock transfected control cells was also observed, with the increase in

*bcl-x*$_L$ proving to be significant (Figure 4 4B) Therefore a reciprocal change in *bax* and *bcl-x*$_L$, but not *bad* and *bcl-2*, was evident following CBF-1-dependent stimulation and inhibition of the Notch signalling pathway This was confirmed by simultaneous over-expression and inhibition of the Notch signalling pathway, which decreases any change exerted by individual manipulation of the Notch signalling pathway on both *bax* and *bcl-x*$_L$ expression to 1 08±0 07 and 0 9±0 09 as compared to mock transfected control (Figure 4 4C)

**Figure 4.1 Inhibition of Notch in Cycling RVSMC causes increased Apoptosis, as measured by the Annexin V/Propidium Iodide Dual Stain**

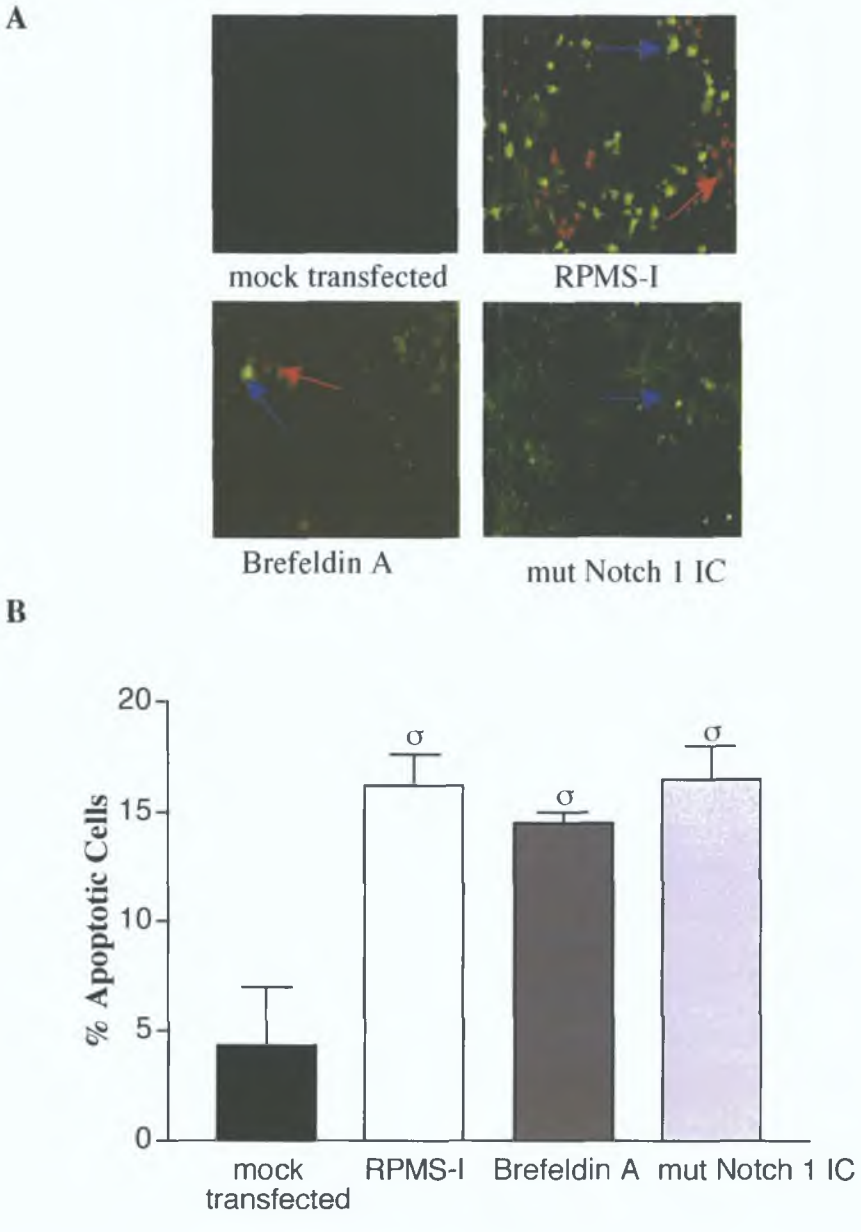
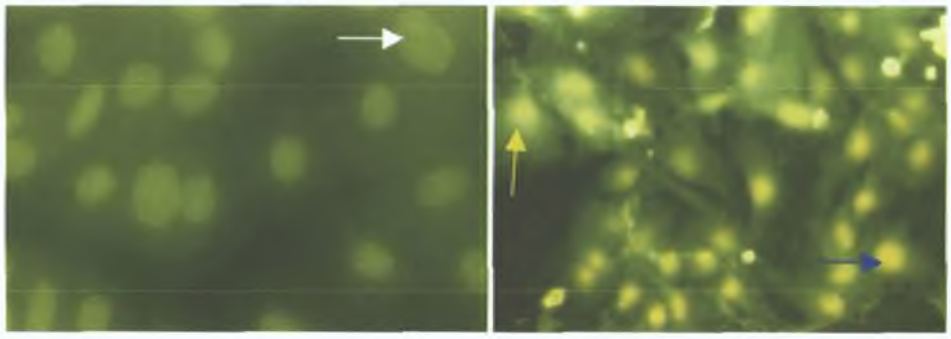



**Figure 4.1 Inhibition of Notch in cycling RVSMC results in increased apoptosis.** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or the Notch inhibitors, RPMS-I or mut Notch 1 IC. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h). Additionally, indicated cells were treated with the pharmacological inhibitor Brefeldin A (0.1μg/ml, 24 h). Cells were stained with the Annexin V/Propidium Iodide dual stain and viewed under a fluorescent microscope. A) Representative images. Apoptotic cells, blue arrow; Necrotic cells, red arrow. B) Graph showing percentage increase in the number of apoptotic cells due to Notch inhibition. Each experiment was performed in duplicate, with the graph representative of n=3. $^{\kappa}$ $p<0.05$ as compared to mock transfected control (student's t test).

**Figure 4.2 Inhibition of Notch in Cycling RVSMC causes increased Apoptosis, as measured by the Acridine Orange/Ethidium Bromide Dual Stain**

A




B

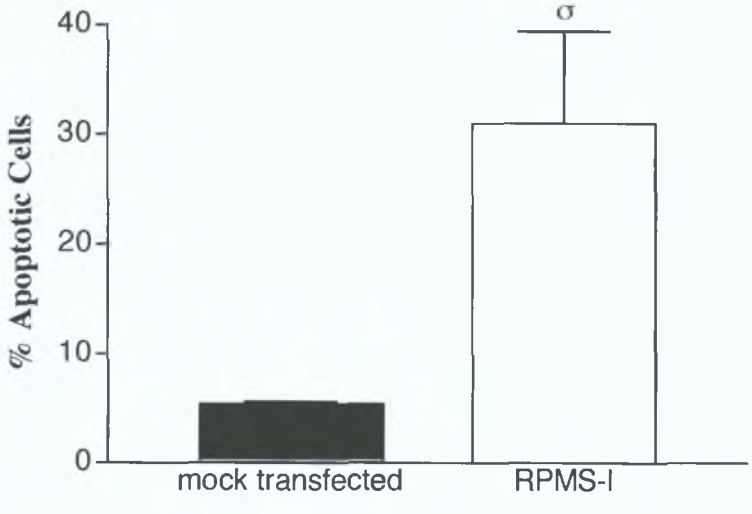



**Figure 4.2 Inhibition of Notch in cycling RVSMC results in increased apoptosis.** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or the Notch inhibitor, RPMS-I. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h). Cells were stained with the Acridine Orange/Ethidium Bromide dual stain and viewed under a fluorescent microscope. A) Representative images. Viable cell, white arrow; Early apoptotic cell, yellow arrow; Late apoptotic cell, blue arrow. B) Graph showing percentage increase in the number of apoptotic cells due to Notch inhibition. Each experiment was performed in duplicate, with the graph representative of n=2. $^{\kappa}$ $p<0.05$ as compared to mock transfected control (student's t test).

**Figure 4 3 Inhibition of Notch in Cycling RVSMC causes increased Apoptosis, as measured by Caspase 3 Activity**

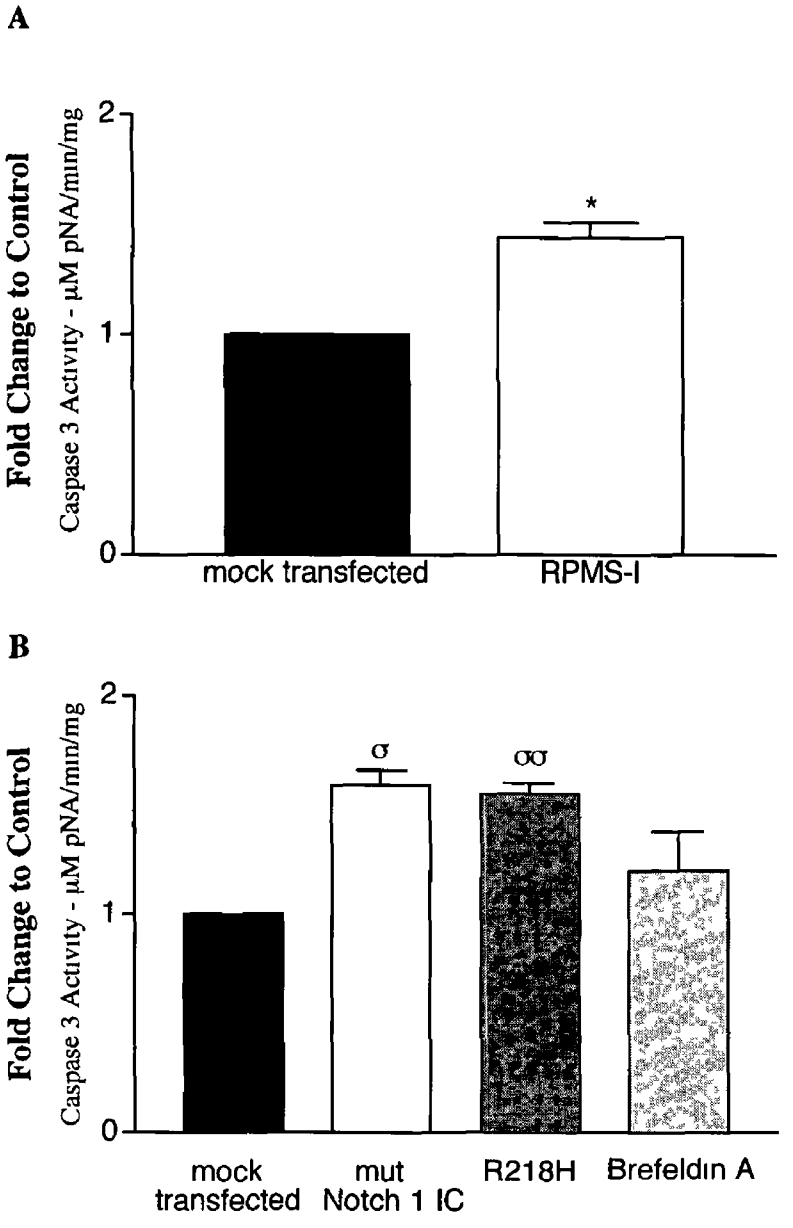
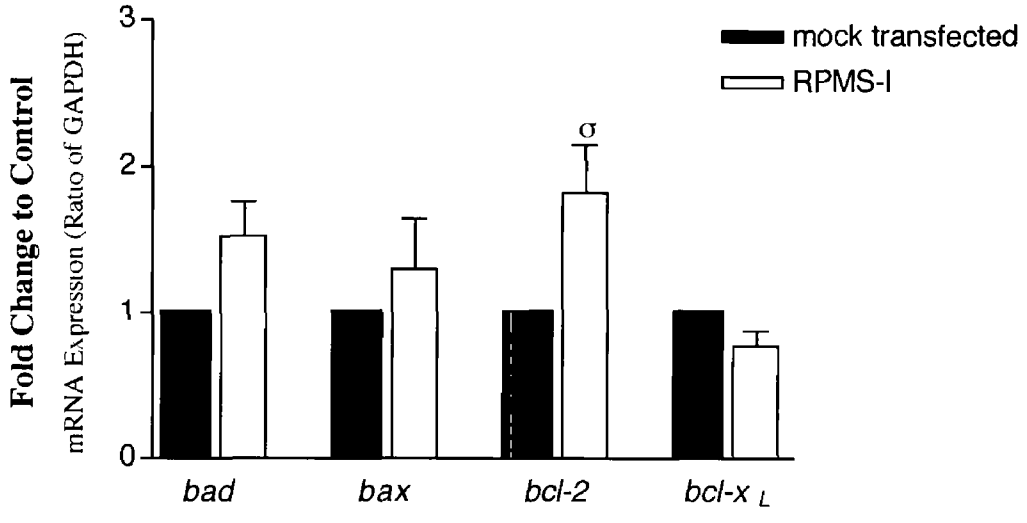


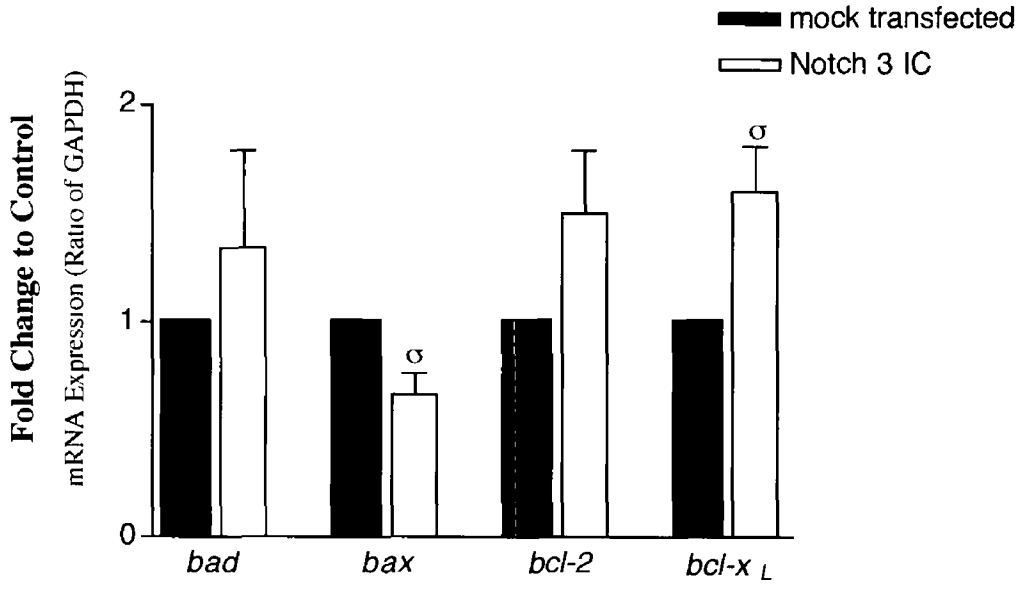

**Figure 4 3 Inhibition of Notch in cycling RVSMC results in increased apoptosis** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or the Notch inhibitors, RPMS-I, mut Notch 1 IC or R218H as indicated Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 µg/ml, 48 h) Additionally, indicated cells were treated with the pharmacological inhibitor Brefeldin A (0 1µg/ml, 24 h) Protein was isolated and assayed for caspase 3 activity Caspase assays were normalized to protein levels, and expressed as fold increase over control (= the value obtained with mock transfected cells arbitrarily assigned a value of 1) A) n=6, * $p<0\ 05$ as compared to mock transfected control (rank test) B) Representative experiment performed in triplicate $^{\sigma}$ $p<0\ 05$, $^{\sigma\sigma}$ $p<0\ 005$ as compared to mock transfected control (student's t test)

**Figure 4 4 The Effect of Endogenous Inhibition or Over-expression of Notch 3 IC on Bcl-2 Family mRNA Expression**

**A RPMS-I**




**B Notch 3 IC**



C Notch 3 IC + RPMS-I

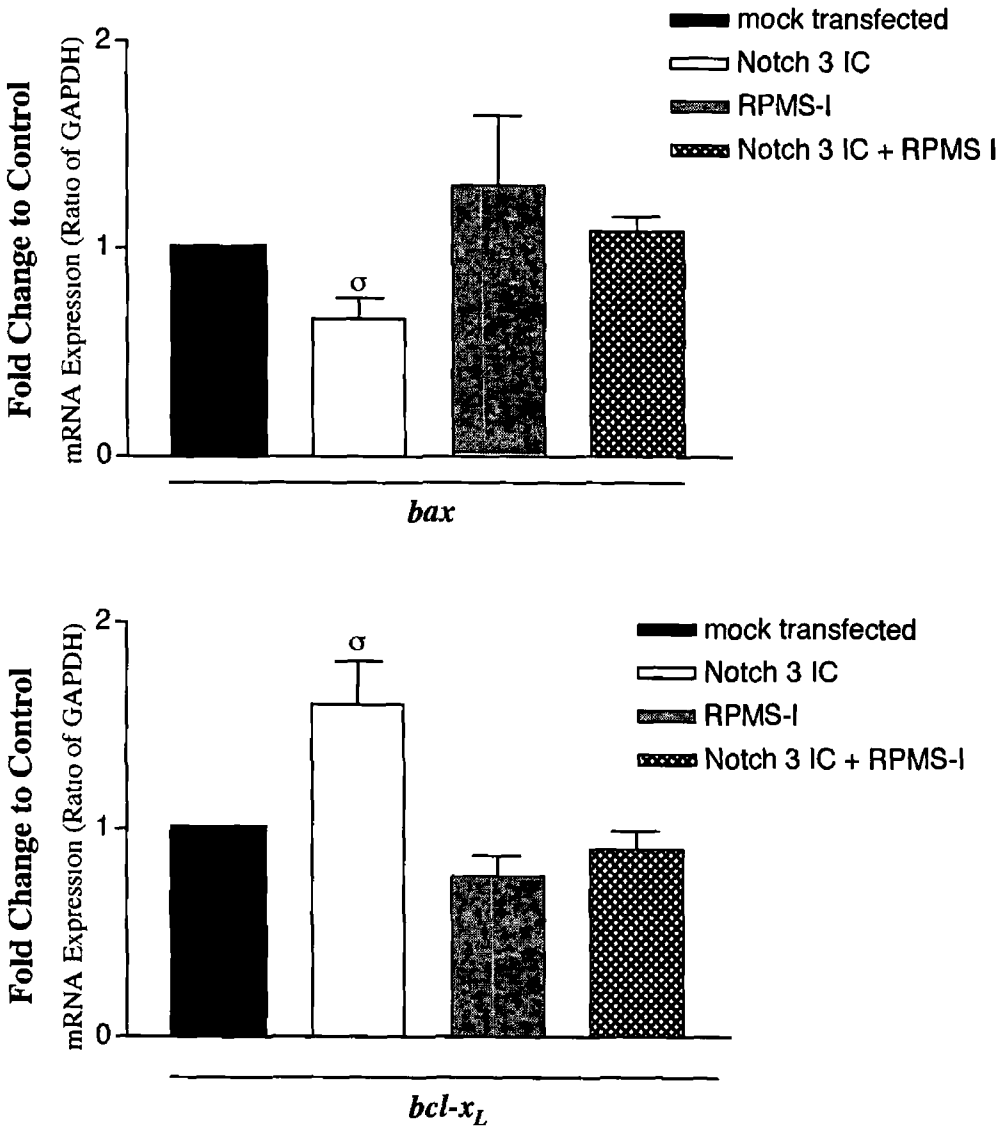



**Figure 4 4 Inhibition of Notch in cycling RVSMC results in increased apoptosis** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, the Notch inhibitor, RPMS-I (A) or Notch 3 IC (B), or both (C) as indicated Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 μg/ml, 48 h) RNA was isolated and assayed for *bad*, *bax*, *bcl-2* and *bcl-$x_L$* expression as indicated using quantitative real time PCR analysis Expression was normalized to GAPDH levels, and expressed as fold increase over control (= the value obtained with mock transfected cells arbitrarily assigned a value of 1) n=3, $^{\sigma}$ $p<0$ 05 as compared to mock transfected control

### 4 2 2 Effect of Notch Signalling Pathway Over-expression on Serum Deprivation-induced Apoptosis in RVSMC

#### 4 2 2 1 Serum Deprivation-induced Apoptosis in RVSMC

Several studies have established the fact that serum deprivation induces VSMC apoptosis (Bennett *et al*, 1995, Kono *et al*, 2002) This is confirmed in the present study through analysis of the apoptotic profile of serum deprived RVSMC, again utilizing histochemical analysis and caspase 3 activity assays

Apoptosis was measured during a serum deprivation timecourse over 96 h *(data not shown)* A period of 72 h serum deprivation was necessary to induce apoptosis, as measured with the annexin V/propidium iodide and the acridine orange/ethidium bromide dual stains (Figure 4 5A and 4 5B respectively) Serum deprivation resulted in a significant increase in the percentage of apoptotic cells, as measured using the annexin V/propidium iodide dual stain, with control cells exhibiting 2 5±0 6% apoptosis compared to 67 75±4 5% in serum deprived cells (Figure 4 5A) Similarly, measurement of apoptosis using the acridine orange/ethidium bromide dual stain revealed a significant increase in the percentage of apoptotic cells in serum deprived (71 1±7 0%) versus control cells (2 0±0 5%) (Figure 4 5B) Using increase in caspase 3 activity as a measure of apoptosis, 6 h serum deprivation was shown to be optimal to induce apoptosis *(data not shown)* Serum deprivation resulted in a significant fold increase in caspase 3 activity (1 4±0 06) over serum control cells (Figure 4 5C)

The anti-apoptotic effect of the Notch signalling pathway revealed in section 4 2 1 was further analyzed through over-expression of either Notch 1 IC or Notch 3 IC in RVSMC The effect of this over-expression on serum deprivation induced apoptosis was investigated

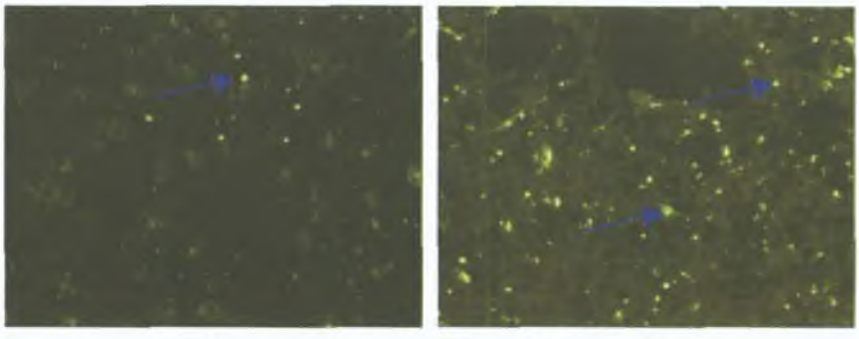
#### 4 2 2 2 Effect of Notch 3 IC Over-expression on Serum Deprivation-induced Apoptosis in RVSMC

RVSMC were transfected with an empty vector or Notch 3 IC, and co-transfected with the Notch signalling pathway inhibitors, RPMS-I or mutant Notch 1 IC Apoptosis was induced by serum deprivation and apoptosis measured using the annexin V/propidium iodide dual stain (Figure 4 6) Transfection of Notch 3 IC significantly attenuated the serum deprivation-induced percentage increase in apoptotic cells

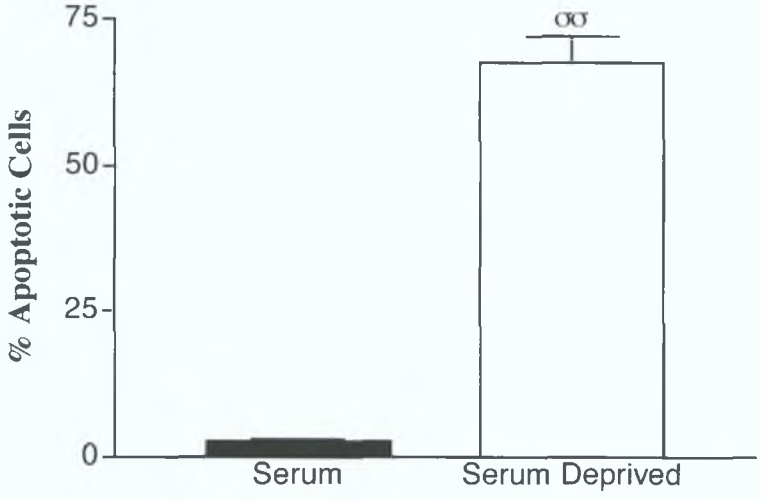
from 67 75±4 5% in mock transfected control cells to 38 53±2 0% Co-transfection of the Notch inhibitors, RPMS-I and mutant Notch 1 IC, decreased this attenuation to 50 53±3 85% and 60 91±6 12% respectively (Figure 4 6B) Similarly, transfection of Notch 3 IC proved to be anti-apoptotic in cells analyzed using the acridine orange/ethidium bromide dual stain (Figure 4 7) Serum deprived cells exhibited 71 0±7 0 percentage apoptosis (Figure 4 7B) Transfection of Notch 3 IC significantly attenuated the serum deprivation-induced increase in apoptosis to 39 2±3 8% Histochemical analysis suggests that although Notch 3 IC demonstrated an anti-apoptotic effect on serum deprivation-induced apoptosis, the presence of apoptotic bodies in the Notch 3 IC transfected cells suggests that Notch retards but not completely inhibits apoptosis The anti-apoptotic effect of Notch 3 IC was further confirmed by caspase 3 activity assays on mock and Notch 3 IC transfected cells (Figure 4 8) Notch 3 IC transfection resulted in a small but significant attenuation of the fold increase in caspase 3 activity to 0 76±0 04 over control (serum deprived mock transfected cells, arbitrarily assigned a value of 1), which represents a 24 0±4 0% decrease in caspase 3 activity Co-transfection of Notch 3 IC with the Notch inhibitor, RPSM-I, restored caspase 3 activity to 1 29±0 21 fold over control, which is significantly increased over the level of caspase 3 activity in cells transfected with Notch 3 IC alone

**Figure 4.5 Serum Deprivation Induces Apoptosis in RVSMC**

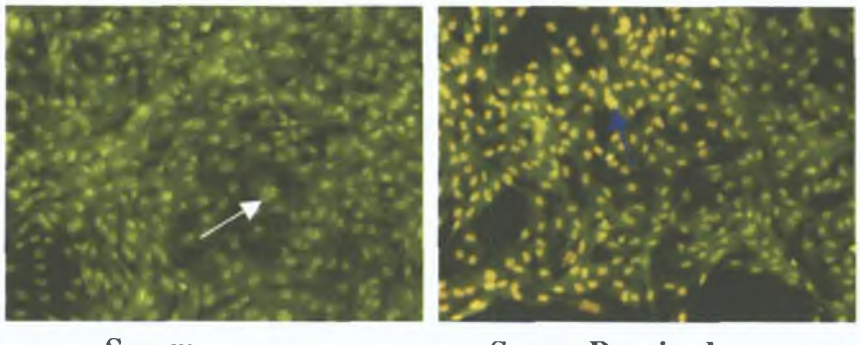
**A Annexin V/Propidium Iodide**



**Serum** **Serum Deprived**

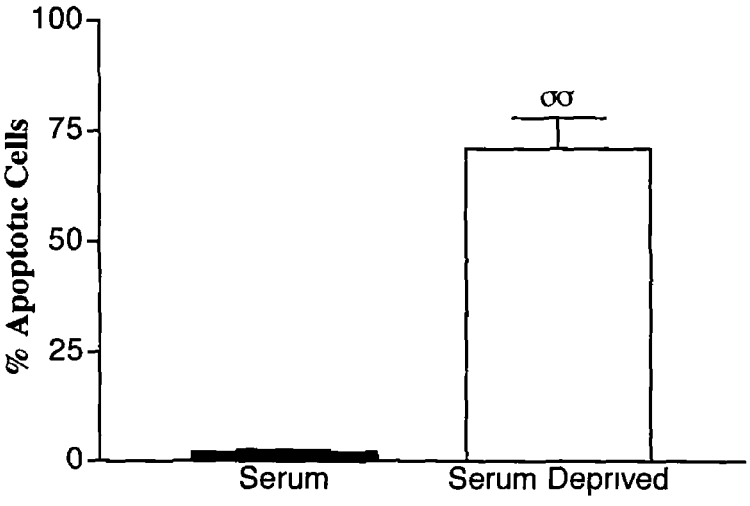



**B Acridine Orange/Ethidium Bromide**



**Serum** **Serum Deprived**

**B Acridine Orange/Ethidium Bromide (continued)**




**C Caspase 3 Activity**

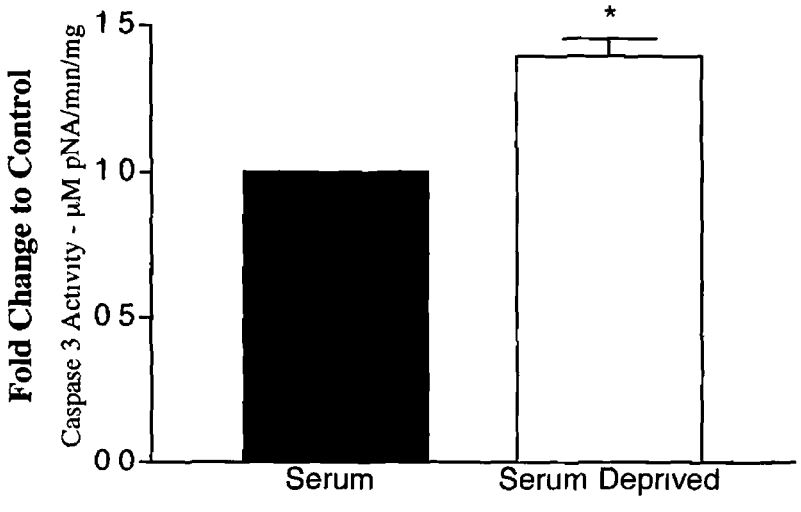



**Figure 4 5 Serum deprivation induces apoptosis in RVSMC** A and B) RVSMC were serum deprived for 72 h Cells were stained with the annexin V/propidium iodide dual stain (A) or the acridine orange/ethidium bromide dual stain (B) and viewed under a fluorescent microscope Representative images, viable cells, white arrow, apoptotic cells, blue arrow Histograms represent cumulative data for the annexin V/propidium iodide dual stain (A) or the acridine orange/ethidium bromide dual stain (B), n=3, $^{\sigma\sigma}$ $p<0\ 005$ as compared to serum control (student's t test) C) RVSMC were serum deprived for 6 h Protein was isolated and assayed for caspase 3 activity Caspase assays were normalized to protein levels, and expressed as fold increase over control (= the value obtained with cells exposed to 10% FCS, arbitrarily assigned a value of 1), n=6, * $p<0\ 05$ as compared to serum control (rank test)

**Figure 4.6 Effect of Notch 3 Over-expression on Serum Deprivation-induced Apoptosis in RVSMC**

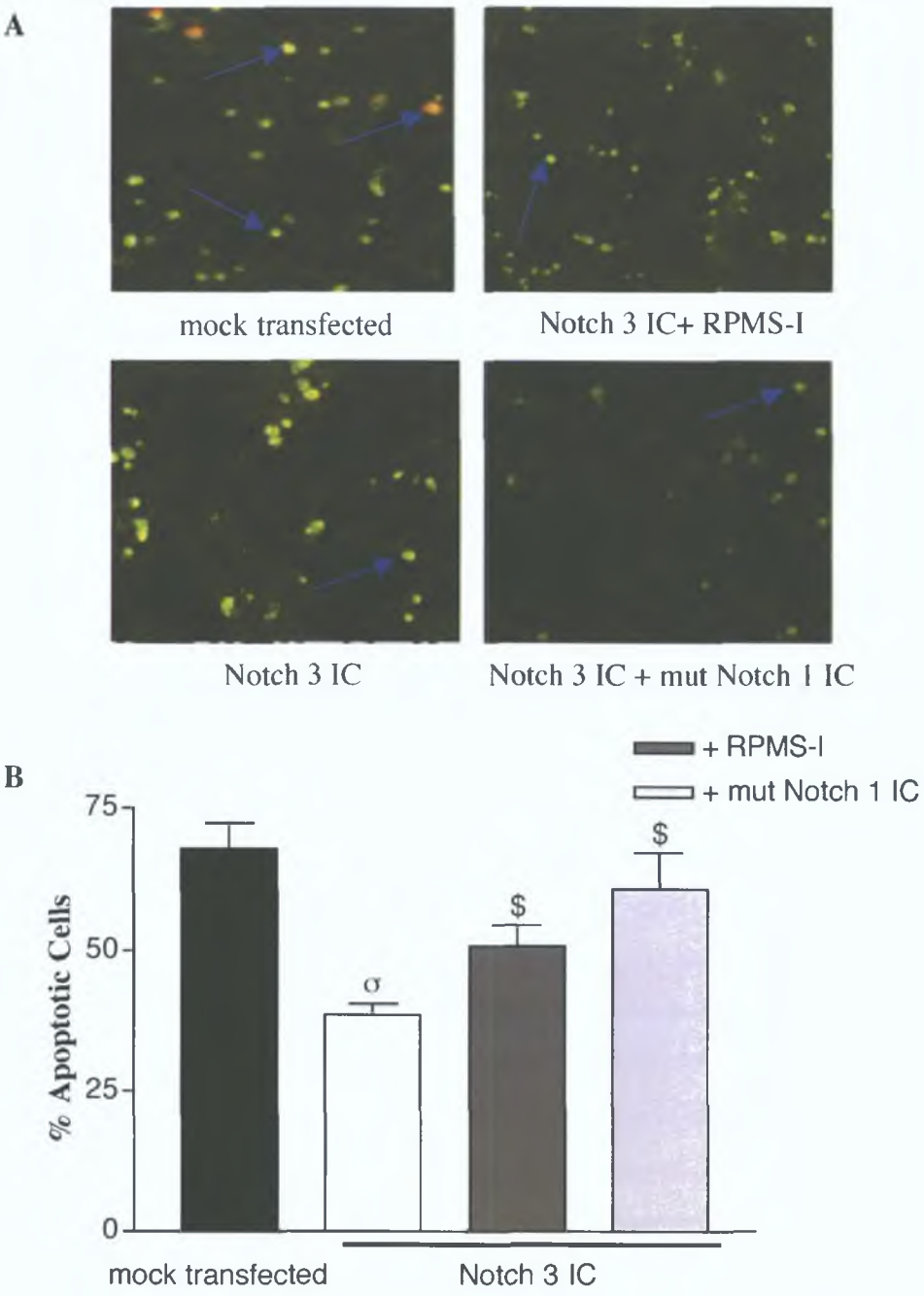
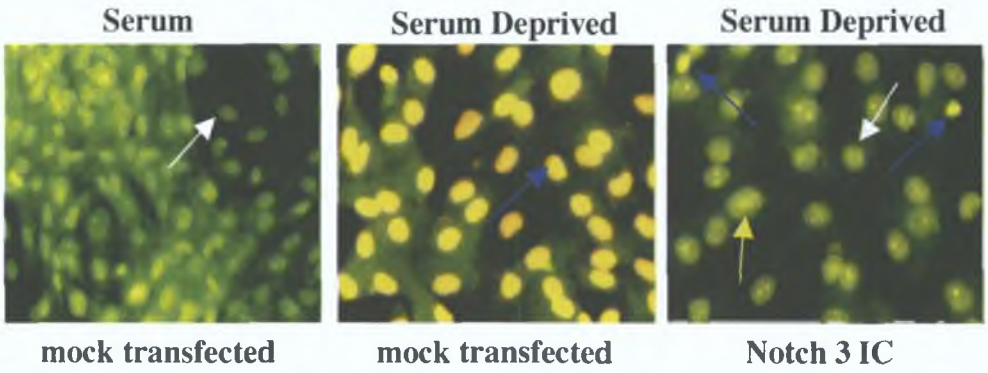



**Figure 4.6 Effect of Notch 3 on serum deprivation-induced apoptosis in RVSMC.** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, Notch 3 IC or the Notch inhibitors, RPMS-I or mut Notch 1 IC as indicated. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h). RVSMC were serum deprived for 72 h. Cells were stained with the Annexin V/Propidium Iodide dual stain and viewed under a fluorescent microscope, n=3. A) Representative images; apoptotic cells, blue arrow. B) Graph showing percentage apoptotic cells. $^{\kappa}$ $p<0.05$ as compared to mock transfected control, $^{\$}$ $p<0.05$ as compared to cells over-expressing Notch 3 IC (student's t test).

**Figure 4.7 Effect of Notch 3 Over-expression on Serum Deprivation-induced Apoptosis in RVSMC**

A




B

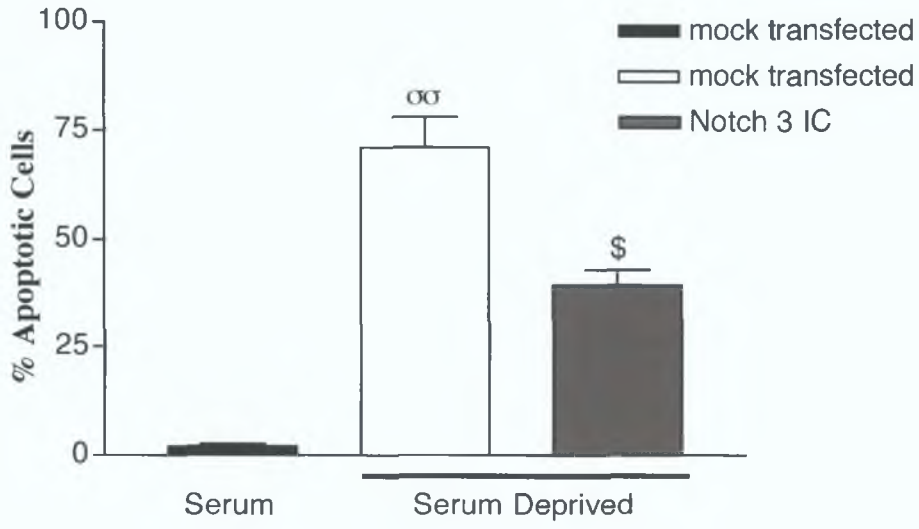



**Figure 4.7 Effect of Notch 3 on serum deprivation induced apoptosis in RVSMC.** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC as indicated. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h). RVSMC were serum deprived as indicated for 72 h. Cells were stained with the Acridine Orange/Ethidium Bromide dual stain and viewed under a fluorescent microscope, n=3. A) Representative images; Viable cell, white arrow; Early apoptotic cell, yellow arrow; Late apoptotic cell, blue arrow. B) Graph showing percentage apoptotic cells. $^{\text{ŏŏ}}$ $p<0.005$ as compared to mock transfected control, $^{\$}$ $p<0.05$ as compared to cells over-expressing Notch 3 IC (student's t test).

**Figure 4 8 Effect of Notch 3 Over-expression on Serum Deprivation-induced Apoptosis in RVSMC**

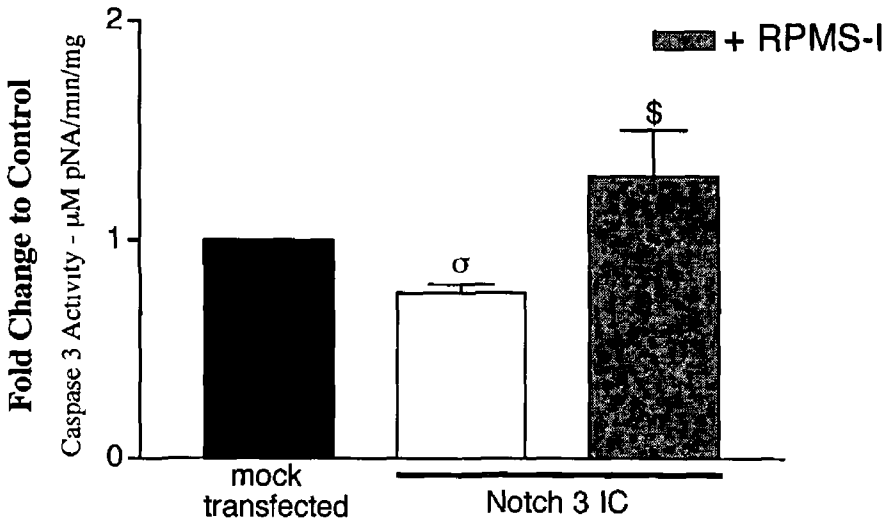


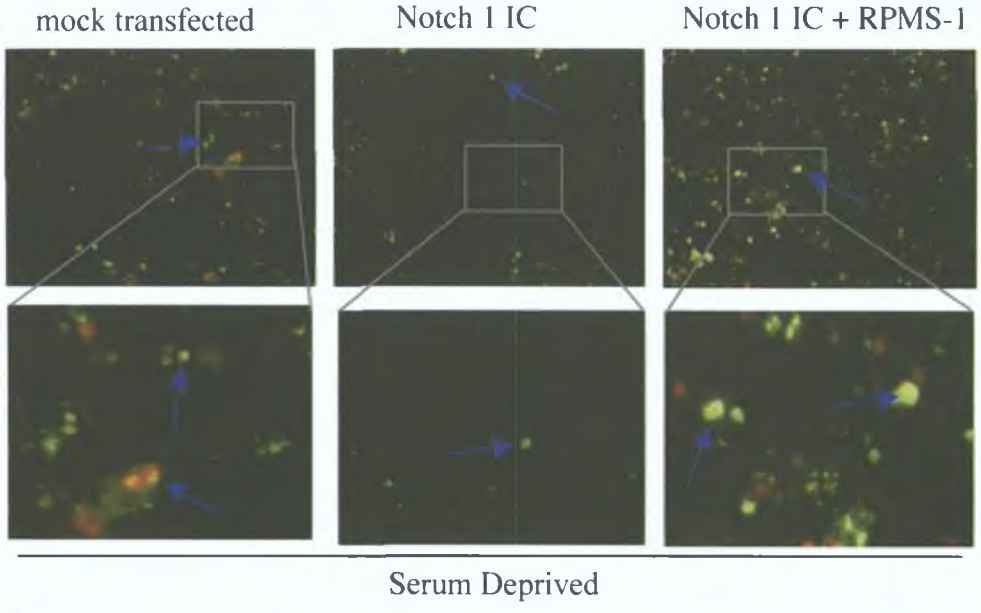

**Figure 4 8 Effect of Notch 3 on serum deprivation-induced apoptosis in RVSMC** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, Notch 3 IC or the Notch inhibitor RPMS-I, as indicated Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 µg/ml, 48 h) RVSMC were serum deprived for 6 h Protein was isolated and assayed for caspase 3 activity Caspase assays were normalized to protein levels, and expressed as fold increase over control (= the value obtained with mock (p7pCMV) transfected cells, arbitrarily assigned a value of 1), n=3 $^{\sigma}$ p<0 05 as compared to mock transfected control, $^{\$}$ p<0 05 as compared to cells over-expressing Notch 3 IC (student's t test)

### 4 2 2 3 Effect of Notch 1 IC Over-expression on Serum Deprivation-induced Apoptosis in RVSMC

In addition to Notch 3 IC, the effect of Notch 1 IC on serum deprivation-induced apoptosis was also investigated RVSMC were transfected with an empty vector or Notch 1 IC, and co-transfected with the Notch inhibitor RPMS-I Apoptosis was induced through serum deprivation, and analyzed using the annexin V/propidium iodide dual stain (Figure 4 9) Results obtained clearly demonstrate the anti-apoptotic effect of Notch 1 IC against serum deprivation-induced apoptosis Notch 1 IC significantly attenuated the serum deprivation-induced increase in apoptotic cells from 68 0±4 7% to 40 0±2 88% Co-transfection of Notch 1 IC with RPMS-I resulted in a restoration of the level of apoptosis over control level, to 78 0±7 2%, which was significantly increased over the level of apoptosis apparent in Notch 1 IC transfected cells alone The anti-apoptotic effect of Notch 1 IC was further confirmed through analysis of serum deprivation-induced apoptosis with the acridine orange/ethidium bromide dual stain (Figure 4 10) Serum-deprivation significantly increased the percentage of apoptotic cells from 3 2±0 7% to 73 2±5 9% This increase in apoptosis was significantly attenuated with the transfection of Notch 1 IC to 41 3±4 5% (Figure 4 10B) Caspase 3 activity analysis also established the anti-apoptotic effect of Notch 1 IC on serum deprivation-induced apoptosis Similar to the effect of Notch 3 IC, Notch 1 IC transfection resulted in a small but significant attenuation of the fold increase in caspase 3 activity to 0 80±0 045 to control (serum deprived mock transfected cells, arbitrarily assigned a value of 1), which represents a 20 0±4 5% decrease in caspase 3 activity Over-expression of the Notch inhibitors, RPMS-I and R218H-RBP-Jα, resulted in caspase 3 activities of 1 11±0 08 and 1 27±0 11 respectively over control

**Figure 4.9 Effect of Notch 1 Over-expression on Serum Deprivation-induced Apoptosis in RVSMC**

A




B

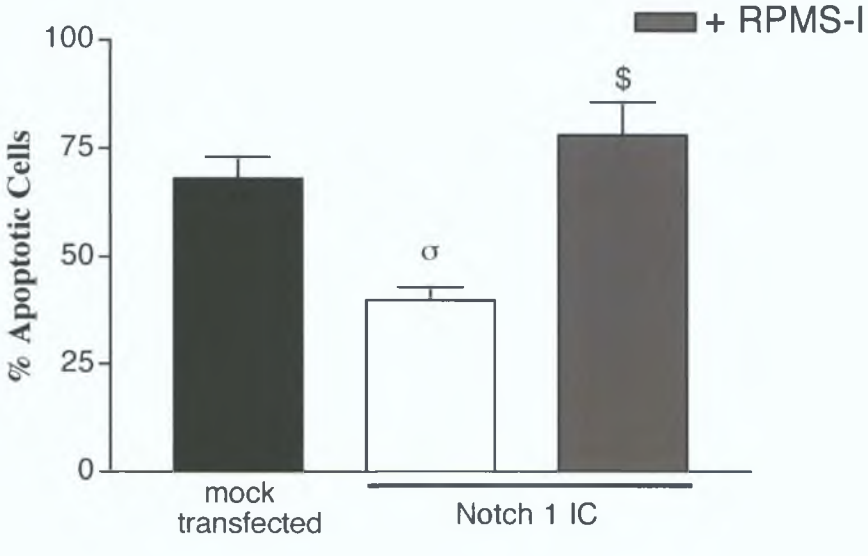



**Figure 4.9 Effect of Notch 1 on serum deprivation-induced apoptosis in RVSMC.** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, Notch 1 IC or the Notch inhibitor, RPMS-I. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h). RVSMC were serum deprived for 72 h. Cells were stained with the Annexin V/Propidium Iodide dual stain and viewed under a fluorescent microscope, n=3. A) Representative images; Blue arrows, apoptotic cells. B) Graph showing percentage apoptotic cells. $^{\kappa}$ $p<0.05$ as compared to mock transfected control, $^{\$}$ $p<0.05$ as compared to cells over-expressing Notch 1 IC (student's t test).

**Figure 4.10 Effect of Notch 1 on Serum Deprivation-induced Apoptosis in RVSMC**

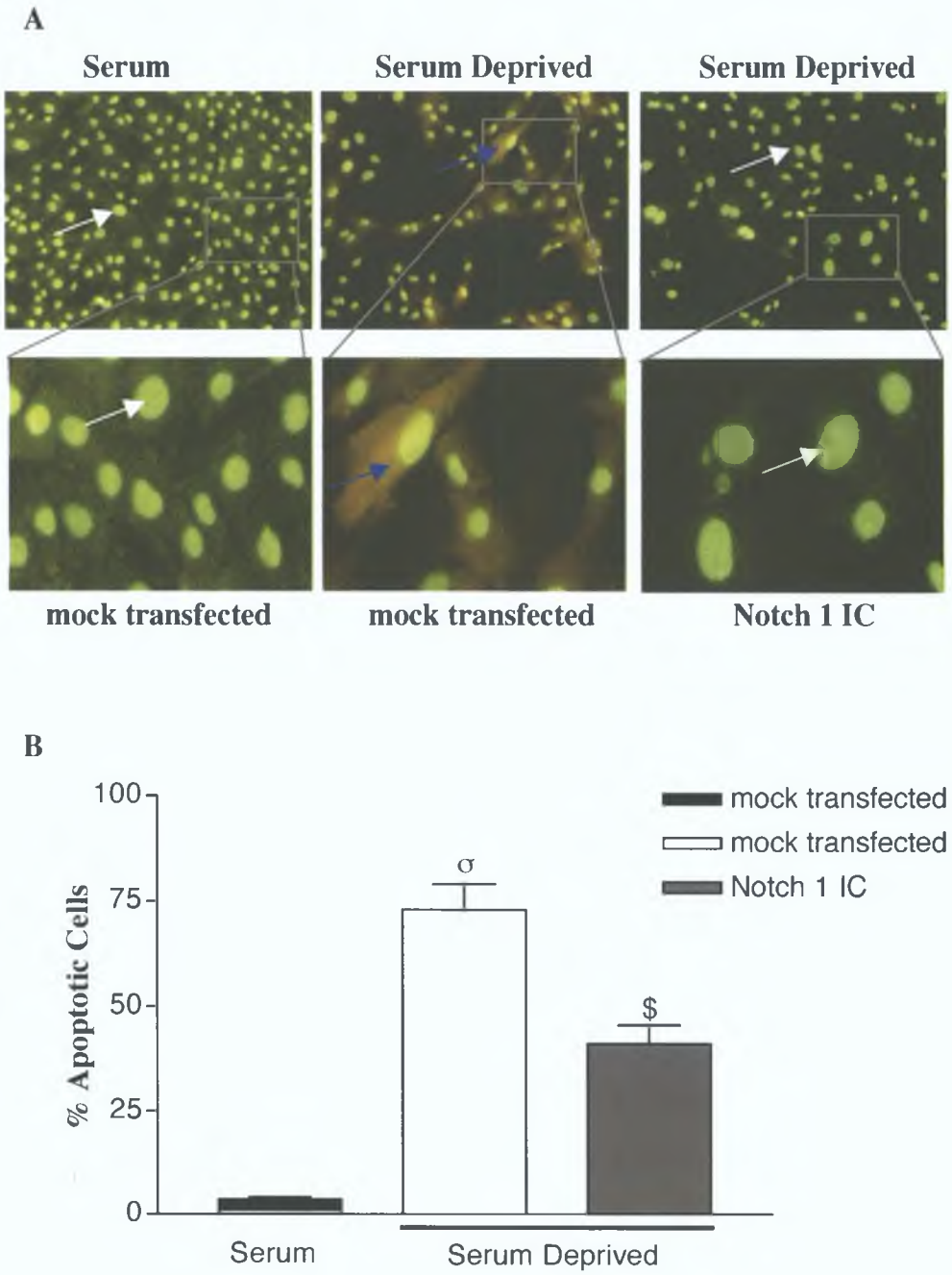



**Figure 4.10 Effect of Notch 1 on serum deprivation-induced apoptosis in RVSMC.** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 1 IC as indicated. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h). RVSMC were serum deprived as indicated for 72 h. Cells were stained with the Acridine Orange/Ethidium Bromide dual stain and viewed under a fluorescent microscope, n=3. A) Representative images; White arrows, viable cell; Blue arrow, apoptotic cell. B) Graph showing percentage apoptotic cells. $^{\kappa}$ $p<0.05$ as compared to mock transfected serum control, $^{\$}$ $p<0.05$ as compared to mock transfected serum deprived cells (student's t test).

**Figure 4 11 Effect of Notch 1 Over-expression on Serum Deprivation-induced Apoptosis in RVSMC**

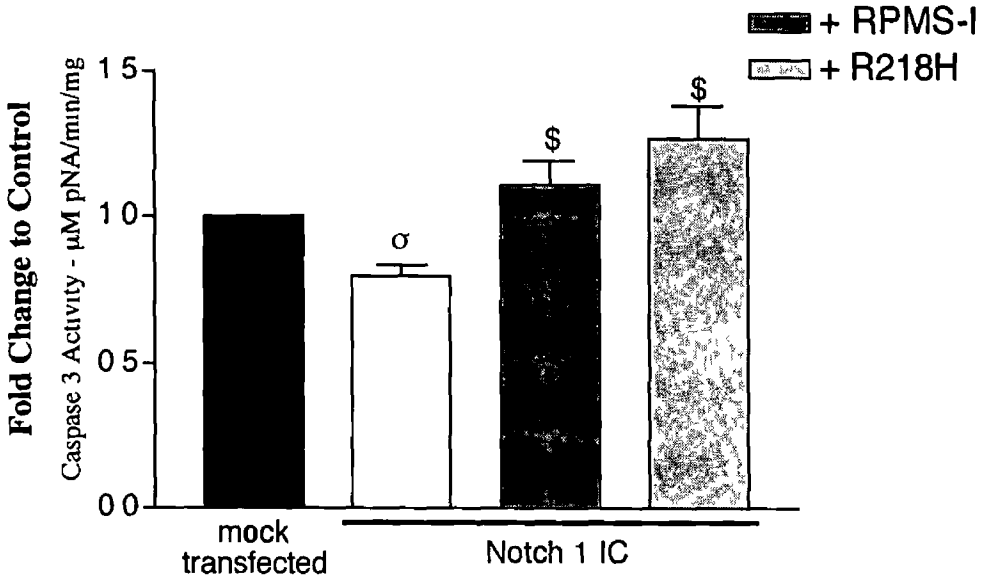



**Figure 4 11 Effect of Notch 1 on serum deprivation-induced apoptosis in RVSMC** RVSMC were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV, Notch 1 IC or the Notch inhibitors RPMS-I or R218H, as indicated Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 µg/ml, 48 h) RVSMC were serum deprived for 6 h Protein was isolated and assayed for caspase 3 activity Caspase assays were normalized to protein levels, and expressed as fold increase over control (= the value obtained with mock transfected cells, arbitrarily assigned a value of 1), n=3 $^{\sigma}$ p<0 05 as compared to mock transfected control, $^{\$}$ p<0 05 as compared to cells over-expressing Notch 1 IC (student's t test)

### 4 2 3 Effect of Serum Deprivation on the Notch Signalling Pathway in RVSMC

To elucidate an explanation for serum deprivation-induced apoptosis in RVSMC, and to further confirm the anti-apoptotic effect of the Notch signalling pathway on such apoptosis, the effect of serum deprivation on components of the Notch signalling pathway was examined

Expression of *notch 3* was determined using quantitative real time PCR analysis at 6 h, 24 h, and 72 h post-serum deprivation, and compared to cycling control cells at each timepoint (Figure 4 12A) Serum deprivation resulted in a decrease in *notch 3* mRNA expression levels at 6 h, 24 h, and 72 h post-serum deprivation by 48 0±3 0%, 46 0±2 0% and 37 0±7 0% respectively as compared to control cycling cells The decrease evident at 6 h and 24 h post-serum deprivation was significant with respect to cycling control cells A corresponding serum deprivation-induced decrease in Notch 3 IC expression was also observed in RVSMC (Figure 4 12B) Serum deprivation significantly decreased Notch 3 IC protein expression by 48 0±11 0% as compared to control cycling cells In addition, serum deprivation resulted in a decrease in Notch target gene and Notch ligand mRNA expression (Figure 4 12C) Serum deprivation for 6 h resulted in a significant decrease in both *notch 1* (41 0±5 0%) and *hrt-2* (40 0±14 0%) mRNA expression compared to serum control mRNA expression levels Similar significant decreases in *hes-5* (31 0±19 0%) and *jagged* (35 0±13 0%) expression levels was also noted

The effect of serum deprivation on CBF-1-dependent promoter activity in RVSMC was also investigated RVSMC were co-transfected with a CBF-1 luciferase-tagged reporter plasmid and Notch 1 IC, and, following overnight recovery from transfection, cells were maintained in either serum containing or serum deprived media as indicated (Figure 4 13) A pattern of CBF-1 activity reminiscent of that due to serum stimulation (Figure 3 13) was evident in cycling cells, and can perhaps be attributable to exposure of the cells to fresh serum Serum deprivation resulted in a decrease in CBF-1 activity at each timepoint, with respect to its serum-containing counterpart A significant decrease of 34 3±4 5% was evident 4 h post serum deprivation, with similar levels of inhibition of 37 0±20 6% and 22 3±39 0% seen due to serum deprivation at 8 h and 24 h (Figure 4 13)

### 4.2.4 Effect of the Notch Signalling Pathway on *NFκB* Promoter Activity in RVSMC

Interactions between the Notch signalling pathway, EBV-related proteins, and the NFκB pathway have been demonstrated (Wang *et al*, 2001, D'Souza *et al*, 2004) in many cell types, but not VSMC Therefore, we investigated whether the components of the Notch signalling pathway or EBNA-2 could transactivate a luciferase tagged NFκB reporter plasmid in VSMC

RVSMC were transfected with the luciferase-tagged *NFκB* reporter plasmid, and co-transfected with either p7pCMV, the known NFκB inhibitor, IκB, Notch 1 IC, Notch 3 IC or EBNA-2 (Figure 4 14) The baseline level of transactivation of the NFκB plasmid in the presence of serum was high, and arbitrarily assigned a value of 1 Co-transfection of IκB resulted in a significant attenuation of 79 0±2 0% in basal NFκB activity in VSMC Similarly, both Notch 1 IC and Notch 3 IC resulted in a significant attenuation of 69 0±14 0% and 72 0±7 0% respectively in basal NFκB activity Transfection of EBNA-2 however, did not affect the level of NFκB activity in VSMC

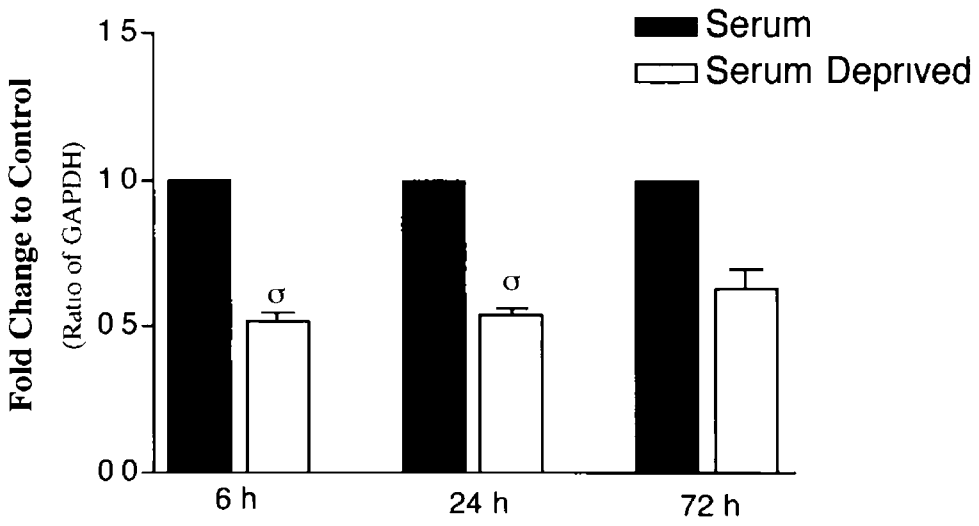
### 4 2.5 Effect of the Notch Signalling Pathway on *bfl-1* Promoter Activity in RVSMC

It has previously been shown that ectopic expression of the anti-apoptotic Bfl-1 protein can protect a Burkitts lymphoma cell line from serum deprivation-induced apoptosis *in vitro* (D' Souza *et al*, 2000) Additionally it has been shown that EBNA-2 can up-regulate *bfl-1* mRNA expression levels, and regulate *bfl-1* promoter activity in a Burkitts lymphoma cell line through interaction with components of the cellular Notch signalling pathway (D'Souza *et al*, 2004) Therefore, this study addressed the question whether components of the Notch signalling pathway activated the *bfl-1* promoter in RVSMC

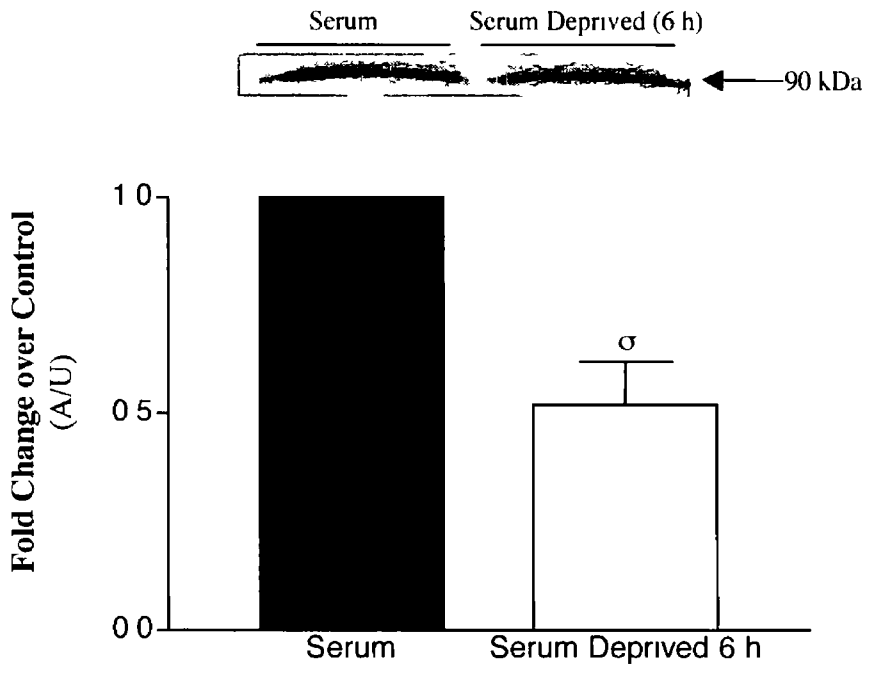
RVSMC were transfected with the luciferase-tagged *bfl-1* promoter, and co-transfected with either p7pCMV, Notch 1 IC, Notch 3 IC or EBNA-2 (Figure 4 15) Neither Notch 1 IC (0 99±0 26) nor Notch 3 IC (0 97±0 34) transfected cells showed any fold change in *bfl-1* luciferase activity with respect to mock transfected cells (arbitrarily assigned a value of 1) EBNA-2 transfection, however, resulted in a 3 13±1 2 fold increase in *bfl-1* luciferase activity compared to mock transfected control

**Figure 4 12 Effect of Serum Deprivation on the Notch Signalling Pathway in RVSMC**

**A Notch 3 mRNA Expression**




**B Notch 3 Protein Expression**



C

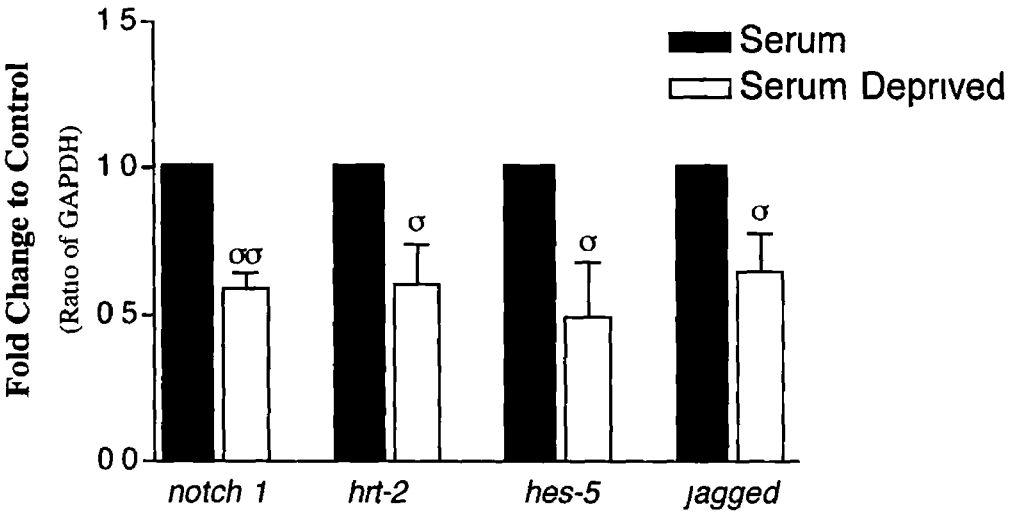



**Figure 4 12 Effect of serum deprivation on the Notch signalling pathway in RVSMC** A) RVSMC were maintained in serum containing media (10% FCS) or serum deprived media (0% FCS) for 6 h, 24 h or 72 h as indicated mRNA levels were measured using quantitative RT-PCR for Notch 3, and expressed as fold increase over control (= the levels of target gene present in cells exposed to serum containing media at each timepoint, arbitrarily assigned a value of 1) Representative experiment, assayed in triplicate B) RVSMC were maintained in serum containing media (10% FCS) or serum deprived media (0% FCS) for 6 h, assayed for Notch 3 protein expression, and expressed as fold increase over control (= the level Notch 3 protein in cells exposed to serum containing media, arbitrarily assigned a value of 1), n=2 C) RVSMC were maintained in serum containing or serum deprived media for 6 h as indicated mRNA levels were measured using quantitative RT-PCR for Notch 1, Notch 3, HRT-2, Hes-5 and Jagged, and expressed as fold increase over control (= the levels of target gene present in cells exposed to serum containing media, arbitrarily assigned a value of 1), n=3 $^{\sigma}$ $p<0\,05$, $^{\sigma\sigma}$ $p<0\,005$ as compared to serum control (student's t test)

**Figure 4 13 Effect of Serum Deprivation on the Notch Signalling Pathway Activity in RVSMC**

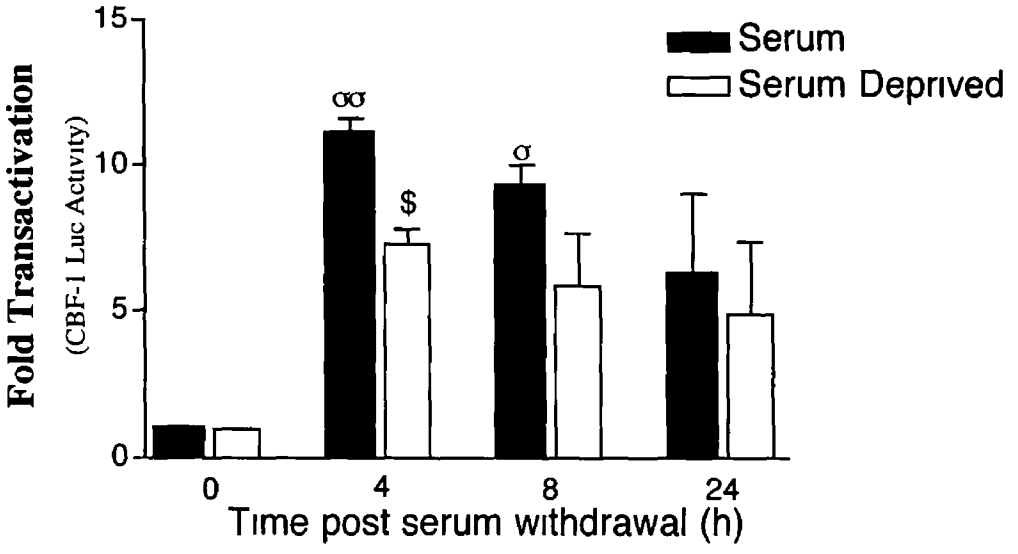



**Figure 4 13 Effect of serum deprivation on the Notch signalling pathway activity in RVSMC** RVSMC were transiently transfected with the CBF-1 luciferase-tagged reporter plasmid and Notch 1 IC (B) Following overnight recovery from transfection, cells were maintained in serum containing or serum deprived media as indicated (0 h) Luciferase assays were normalized to β-galactosidase activities and protein levels, n=2, and expressed as fold increase over control (= the value obtained with transfected cells at 0 h arbitrarily assigned a value of 1) $^{\sigma}$ $p<0\ 05$, $^{\sigma\sigma}$ $p<0\ 005$ as compared to 0 h serum control, $^{\$}$ p,0 05 as compared to serum control at that timepoint (student's t test)

**Figure 4 14 Effect of Notch Signalling Pathway Activation on *NFκB* Promoter Activity in RVSMC**

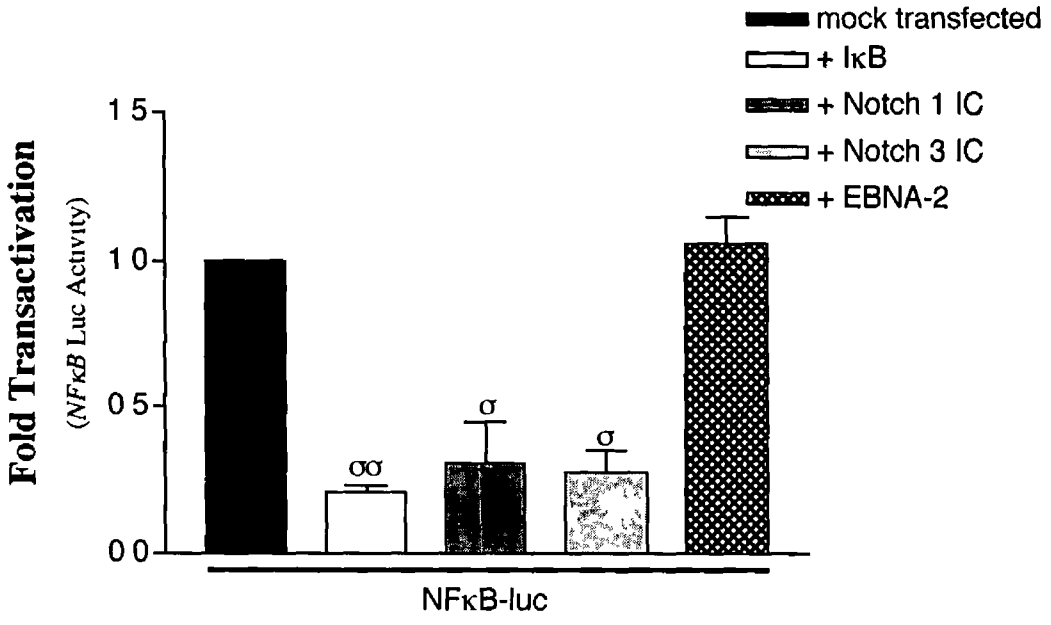



**Figure 4 14 Effect of Notch signalling pathway activation on *NFκB* promoter activity in RVSMC** RVSMC were transiently transfected with the *NFκB* reporter plasmid and co-transfected with either Notch 1 IC, Notch 3 IC or EBNA-2 Following overnight recovery from transfection, protein samples were isolated and assayed for luciferase activity Luciferase assays were normalized to β-galactosidase activities and protein levels, n=2, and expressed as fold increase over control (= the value obtained with *NFκB* reporter plasmid transfected cells arbitrarily assigned a value of 1), $^{\sigma}$ $p<0\ 05$, $^{\sigma\sigma}$ $p<0\ 005$ as compared to *NFκB* reporter plasmid transfected cells (student's t test)

**Figure 4 15 Effect of Notch Signalling Pathway Activation on *bfl-1* Promoter Activity in RVSMC**

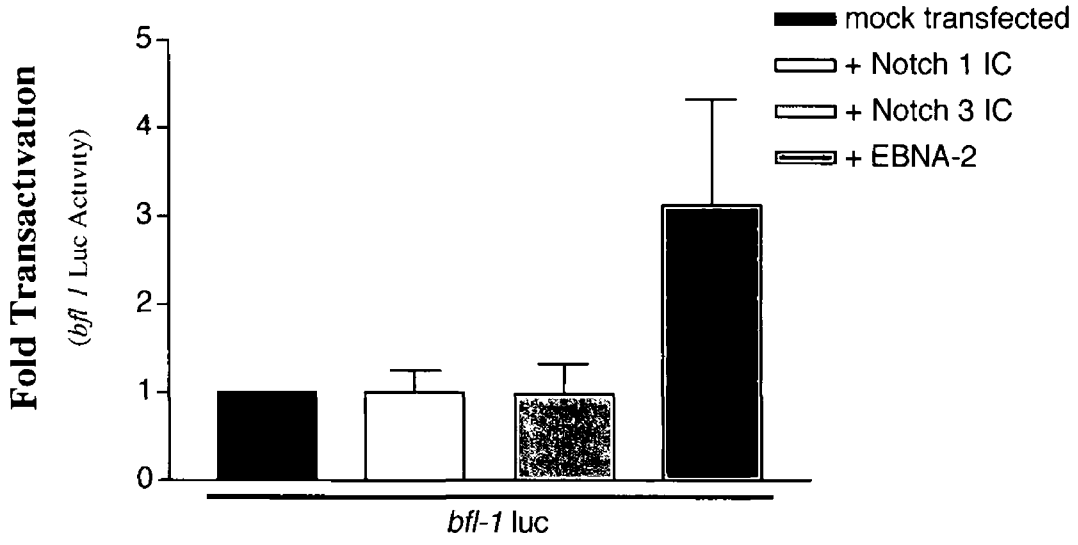



**Figure 4 15 Effect of Notch signalling pathway activation on *bfl-1* promoter activity in RVSMC** RVSMC were transiently transfected with the *bfl 1* reporter plasmid and co-transfected with either Notch 1 IC, Notch 3 IC or EBNA-2 Following overnight recovery from transfection, protein samples were isolated and assayed for luciferase activity Luciferase assays were normalized to κ-galactosidase activities and protein levels, n=3, and expressed as fold increase over control (= the value obtained with *bfl 1* reporter plasmid transfected cells arbitrarily assigned a value of 1)

## 4 3 Discussion

This study clearly establishes the anti-apoptotic effect of the Notch signalling pathway in VSMC, and proves that this anti-apoptotic effect is, at least in part, CBF-1-dependent

Inhibition of the Notch signalling pathway clearly increases apoptosis in cycling VSMC, suggesting that Notch exerts a net anti-apoptotic effect in these cells This concurs with the anti-apoptotic role of the Notch signalling pathway established in many other cell types (Kaneta *et al*, 2000, MacKenzie *et al*, 2004), and confirms the addition of VSMC to this list In concurrence with this study, Wang *et al*, (2002b) recently published a report citing that Notch 3 IC promotes VMSC survival in response to the pro-apoptotic FasL, through induction of cFLIP Contrary to this study however, they find that Notch 3 inhibits VSMC apoptosis in a CBF-1-independent manner, whereas we have established a CBF-1-dependent anti-apoptotic effect of the Notch signalling pathway It is likely, therefore, that Notch can inhibit apoptosis in both a CBF-1-dependent and -independent manner in VSMC To concur with this theory, a recent report details that Notch 4 inhibits apoptosis in both a CBF-1-dependent and -independent manner in EC (MacKenzie *et al*, 2004) The method of execution of Notch signalling pathway-mediated events in VMSC is, to date, largely undefined Several reports suggest that Notch, in other cell types, may interact with several signalling pathways, some of which are involved in the regulation of apoptosis (Jang *et al*, 2004) Wang *et al*, for example, established the involvement of the ERK/MAPK pathway in Notch-mediated cell survival in VSMC, as the induction of cFLIP occurred in an ERK/MAPK-dependent manner This suggests methods through which the anti-apoptotic effect of the Notch signalling pathway in VMSC could be mediated, and will be discussed in further detail later

This study also demonstrates that serum deprivation induces apoptosis in VSMC This has been widely demonstrated in many cell types (Pollman *et al*, 1999b, Shichiri *et al*, 2000), including VSMC (Wang *et al*, 2002b) In addition, we also present the novel finding that both Notch 1 IC and Notch 3 IC act to protect VSMC against serum deprivation-induced apoptosis A similar anti-apoptotic effect of Notch 1 has been recently demonstrated in serum deprived arterial EC (Liu *et al*, 2003) This report found that both Notch 1 IC and Hes-1 conferred a strong resistance to serum deprivation-induced apoptosis in EC, indicating that these components of the Notch signalling pathway play a role in regulating EC survival The function of the Notch

signalling pathway has oftentimes been demonstrated as being cell type specific (Yang *et al*, 2004) We have demonstrated that Hes-1 is not a primary Notch target gene in VSMC, therefore it is unlikely that this target gene plays a major role in the Notch-mediated anti-apoptotic effect in VSMC

Notch 1 IC and Notch 3 IC appear to exert a similar level of resistance to apoptosis in all assays It is interesting to note that the attenuation of serum deprivation-induced apoptosis by Notch 3 IC, measured by the annexin V/propidium iodide dual stain, is only partially reversed with the CBF-1 inhibitors, RPMS-I and mutant Notch 1 IC (Figure 4 6) This suggests that the anti-apoptotic effect of Notch 3 IC may occur in both a CBF-1-dependent and -independent manner, perhaps due to interaction with the ERK/MAPK pathway, as shown by Wang *et al*, (2002b) However, the Notch 3 IC-induced decrease in caspase 3 activity is completely reversed by RPMS-I, suggesting that (i) the caspase 3-mediated aspect of serum deprivation-induced apoptosis is CBF-1-dependent, and (ii) that any anti-apoptotic effect mediated in a CBF-1-independent manner is also caspase 3-independent In contrast, the anti-apoptotic effect mediated by Notch 1 is completely reversed using CBF-1 inhibitors in all assays studied, suggesting that Notch 1 regulates apoptosis in a CBF-1-dependent, and not -independent, manner

The mechanisms of serum deprivation-induced apoptosis are complex and are, to date, poorly defined As we have reported that both inhibition of the Notch signalling pathway and serum deprivation induce apoptosis in VSMC, we investigated the effect of serum deprivation on Notch signalling pathway component expression This study reports the novel finding that serum deprivation results in a decrease in expression of Notch signalling pathway components, and also causes a decrease in CBF-1 reporter activity The mechanism of this serum deprivation-induced decrease in Notch signalling pathway components and activity in VSMC remain to be defined, however, as mentioned previously, several serum mitogens have been shown to influence expression of Notch signalling pathway components in both vascular and non-vascular cells (Artavanis-Tsakonas *et al*, 1999, Nickoloff *et al*, 2002, Liu *et al*, 2003)

Whilst the mechanism of Notch-mediated cell survival is not immediately apparent, there are numerous possible anti-apoptotic pathways whereby Notch could regulate apoptosis in VSMC These include interaction with, or regulation of expression of the Bcl-2 family, the NFκB family, c-myc, p53, or regulation of JNK, PI3K or MAPK activity It is possible that Notch interacts with one or all of these components, and interaction may depend on cellular context, for example, the method

of apoptosis induction Whilst in-depth investigation into the interaction of the Notch signalling pathway with all of these anti-apoptotic mechanisms is beyond the scope of this project, this study investigates the interaction of the Notch signalling pathway with the members of the Bcl-2 and NFκB families in VSMC In addition, this study presents evidence from current literature that could rationalize further investigative work within the laboratory into Notch signalling pathway interactions with other known anti-apoptotic pathways, and will be discussed further in Chapter 7

The NFκB signalling pathway plays a central role in many vascular diseases, including arteriosclerosis, restenosis, and reperfusion injury The beneficial and usually transient effect of this pathway may be exaggerated in pathological situations within the vasculature, resulting in damage to the vessel wall and impaired vascular cell function (De Martin *et al*, 2000) NFκB generally mediates cell survival signals and opposes apoptotic processes by regulating the expression of anti-apoptotic proteins Interactions between the Notch signalling pathway, EBV-related proteins, and the NFκB pathway have been demonstrated (Wang *et al*, 2001, D'Souza *et al*, 2004) Wang *et al*, have shown that Notch 1 inhibits NFκB activity in T-cells through physically interacting with the p50 subunit of NFκB and exerting the same effect as an IκB molecule Additionally, a recent paper by Oakley *et al*, (2003) reports that expression of the NFκB inhibitor, IκBα, is under the control of CBF-1 and subsequently Notch 1 in both liver and kidney cells CBF-1 is reported to interact with a dual NFκB/CBF-1 binding site (κB2 site) in the IκBα promoter, repressing IκBα expression resulting in increased levels of NFκB activity Over-expression of Notch 1 IC is reported to reverse the repressive effects of CBF-1 on IκBα expression, thus decreasing NFκB activity within the cell The effect of Notch 1 is CBF-1-dependent, as over-expression of Notch 1 IC without the CBF-1 binding RAM domain, or Notch 1 IC in the presence of the CBF-1 inhibitor, R218H-RBPJκ, does not result in increased IκBα expression As this mechanism is conserved across kidney and liver cells, it is likely that Notch interacts with the NFκB pathway in VSMC in a similar manner

**Figure 24 Model for the Regulation of NFκB Activity by CBF-1 and Notch**

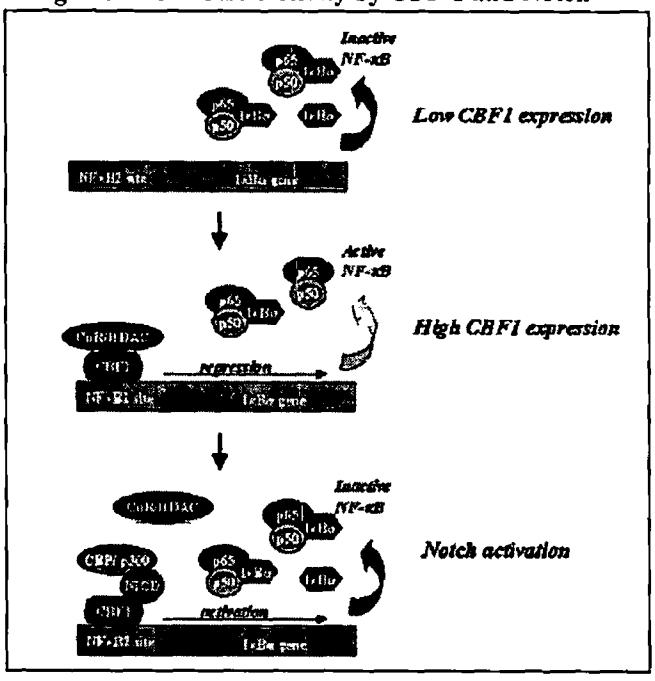



**Figure 24** *Top panel* Under conditions of low CBF-1 expression the IκBα gene is transcribed in a de-repressed state, resulting in excess IκBα protein, and low levels of NFκB activity *Middle panel* Under conditions of high CBF-1 expression, CBF-1 binds to the κB2 site of the IκBα promoter, recruits co-repressor complexes, resulting in repression of IκBα transcription, and increased NFκB activity *Bottom panel* Activated Notch 1 IC stimulates release of the co-repressor complex, activates CBF-1, and recruits the transcriptional co-activator CBP/p300, resulting in activation of IκBα gene transcription, and reduced NFκB activity (Oakley *et al*, 2003)

We provide previously unreported evidence that both Notch 1 IC and Notch 3 IC inhibit NFκB activity in VSMC, whereas EBNA-2 does not affect the activity of the NFκB signalling pathway in these cells This is supported by the observations that both Notch expression is decreased, and NFκB expression increased in arterial VSMC following balloon injury (De Martin *et al*, 2000, Wang *et al*, 2002a) Whilst our results concur with the effect of Notch expression on NFκB expression in T-cells, it is somewhat surprising as the Notch signalling pathway has been implicated in cell survival in VSMC However, whilst the NFκB is generally considered to exert an anti-apoptotic effect in VSMC, inhibition of this pathway by IκB resulted in apoptosis in low- but not high-density cultures (De Martin *et al*, 2000) It is interesting to note that a similar bi-functional effect was documented on the regulation of VSMC growth due to Notch 3 IC over-expression (Campos *et al*, 2002) This study revealed a decrease in cell accumulation in low-density cultures, and a lack of growth arrest in high-density cultures As Notch exerts a differential effect on the proliferation pathway in VSMC, it is possible that a similar differential effect is also exerted on the NFκB pathway Jagged

2 expression results in a rapid and transient activation of the NFκB family in murine erythroleukaemia cells, followed by an inhibition of the pathway (Jang *et al*, 2004) The activation of the NFκB pathway occurred 30 min following Notch signalling pathway activation, however this effect was reversed, and NFκB activity dramatically down-regulated 4 h post-Notch activation The crosstalk between the Notch and NFκB pathways appears to be bi-directional, as NFκB up-regulates Jagged 1 expression in T-cells (Robey 1999) The interaction between the Notch and NFκB pathways in VSMC, and the resulting effects on apoptosis, remain incompletely defined This study confirms an interaction between these two pathways in VSMC, thus providing a basis upon which further research can be established

In an attempt to elucidate the mechanisms through which the Notch signalling pathway exerts its 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 and EBNA-2 have been shown to regulate members of the Bcl-2 family in both vascular and non-vascular cells Notch 1 and Notch 4 up-regulate Bcl-2 expression in T-cells and EC respectively, whereas EBNA-2 has been shown to up-regulate Bid in B cells (Deftos *et al*, 1998, MacKenzie *et al*, 2004, Schlee *et al*, 2004) In addition, EBNA-2 up-regulates *bfl-1* mRNA expression and promoter activity in a BL cell line in a CBF-1-dependent manner *(P Pegman personal communication)* This study established that EBNA-2, but neither Notch 1 IC nor Notch 3 IC, activated the *bfl-1* promoter in VSMC, suggesting that the anti-apoptotic of Notch is not mediated through Bfl-1 in VSMC

As the Bcl-2 family is central to the regulation of apoptosis in many cell types, and changes in Bcl-2 family expression are oftentimes observed during the pathogenesis of vascular disease, it is likely that the Notch signalling pathway regulates at least some members of the Bcl-2 family Therefore, this study examined the effect of the Notch signalling pathway on mRNA expression of both pro- and anti-apoptotic members of the Bcl-2 family Following inhibition of the Notch signalling pathway, we observed increased pro-apoptotic *bad* and *bax* expression, decreased anti-apoptotic *bcl-x*$_L$ expression but also increased anti-apoptotic *bcl-2* expression Conversely, following Notch 3 IC-mediated stimulation of the Notch signalling pathway increased *bad* and *bcl-2* expression was maintained, whereas decreased *bax* and increased *bcl-x*$_L$ expression was observed The opposing effects of stimulation and CBF-1-dependent inhibition of the Notch signalling pathway on *bax* and *bcl-x*$_L$ suggest that these genes,

but not *bad* or *bcl-2*, are regulated by the Notch signalling pathway in a CBF-1-dependent manner This study has shown that serum-deprivation results in decreased expression of both Notch 1 IC and Notch 3 IC In addition, recent studies within the laboratory have revealed a serum deprivation-induced decrease in Bcl-$x_L$ expression, further suggesting a Notch-mediated regulation of Bcl-$x_L$ expression in VSMC This regulation of *bax* and *bcl-$x_L$* was confirmed in a preliminary experiment examining gene expression in cells in which the Notch signalling pathway was simultaneously activated and inhibited, and compared to control cells The level of both *bax* and *bcl-$x_L$* expression in both sets of cells was very similar, thus confirming CBF-1-dependent Notch 3 regulation of these apoptotic proteins in VSMC In addition, using bioinformatics, this study examined whether any Notch target gene promoter sites are present on either the *bax* or *bcl-$x_L$* genes Nakagawa *et al*, (2000) have determined that the HRT family of Notch target genes preferentially bind to an E box motif, CACGTG, but also bind to other E box motifs (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 have determined that the Notch family HRT target genes can bind to, and therefore possibly directly regulate, both the *bax* and *bcl-$x_L$* genes (using MultAlin) The HRT family can bind to the CACGTG or CATCTG sequences of *bax*, and the CACCTG or CACTTG sequences of *bcl-$x_L$*, however, this would have to be further confirmed with mutational analysis Therefore, it is likely that the HRT family of Notch target genes act as effectors of the Notch signalling pathway, at least in part, by repressing *bax* expression and promoting *bcl-$x_L$* expression, which has been confirmed in additional experiments within the laboratory

This study has provided the basis for further analysis of the mechanism of Notch signalling pathway regulation of *bax* and *bcl-$x_L$* Using siRNA knockdown technology preliminary results indicate that the down-regulation of *hrt-1*, *hrt-2* and *hrt-3* results in increased *bax* expression, with the most significant effect being mediated by *hrt-3* In contrast, down-regulation of *hes-1* results in decreased *bax* expression In addition, targeted inhibition of *hrt-1*, *hrt-2* and *hrt-3* results in a decrease in *bcl-$x_L$* expression in VSMC, whereas *hes-1* down-regulation results in a slight increase in *bcl-$x_L$* expression *(D Morrow personal communication)* This suggests a differential role for members of the HRT and Hes families in mediating VSMC apoptosis, and may partially account for the relatively small fold changes observed in this study, as RPMS-I inhibits both the HRT and Hes families Therefore, this study has provided a solid basis for the

identification of both *bax* and *bcl-$x_L$* as important Notch target genes in the regulation of VSMC apoptosis, which has been confirmed by ongoing research within the laboratory

It is also interesting to note that both *bax* and *bcl-$x_L$* are transcriptional targets for NFκB NFκB meditates up-regulation of *bcl-$x_L$* expression (Lee *et al*, 1999) and inhibition of NFκB results in decreased *bax* expression (de Martin *et al*, 2000) Therefore, activation of the NFκB and Notch signalling pathways both result in a decrease in *bax* and an increase in *bcl-$x_L$* expression However, we have demonstrated that Notch inhibits NFκB activity in VSMC Therefore, we must consider the possibility of an NFκB-independent Notch-mediated regulation of these genes, or the bi-phasic regulation of Notch on the NFκB signalling pathway

The importance of *bax* and *bcl-$x_L$* in regulating VSMC apoptosis is unsurprising as these genes have been implicated in both physiological apoptosis and the pathogenesis of vascular disease, and in some instances their regulation has been coupled to the Notch signalling pathway in other cell types Numerous papers, for example, indicate a specific role for both Bax and Bcl-$x_L$ during both neo-natal and post-injury vascular remodelling (Pollman *et al*, 1999a, Gibbons and Pollman 2000, Kim *et al*, 2000)

Bcl-$x_L$ is an important modulator of intimal and medial VSMC apoptosis, and is a critical determinant of vascular lesion formation Vascular lesion formation is associated with an up-regulation of Bcl-$x_L$ within intimal VSMC, resulting in decreased cellular apoptosis Bai *et al*, (1999), and others (Pollman *et al*, 1998) have demonstrated that down-regulation of Bcl-$x_L$ expression using anti-sense oligonucleotides results in increased intimal VSMC apoptosis and regression of vascular lesions Therefore, it is conceivable that proposed future anti-apoptotic gene-based therapies targeting Bcl-$x_L$ could utilize the demonstrated Notch signalling pathway regulation of this gene Interestingly, Bai *et al*, (1999) also reports increased medial VSMC apoptosis following injury, whilst Perlman *et al*, (1997) reports decreased Bcl-$x_L$ expression in the media of injured arteries To concur with our finding that Notch pathway stimulation up-regulates Bcl-$x_L$ expression, Wang *et al*, (2002a) reports a balloon injury-induced decrease in Notch signalling pathway expression in the same cells In addition, Notch 1 over-expression has recently been shown to preferentially up-regulate Bcl-$x_L$ expression in T-cells (Jang *et al*, 2004)

In addition, a recent study has demonstrated that up-regulation of Bcl-$x_L$ by serum is critical in protecting cells from serum deprivation-induced apoptosis in a

prostate cancer cell line (Yang *et al*, 2003) As we have demonstrated both that serum deprivation decreases and serum stimulation increases Notch expression, it is probable that Bcl-$x_L$ plays a similar anti-apoptotic role in Notch-mediated protection against serum deprivation-induced apoptosis

Bax is also increasingly recognized as an important mediator of apoptosis within the vasculature Physiological apoptosis that occurs in human umbilical vein EC during delivery is associated both with a strong expression of Bax and caspase 3 activation (Kim *et al*, 2000) Bax is also up-regulated in SMC within human fatty streaks, and is up-regulated following coronary occlusion in the rat heart and following myocardial infarction in the human heart (Misao *et al*, 1996, Kockx *et al*, 1998) In addition, it is postulated that Bax over-expression in ventricles of spontaneously hypertensive rats may contribute to apoptosis (Fortuno *et al*, 1998)

In this study, the observed increase in *bcl-2* expression due to Notch inhibition was somewhat surprising, as we have clearly established that inhibition of the Notch signalling pathway results in VSMC apoptosis However, the rationale for this could be that Bcl-2 has been shown to have variable anti-apoptotic effects within a cell, and that this is determined by the ratio of other apoptotic factors within a cell Bcl-2, for example, protects against Fas-mduced apoptosis in some cells but not in others (Yeh 1997) and increased Bcl-2 expression observed in failing hearts is insufficient to overcome death-promoting signals (Hetts 1998) The question of Notch signalling pathway regulation of Bcl-2 expression is complex, and may be cell type specific Notch 1 increases Bcl-2 expression in a thymic lymphoma cell line (AKR1010) but not in a T-cell hybridroma line (2B4 11), however Notch 1 confers resistance to glucocorticoid-induced apoptosis in both cell types Interestingly, a number of recent papers suggest that Notch promotes Bcl-2 expression, and that this is achieved in a CBF-1-independent manner Notch 1 and Notch 4 up-regulate Bcl-2 expression in T-cells and EC respectively (Jang *et al*, 2004, MacKenzie *et al*, 2004) and inhibit induction of apoptosis in the respective cells Notch may therefore up-regulate Bcl-2 expression in VSMC in a CBF-1-independent manner, thus explaining the sustained increase in Bcl-2 expression following inhibition with RPMS-I

## 4 4 Conclusion

This study establishes some of the mechanisms of Notch-mediated cell survival in VSMC It is clear that the Notch signalling pathway can regulate apoptosis, and

indeed other cell fate decisions, through interactions with multiple signalling pathways, which will be discussed in further detail in Chapter 7 Understanding the process of VSMC apoptosis at a molecular level affords insights into cardiovascular pathogenesis, and also opens new avenues for the development of diagnostic, prognostic and therapeutic tools

# Chapter 5

# Results

## Effect of Cyclic Strain on the Notch Signalling Pathway and Apoptosis *in vitro*

## 5 1 Introduction

An important aspect of this study was to investigate the effect of cyclic strain on both the Notch signalling pathway and apoptosis in VSMC, and additionally determine whether the Notch signalling pathway regulates cyclic strain-mediated apoptosis in these cells

Cells of the vasculature are exposed to two principal haemodynamic forces, shear stress and cyclic strain, generated as a result of blood flow Shear stress is described as the dragging frictional force created due to blood flow, and primarily affects EC under normal conditions Arterial blood vessels are additionally exposed to an oscillating transmural pressure due to the pulsatile nature of arterial blood flow Therefore, arterial blood vessels are exposed to a pulse pressure-induced cyclic strain event, resulting in the "stretching" of the vascular cells in multiple planes VSMC, which constitute the major component of the blood vessel wall, absorb most of the pressure-induced cyclic strain Therefore, this study focuses on the effect of alterations in cyclic strain on VSMC As arterial SMC reside in a mechanically active environment and are subjected to variable mechanical loads, the response of these cells to deformation may represent an important defense mechanism against excess mechanical load

Mechanical forces associated with blood flow play an important role in the regulation of vascular tone, vascular remodelling and the maintenance of vascular homeostasis Blood vessels are continuously exposed to mechanical forces which is necessary, for example, to develop and maintain a differentiated and functional VSMC phenotype (Birukov *et al*, 1998) Altered or elevated biomechanical stress is a common feature of vascular disease (Bennett 1999, Xu 2000) Spontaneous atherosclerotic lesions, for example, are prone to form in arterial branching regions where cyclic strain is elevated In addition, many studies have demonstrated that venous vessels do not develop atherosclerosis in their normal low-pressure environment However, accelerated atherosclerosis is observed when veins are grafted to arteries, and the vessels are exposed to increased mechanical forces (Bennett 1999, Mayr *et al*, 2000)

The pathogenic role of VSMC apoptosis is increasingly recognized in cardiovascular disease SMC apoptosis is described as an early event in vein by-pass graft arteriosclerosis, suggesting that biomechamcal forces play a key role in the regulation of apoptosis (Mayr *et al*, 2000) This has repeatedly been demonstrated by

many studies, both *in vivo* and *in vitro* (Pollman *et al*, 1999, Wernig *et al*, 2003), and this study further establishes this fact However, despite the recognized importance of apoptosis in vascular disease, and potential therapeutic implications for the modulation of VSMC apoptosis, relatively little is known about the regulation of VSMC apoptosis by alterations in biomechanical forces This study has clearly established the anti-apoptotic role of the Notch signalling pathway both in cycling VSMC and against serum deprivation-induced apoptosis It is conceivable, therefore, that the Notch signalling pathway regulates apoptosis induced by other means, for example, due to alterations in biomechanical force

Mechanical forces are an important regulator of structure and function in mammalian cells, and have been implicated in phenotypic changes, including alteration of gene expression in VSMC Cyclic strain, for example, has been shown to increase expression of PDGF-$\alpha$ and Integrin $\beta_3$, whilst concomitantly decreasing MMP-1 expression A study by Feng *et al*, (1999) suggests however, that cyclic strain is a highly specific stimulus both *in vivo* and *in vitro*, with response restricted to a small number of genes Of five thousand genes analyzed in this report, for example, only eleven exhibited changes in expression due to cyclic stress Our study investigates whether components of the Notch signalling pathway should be added to the continuously increasing list of cellular components regulated by cyclic strain

VSMC can sense and transduce mechanical stress signals to cellular responses via receptor-dependent and -independent G protein-dependent pathways, as previously discussed in Chapter 1 A recent report by Wernig *et al*, (2003) indicates that cyclic strain-induced apoptosis in VSMC in mediated by $\beta_1$-integrin-rac-p38-p53 signalling pathways However, as is the case with apoptosis induced by other stimuli, it is possible that additional signalling pathways mediate cyclic strain-induced VSMC apoptosis As we have previously identified *bax* and *bcl-x*$_L$ as targets of the Notch signalling pathway, we investigated members of the *bcl-2* family of genes as possible mediators of apoptosis in mechanically stimulated VSMC

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 *in vitro* Unless otherwise stated, cells in this study were exposed to 10% cyclic strain using a heart pulse pressure waveform This is generally accepted as a level of cyclic strain that VSMC are subjected to under normal physiological conditions *in vivo* Cardiovascular disease states, such as hypertension, can increase the level of cyclic strain exerted on arterial VSMC up

to 30% (Li and Xu 2000). Large deformations *in vitro* (>15%) result in transient VSMC injury, similar to observations following experimental balloon injury *in vivo* (Feng *et al.*, 1999).

**Figure 25: Flexercell Tension Plus™ Strain Unit**

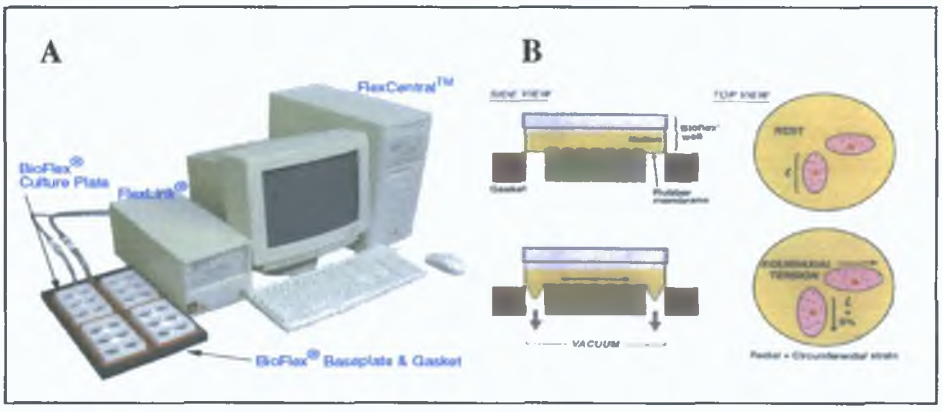



**Figure 25**: A: Flexercell Tension Plus™ Strain Unit is a microprocessor-driven instrument that regulates pressure to BioFlex® culture plates. B: Cartoon detailing the mechanical stretch exerted on cells in vitro due to the Flexercell Tension Plus™ strain unit.

This study provides evidence for a role of the Notch signalling pathway in modulating cyclic strain-induced apoptosis in VSMC. As aberrant regulation of cyclic strain-induced apoptosis plays a pivotal role in many forms of cardiovascular disease states, this has the potential to translate into future therapeutic benefits.

## 5 2 Results

### 5 2 1 Cyclic Strain Induces Apoptosis in RVSMC *in vitro*

The effect of cyclic strain on RVSMC apoptosis *in vitro* was determined using histochemical analysis and caspase 3 activity assays Cyclic strain (10%, 24 h) resulted in a significant increase in annexin V positive (green /apoptotic) cells with respect to static control Representative histochemical analysis are shown in Figure 5 1A, and cumulative data on the percentage of apoptotic (annexin V positive/propidium iodide negative cells) are also presented (Figure 5 1B) Static cells exhibit a low level of apoptotic cells (6 1±1 22%), which is significantly increased, as result of exposure to cyclic strain, to 15 6±2 6% This increase in apoptosis in RVSMC due to cyclic strain was further demonstrated using the acridine orange/ethidium bromide dual stain (Figure 5 2) Cyclic strain significantly increased the percentage of apoptotic cells from 8 25±1 25% in the static control sample to 30 75±8 98% (Figure 5 2B) In addition, cyclic strain-induced apoptosis was confirmed through caspase 3 activity assays (Figure 5 3) Cyclic strain resulted in a significant fold increase in caspase 3 activity to 1 66±0 15 with respect to static control (arbitrarily assigned a value of 1), which corresponds to a 66 0±15% increase The effect of cyclic strain on the *bcl-2* family of apoptotic genes was also investigated (Figure 5 4) Cyclic strain increased the expression of the pro-apoptotic *bad* and *bax* genes by 41 0±28 0% and 31 0±8 0% respectively over unstrained control cells An increase of 68 0±41 0% in *bcl-2* gene expression was also observed in VSMC following cyclic strain, as compared to static control cells In contrast, cyclic strain resulted in a decrease of 29 0±17 0% in expression of the anti-apoptotic *bcl-$x_L$* gene The pattern of *bcl-2* family gene expression is in agreement with our finding that cyclic strain induces apoptosis in VSMC, as the progression of apoptosis is determined by the relative concentration of both pro- and anti-apoptotic mediators within a cell These results suggest that the balance of *bcl-2* family gene expression favours apoptosis following cyclic strain

**Figure 5.1 Cyclic Strain Increases Apoptosis in RVSMC, as measured by the Annexin V/Propidium Iodide Dual Stain**

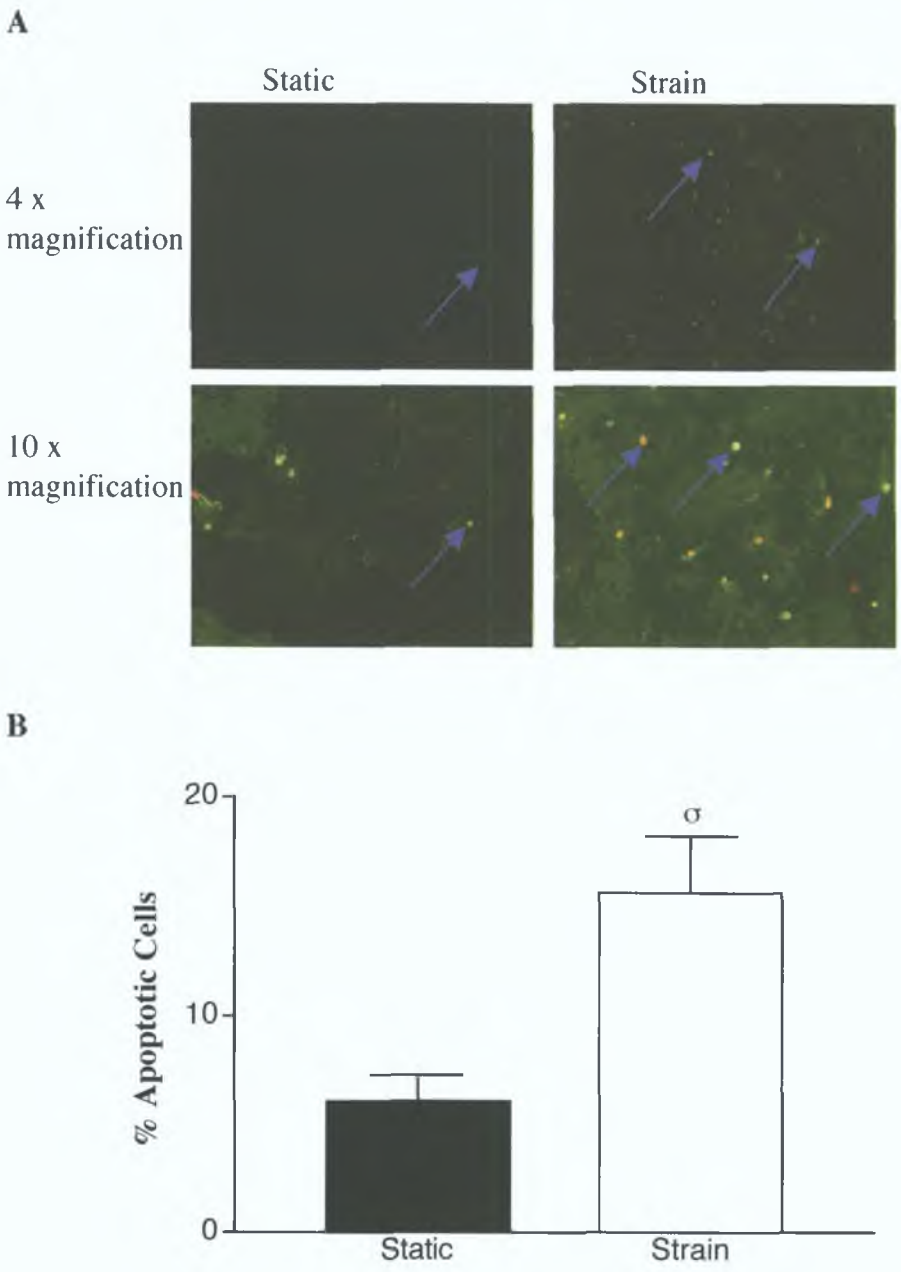
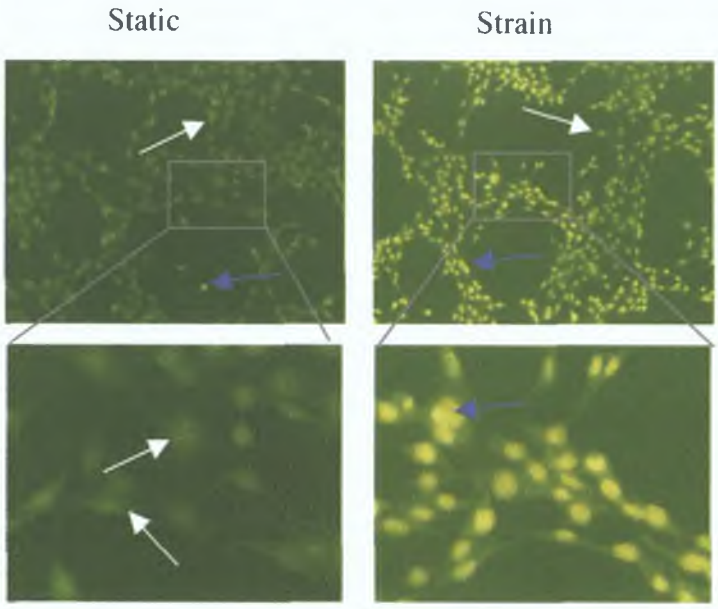



**Fig 5.1 Cyclic strain induces apoptosis in RVSMC.** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well. Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h). Cells were stained with the Annexin V/Propidium Iodide dual stain and viewed under a fluorescent microscope. A) Representative images; Apoptotic cell, blue arrow. B) Graph showing percentage increase in the number of apoptotic cells due to cyclic strain. Each experiment was performed in duplicate, with the graph representative of n=2. $^{\beta}$ $p<0.05$ as compared to static control (student's t test).

**Figure 5.2 Cyclic Strain Increases Apoptosis in RVSMC, as measured by the Acridine Orange/ Ethidium Bromide Dual Stain**

A




B

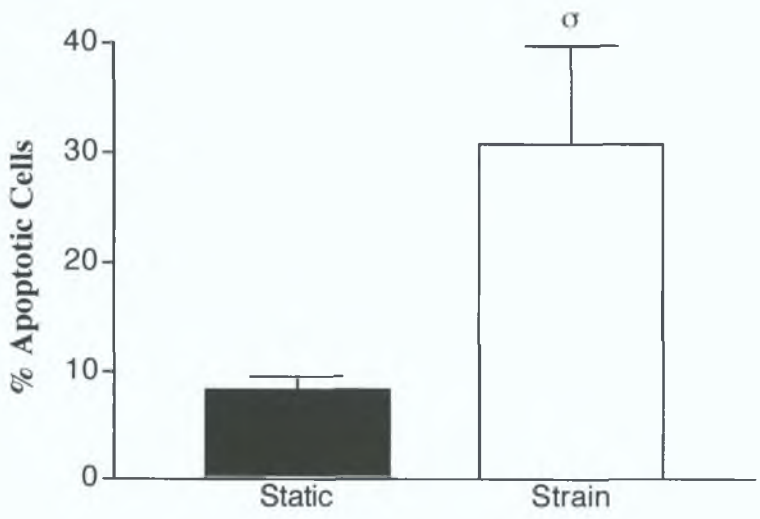



**Fig 5.2 Cyclic strain induces apoptosis in RVSMC.** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well. Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h). Cells were stained with the Acridine Orange/ Ethidium Bromide dual stain and viewed under a fluorescent microscope. A) Representative images; Viable cell, white arrow; Apoptotic cell, blue arrow. B) Graph showing percentage increase in the number of apoptotic cells due to cyclic strain. Each experiment was performed in duplicate, with the graph representative of n=3. $^{\beta}$ $p<0.05$ as compared to static control (student's t test).

**Figure 5 3 Cyclic Strain Increases Apoptosis in RVSMC, as measured by Caspase 3 Activity**

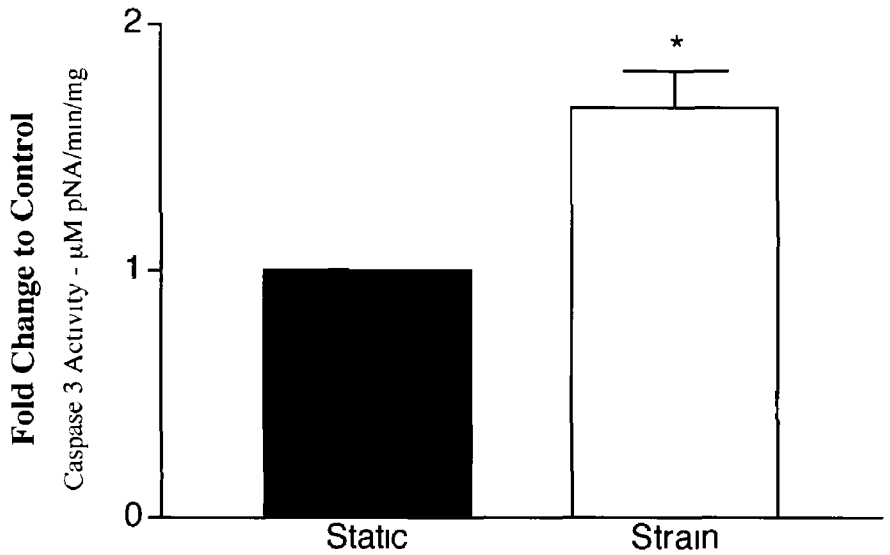



**Fig 5 3 Cyclic strain induces apoptosis in RVSMC** RVSMC were seeded onto Flexercell$^{TM}$ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) Protein was isolated and assayed for caspase 3 activity Caspase assays were normalized to protein levels, and expressed as fold increase over control (= the value obtained with static cells arbitrarily assigned a value of 1), n=6, * $p< 0\ 05$ (rank test) as compared to static control

**Figure 5 4 Effect of Cyclic Strain on Bcl-2 Family mRNA Expression**

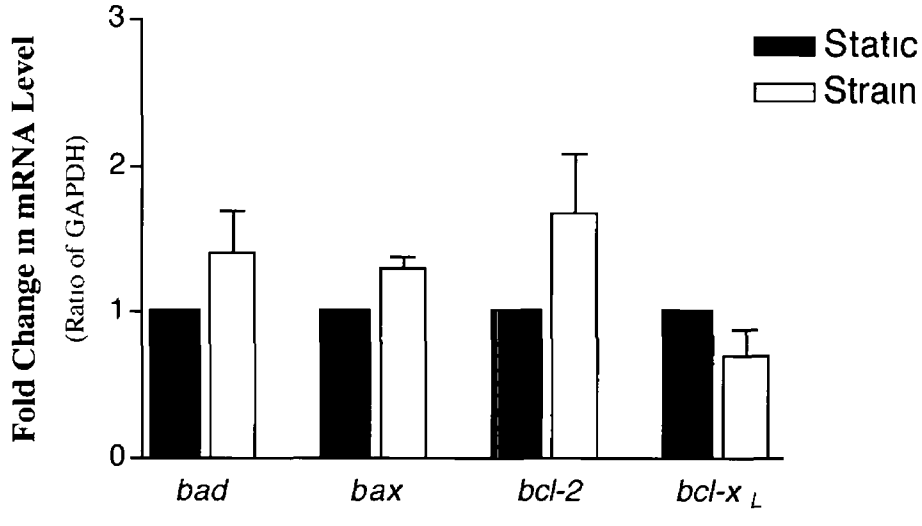



**Fig 5 4 Effect of cyclic strain on Bcl-2 family mRNA expression in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) as indicated Samples were isolated at 24 h post-cyclic strain and subsequently assayed using quantitative PCR for *bad*, *bax*, *bcl-2* and *bcl* $x_L$ mRNA expression as indicated All values were normalized to GAPDH levels, and expressed as a fold change over static control (= the value obtained with static RVSMC mRNA levels, arbitrarily assigned a value of 1), n=3

### 5 2 2 The Effect of Cyclic Strain on Components of the Notch Signalling Pathway in RVSMC

The effect of cyclic strain on components of the Notch signalling pathway was determined in this study by western blot analysis, immunocytochemistry, and quantitative real time PCR analysis Cells were exposed to 10% pulsatile cyclic strain, using the Flexercell™ Strain Unit, for 24 h, unless otherwise stated, and compared to non-strained, static control

Exposure of RVSMC to cyclic strain resulted in a decrease in the level of Notch receptor, ligand and target gene protein expression Notch 1 IC and Notch 3 IC protein expression levels were significantly reduced by 17 0±2 0% (Figure 5 5A) and 25 0±3 0% (Figure 5 5B) respectively as compared to static control expression levels Similarly, exposure to cyclic strain resulted in a significant decrease of 30 0±6 0% in Jagged protein (Figure 5 5C) as compared to static control expression levels The effect of cyclic strain on HRT-1 and HRT-3 protein expression levels in RVSMC was also determined (Figure 5 6) Cyclic strain induced a small but significant decrease in HRT-1 protein expression levels (12 0±1 0%), with a similar decrease in protein expression in evidence for HRT-3 (11 0±2 0%) as compared to static control cells Immunochemical analysis further confirmed the cyclic strain-induced decrease in HRT-1 protein expression (Figure 5 7)

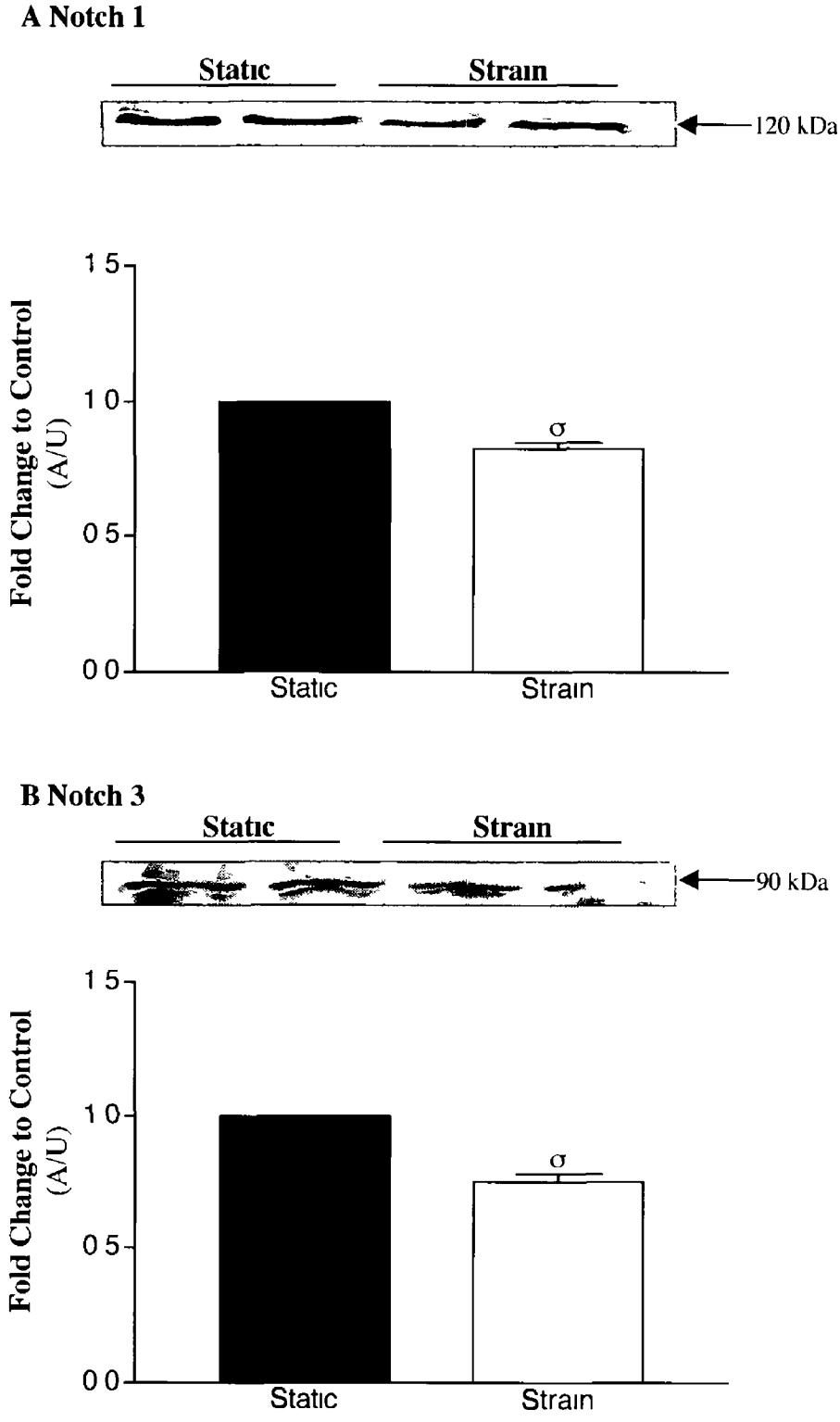
The effect of cyclic strain on the components of the Notch signalling pathway was further confirmed by quantitative real time PCR analysis Cyclic strain caused significant decreases in *notch 1* and *notch 3* mRNA expression levels of 57 0±11 0% and 50 0±2 9% with respect to static control (Figure 5 8A) Similarly, a significant decrease of 35 0±4 0% in *jagged* mRNA levels was also observed due to cyclic strain (Figure 5 8B) The cyclic strain-induced decrease in Notch signalling pathway mRNA expression levels was also evident in analysis of Notch target gene mRNA expression (Figure 5 9) *Hrt* mRNA expression levels were significantly reduced by 39 0±1 0%, 47 0±17 0% and 42 0±12 0% with respect to static control for *hrt-1*, *hrt-2* and *hrt-3* respectively (Figure 5 9A) Similarly, exposure of the cells to cyclic strain resulted in a significant decrease in *hes* 1 (42 0±12 0%) and *hes-5* (57 0±11 0%) mRNA expression levels as compared to static control (Figure 5 9B)

In order to determine whether the effect of cyclic strain on the Notch signalling pathway was force-dependent, RVSMC were subjected to 0, 5, 10 and 15% cyclic strain

and assayed for Notch receptor and Notch target gene mRNA expression (Figure 5 10) Both *notch 3* and *hes-5* exhibit 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 (Figure 5 10A) 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 (Figure 5 10B)

A similar experiment was carried out to determine the effect of time on the cyclic strain-induced changes in the Notch signalling pathway (Figure 5 11) RVSMC were exposed to 10% cyclic strain over a period of 24 h, with timepoints taken at 0 h, 4 h, 8 h, and 24 h post-cyclic strain Quantitative real time PCR analysis revealed a significant time-dependent decrease in *hes-5* mRNA expression, 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)

**Figure 5 5 Effect of Cyclic Strain on Notch Receptor and Notch Ligand Protein Expression in RVSMC**



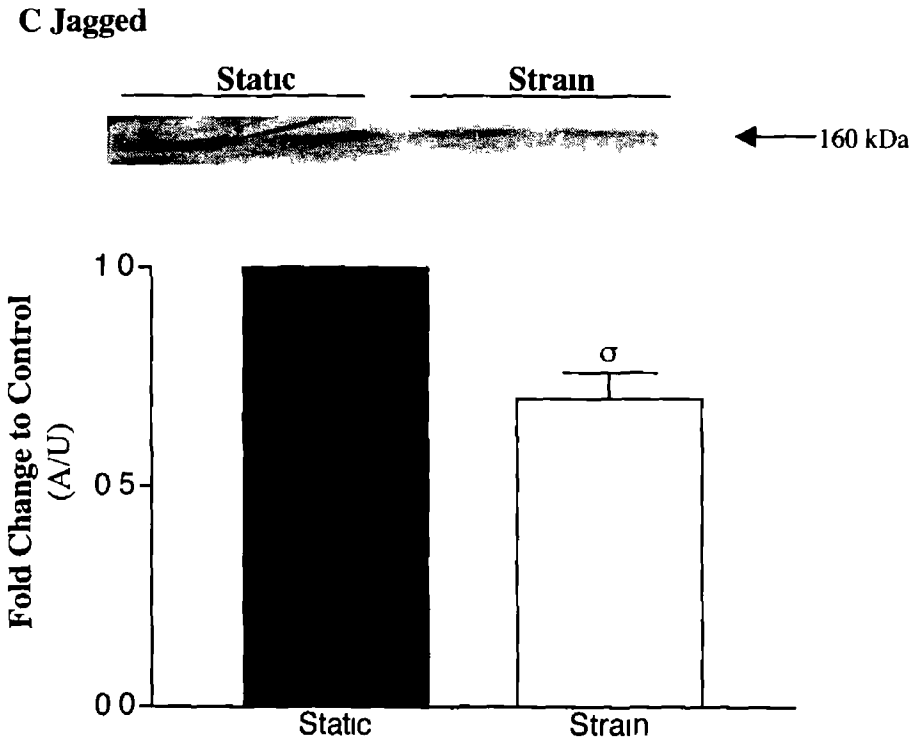



**Fig 5 5 Cyclic strain decreases Notch receptor and ligand protein expression in RVSMC** RVSMC were seeded onto Flexercell$^{TM}$ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) as indicated Protein extracted from both static and strained RVSMC was assayed for Notch 1 (A), Notch 3 (B) and Jagged(C) expression using Western blot analysis Values are expressed as fold change over static (arbitrarily assigned a value of 1), n=3 $^{\sigma}$ p<0 05 as compared to static control (student's t test)

**Figure 5 6 Effect of Cyclic Strain on Notch Target Gene Protein Expression in RVSMC**

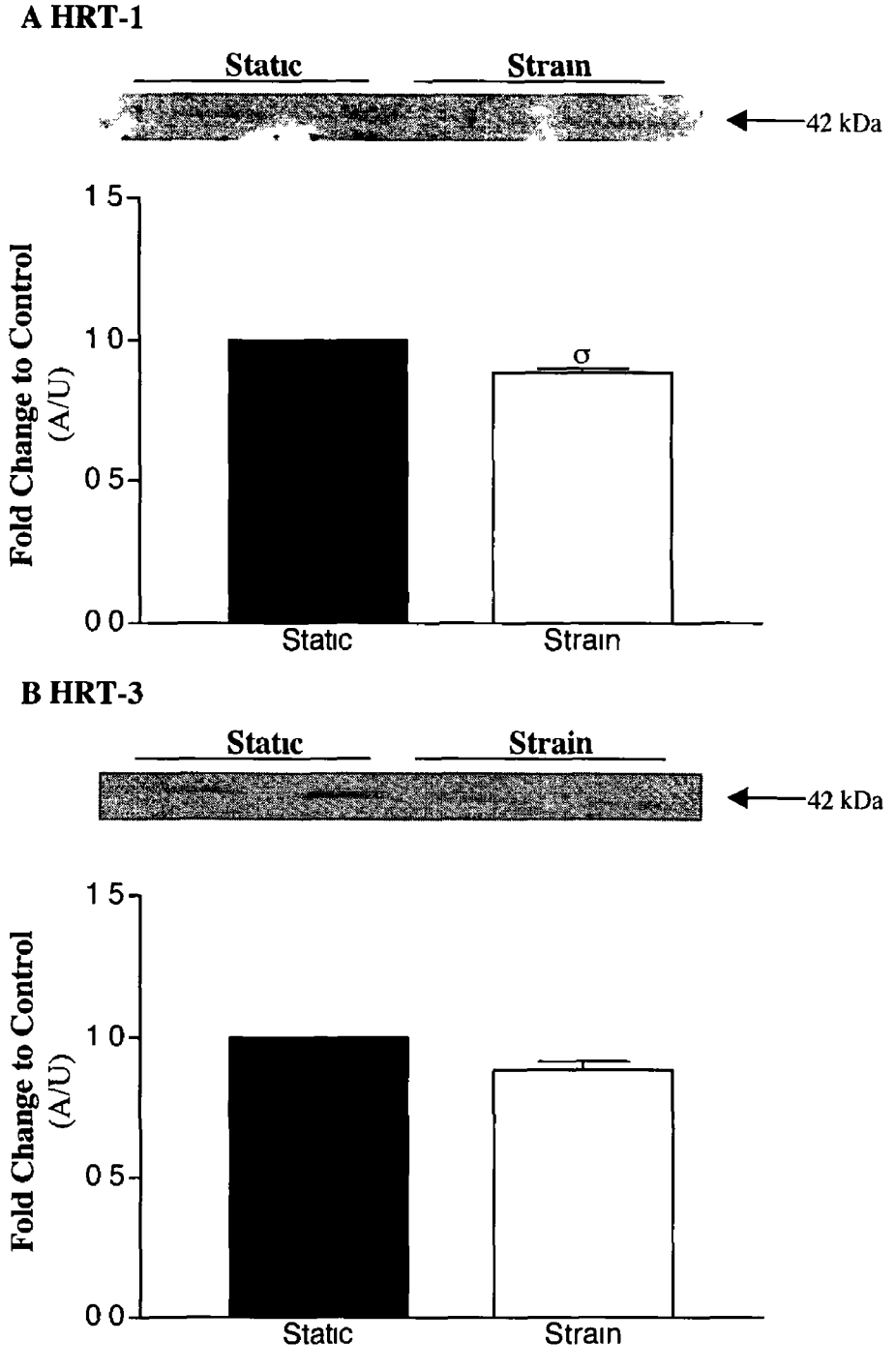



**Fig 5 6 Cyclic strain causes a decrease in Notch target gene protein expression in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) as indicated Protein extracted from both static and strained RVSMC was assayed for HRT-1 (A) or HRT-2 (B) expression using Western blot analysis Values are expressed as fold change over static (arbitrarily assigned a value of 1), n=3 $^{\sigma}$ $p<0\ 05$ as compared to static control (student's t test)

**Figure 5.7 Effect of Cyclic Strain on Notch Target Gene Protein Expression in RVSMC**

**HRT-1**

**Strain**

**Static**

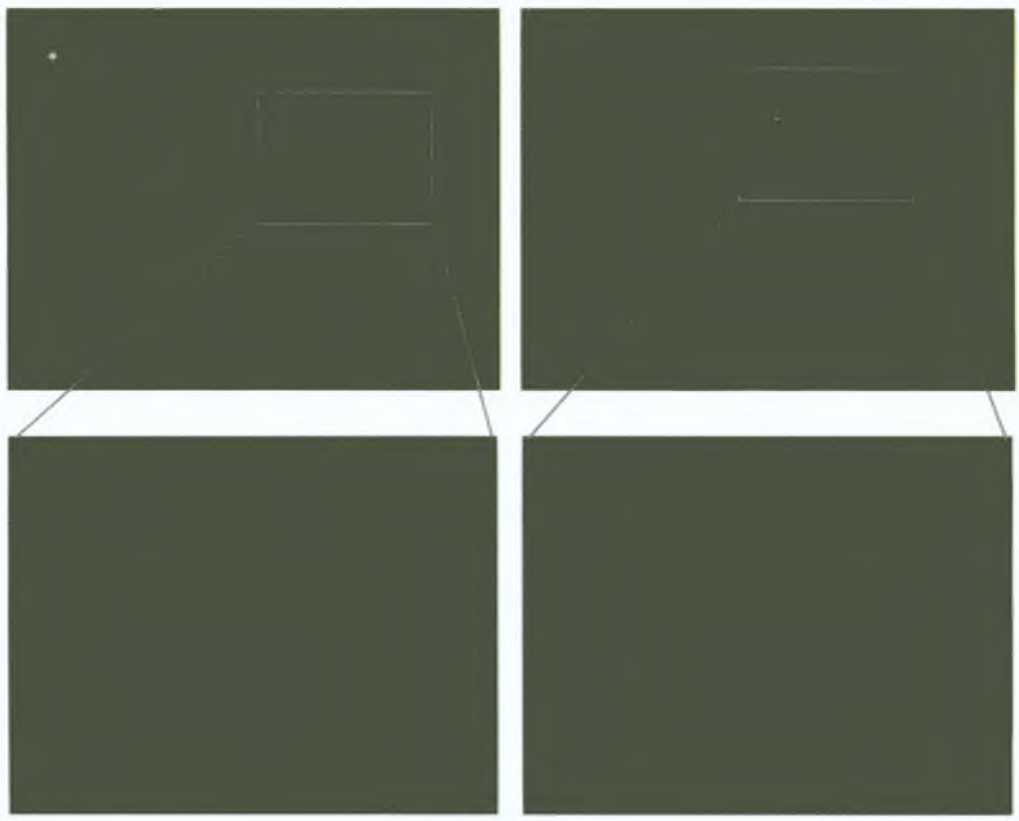
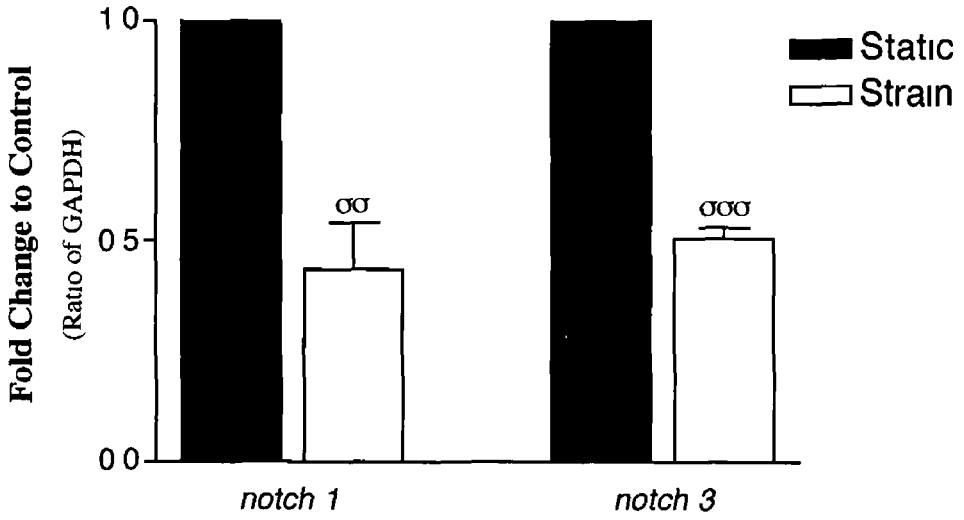


**Fig 5.7 Cyclic strain causes a decrease in Notch target gene protein expression in RVSMC.** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well. Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) as indicated. Cells were fixed and probed for HRT-1 expression using immunocytochemistry, and viewed under a fluorescent microscope, with equal exposure times used for both static and strain images.

**Figure 5 8 Effect of Cyclic Strain on Notch Receptor and Notch Ligand mRNA Expression in RVSMC**

**A Notch Receptor**




**B Jagged**

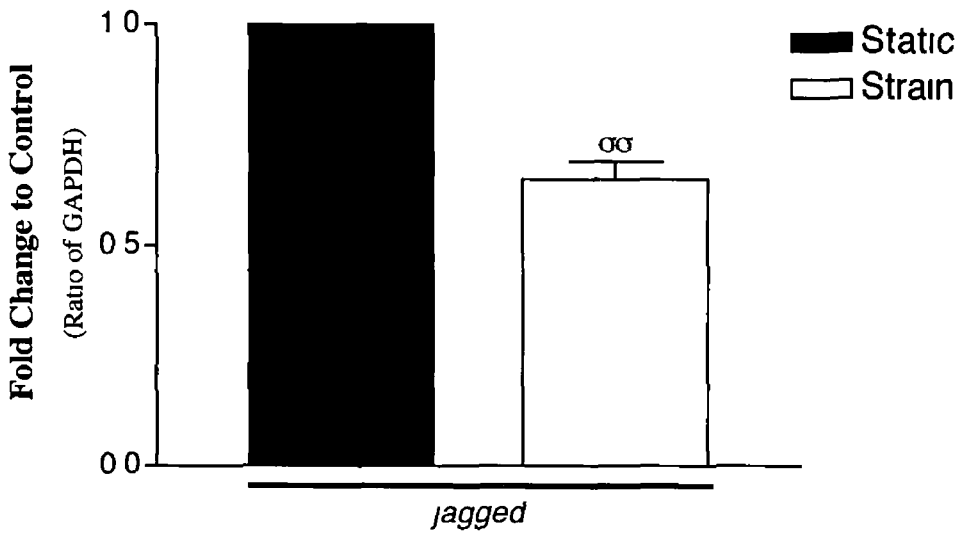
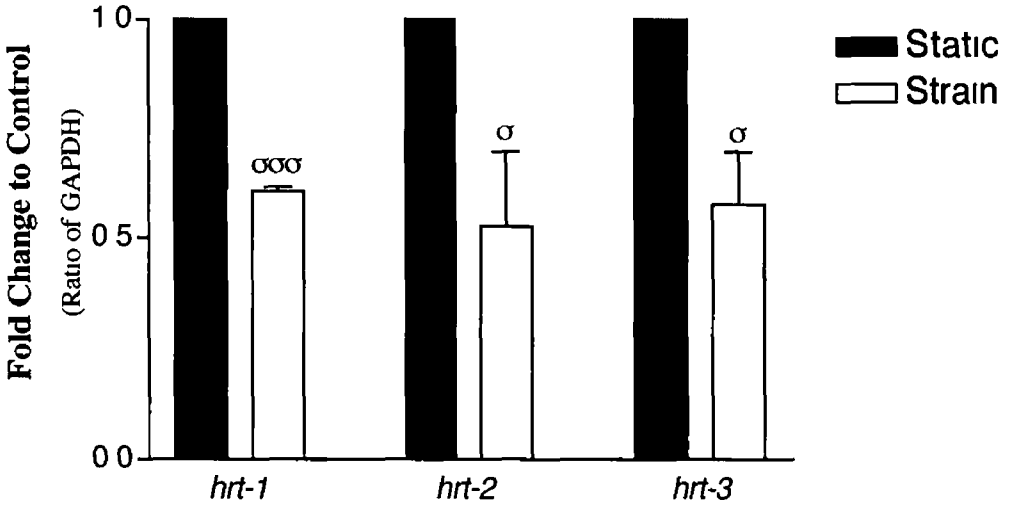



**Fig 5 8 Cyclic strain causes a decrease in Notch receptor and ligand mRNA expression in RVSMC** RVSMC were seeded onto Flexercell$^{TM}$ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) as indicated Samples were isolated at 24 h post-strain and subsequently assayed using quantitative PCR for Notch receptor (A) and Notch ligand (B) mRNA expression All values were normalized to GAPDH levels, and expressed as a fold change over static control (= the value obtained with static RVSMC mRNA levels, arbitrarily assigned a value of 1), n=3 $^{\sigma\sigma}$ $p<0\ 005$, $^{\sigma\sigma\sigma}$ $p<0\ 005$ as compared to static control (student's t test)

**Figure 5 9 Effect of Cyclic Strain on Notch Target Gene mRNA Expression in RVSMC**

**A HRT**




**B Hes**

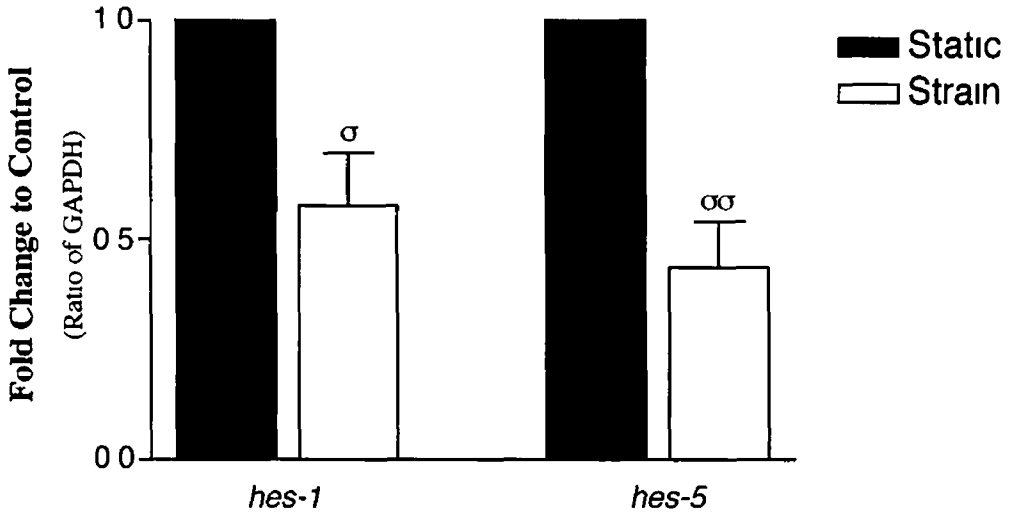
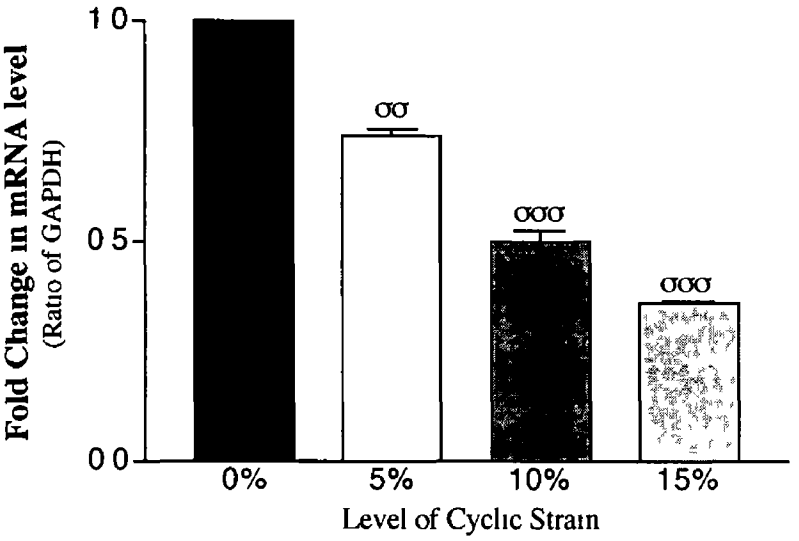



**Fig 5 9 Cyclic strain causes a decrease in Notch target gene mRNA expression in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain (10%, 24 h) as indicated Samples were isolated at 24 h post-strain and subsequently assayed using quantitative PCR for *hrt-1*, *hrt-2*, *hrt-3* (A), *hes-1* and *hes-5* (B) mRNA expression All values were normalized to GAPDH levels, and expressed as a fold change over static control (= the value obtained with static RVSMC mRNA levels, arbitrarily assigned a value of 1), n=3 $^{\sigma}$ p<0 05, $^{\sigma\sigma}$ p<0 005, $^{\sigma\sigma\sigma}$ p<0 0005 as compared to static control (student's t test)

**Figure 5 10 Force Dependent Effect of Cyclic Strain-induced Decrease in Notch Signalling Pathway mRNA Expression in RVSMC**

**A *notch 3* mRNA Expression**




**B *hes-5* mRNA Expression**

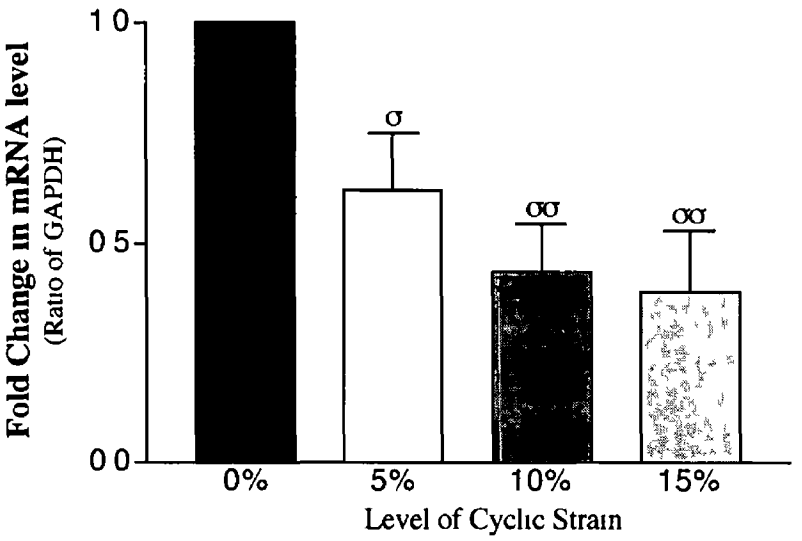



**Fig 5 10 Force-dependent effect of cyclic strain induced decrease in Notch Signalling Pathway mRNA expression in RVSMC** RVSMC were seeded onto Flexercell$^{TM}$ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to differing levels of cyclic strain, 0%, 5%, 10% or 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 $^{\sigma}$ $p<0.05$, $^{\sigma\sigma}$ $p<0.005$, $^{\sigma\sigma\sigma}$ $p<0.0005$ as compared to static control (student's t test)

**Figure 5.11 Effect of Time on Cyclic Strain-induced Decrease in Notch Signalling Pathway mRNA Expression in RVSMC**

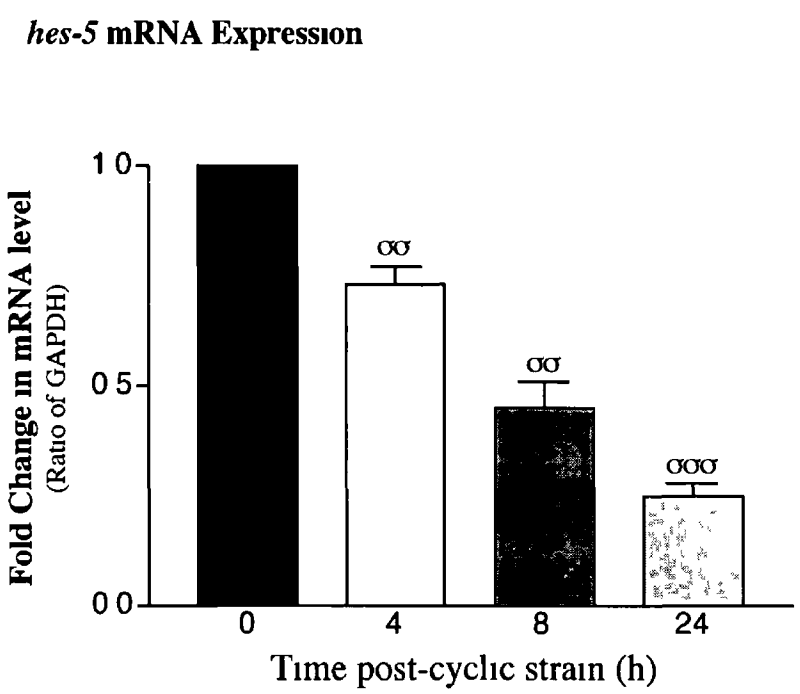



**Fig 5 11 Time-dependent effect of cyclic strain induced decrease in Notch Signalling Pathway mRNA expression in RVSMC** RVSMC were seeded onto Flexercell$^{TM}$ plates at 1 x $10^5$ cells/well Following a 48 h growth period, cells were subjected to cyclic strain over a period of 24 h, as indicated Samples were isolated at 0 h, 4 h, 8 h, and 24 h post-strain and subsequently assayed using quantitative PCR for and *hes-5* 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 $^{\sigma\sigma}$ $p<0$ 005, $^{\sigma\sigma\sigma}$ $p<0$ 0005 as compared to static control (student's t test)

### 5 2 3 Effect of Cyclic Strain on Notch Signalling Pathway Activation in RVSMC

The effect of cyclic strain on Notch signalling pathway activation was investigated over a 24 h period This was determined through transient transfection of RVSMC with luciferase-tagged reporter plasmids, the CBF-1 reporter plasmid, or Notch target gene reporter plasmids, and comparing transactivation in static versus strained cells

Cyclic strain resulted in a decrease in CBF-1 luciferase activity as compared to static control cells at 6 h, 10 h and 24 h post-cyclic strain (Figure 5 12) Static cells analyzed over a 24 h time period exhibited a temporal increase in CBF-1 luciferase activity, reminiscent of the effect of serum stimulation on luciferase activity (Figure 3 13) This can be attributable to the replacement of the cell media at the 0 h timepoint Cyclic strain significantly decreases CBF-1 luciferase activity by 82 36±1 96% 6 h following the onset of cyclic strain (Figure 5 12) Similarly, a decrease of 38 9±22 2% and 26 9±13 0% in CBF-1 luciferase activity was evident at 10 h and 24 h post-cyclic strain respectively, as compared to static control at each timepoint

Exposure of RVSMC to cyclic strain also resulted in a decrease in Notch target gene reporter activity Cells were transfected with the *hrt-1* luciferase tagged reporter, subjected to cyclic strain over a 24 h period, and compared to static controls (Figure 5 13A) A representative experiment revealed a marked decrease in *hrt-1* luciferase activity between static and strained cells at all timepoints analyzed As with the CBF-1 reporter experiment, a temporal increase in *hrt-1* luciferase activity was evident in static but not strained cells Cyclic strain significantly decreased *hrt-1* luciferase activity by 50 0±2 7% and 65 8±0 5% at 8 h and 10 h post-cyclic strain respectively, as compared to static control at each timepoint A similar decrease of 33 4±7 0% was apparent at 24 h post-cyclic strain, as compared to static control cells A more in depth analysis of the effect of cyclic strain on *hrt-1* luciferase activity revealed a similar trend (Figure 5 13B) RVSMC were analyzed at 8 h post-cyclic strain and a significant decrease of 62 0±12 0% in *hrt-1* luciferase activity was evident in strained cells at this timepoint, as compared to static control cells

Similarly, the effect of cyclic strain on *hes-5* luciferase activity in RVSMC was also investigated (Figure 5 14) A representative timecourse revealed a decrease in *hes-5* luciferase activity at all timepoints, reminiscent of the effect of cyclic strain on the CBF-1 and *hrt-1* luciferase reporter plasmids Cyclic strain significantly decreased *hes-*

*5* luciferase activity at 8 h and 24 h post-cyclic strain by 45 84±12 5% and 65 38±3 84% respectively, as compared to appropriate static controls, and decreased *hes-5* luciferase activity at 10 h post cyclic strain by 6 25±18 75% as compared to static control Further analysis of the effect of cyclic strain at 8 h post-onset confirmed this trend (Figure 5 14B) In this case, a significant decrease of 47 0±4 0% in *hes-5* luciferase activity was evident, as compared to static control cells

**Figure 5.12 Effect of Cyclic Strain on CBF-1 Activation in RVSMC**

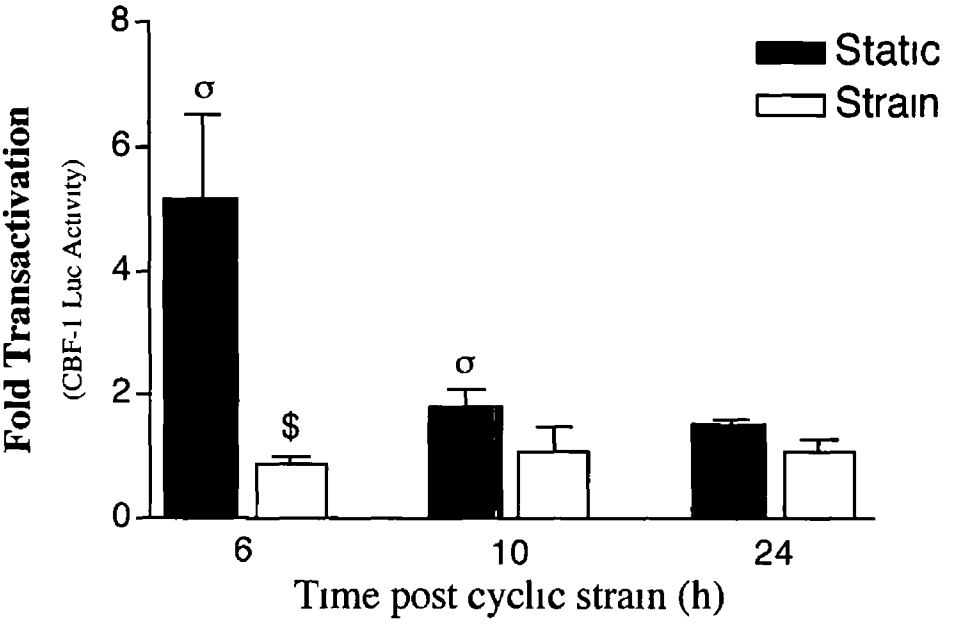
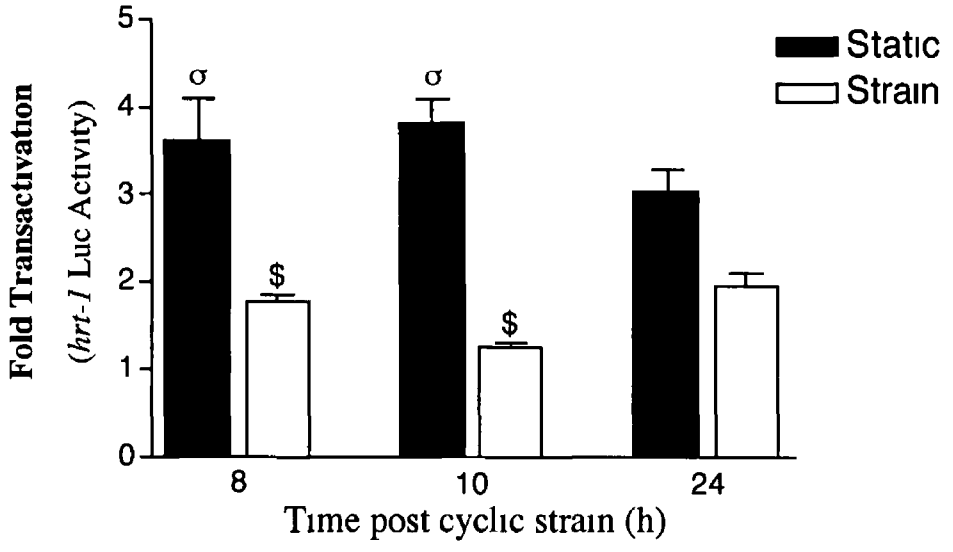



**Fig 5 12 Cyclic strain causes a decrease in Notch Signalling Pathway activation in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well RVSMC were transiently transfected with the CBF-1 luciferase-tagged reporter plasmid Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated Samples were isolated at 0, 6, 10 and 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 CBF-1 reporter plasmid transfected cells at 0 h arbitrarily assigned a value of 1) $^{\sigma}$ $p<0.05$ as compared to 0 h static control, $^{\$}$ $p<0.05$ as compared to static control at that timepoint (student's t test)

**Figure 5 13 Effect of Cyclic Strain on HRT-1 Activation in RVSMC**

**A Representative Timecourse**




**B Cyclic Strain (8 h)**

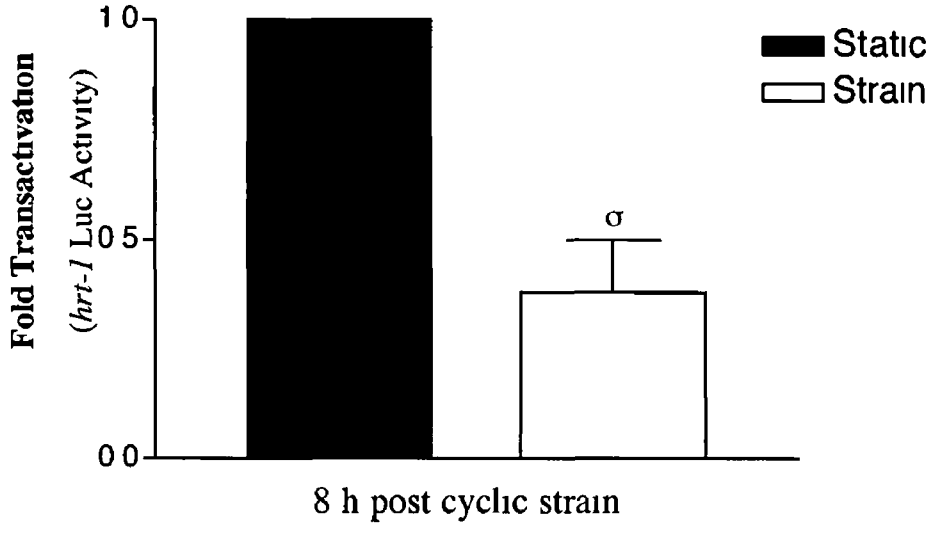
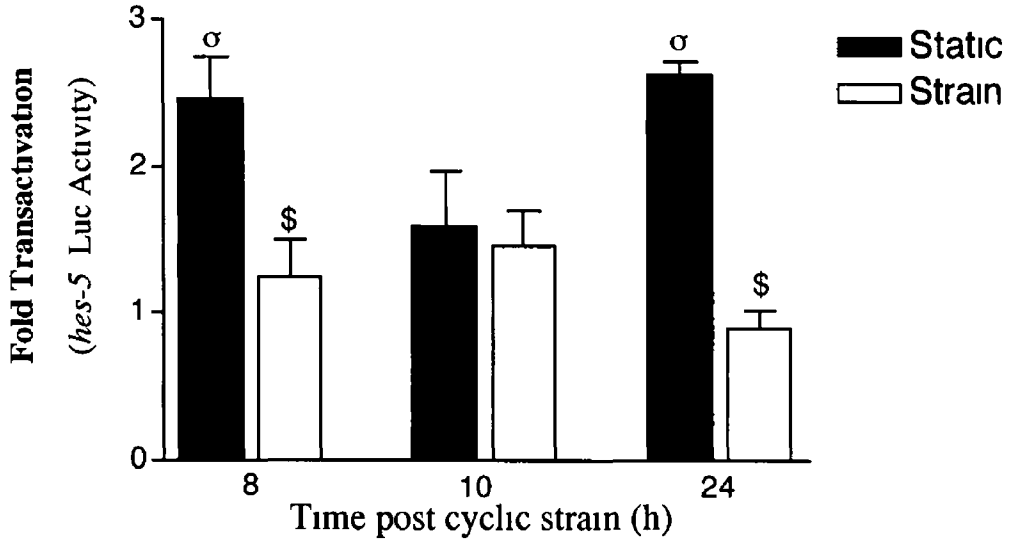



**Fig 5 13 Cyclic strain causes a decrease in HRT-1 activation in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well RVSMC were transiently transfected with the *hrt-1* reporter plasmid Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated A) Samples were isolated at 0, 8, 10 and 24 h post-strain and assayed for luciferase activity Representative timecourse, performed in triplicate B) Samples were isolated 8 h post cyclic strain and assayed for luciferase activity, n=3 Luciferase assays were normalized to β-galactosidase activities and protein levels, and expressed as fold increase over control (= the value obtained with *hrt-1* reporter plasmid transfected cells at 0 h arbitrarily assigned a value of 1 (A), or = the value obtained with static *hrt-1* reporter plasmid transfected cells at 8 h arbitrarily assigned a value of 1 (B) ) $^{\sigma}$ $p<0.05$ as compared to 0 h static control, $^{\$}$ p,0 05 as compared to static control at that timepoint (A), $^{\sigma}$ $p<0.05$ as compared to static control (B) (student's t test)

**Figure 5.14 Effect of Cyclic Strain on Hes-5 Activation in RVSMC**

**A Representative Timecourse**




**B Cyclic Strain (8 h)**

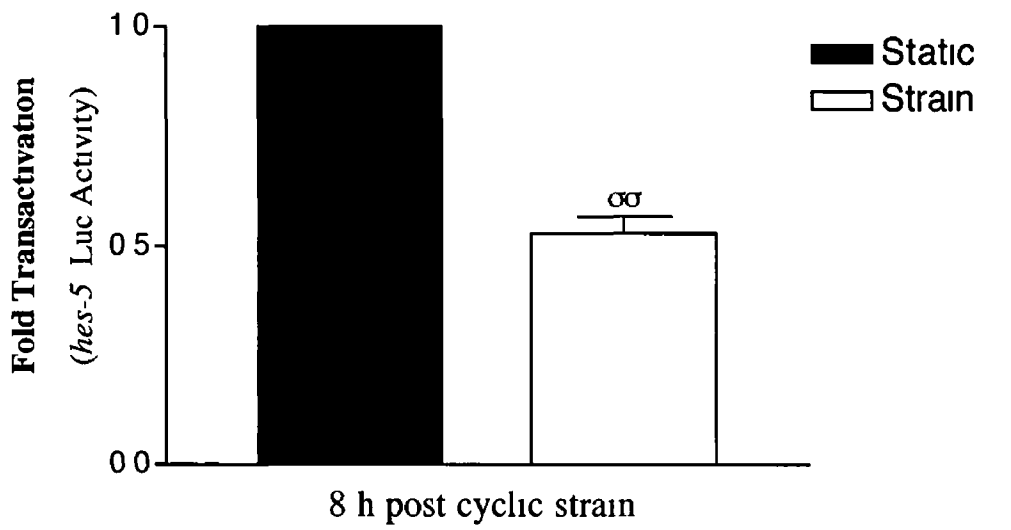



**Fig 5 14 Cyclic strain causes a decrease in Hes-5 activation in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well RVSMC were transiently transfected with the *hes-5* reporter plasmid Following overnight recovery, cells were subjected to cyclic strain (10%, 24 h) as indicated A) Samples were isolated at 0, 8, 10 and 24 h post-strain and assayed for luciferase activity Representative timecourse, performed in triplicate B) Samples were isolated 8 h post cyclic strain and assayed for luciferase activity, n=3 Luciferase assays were normalized to β-galactosidase activities and protein levels, and expressed as fold increase over control (= the value obtained with *hes-5* reporter plasmid transfected cells at 0 h arbitrarily assigned a value of 1 (A), or = the value obtained with static *hes-5* reporter plasmid transfected cells at 8 h arbitrarily assigned a value of 1 (B) ) $^{\sigma}$ $p<0\,05$ as compared to 0 h static control, $^{\$}$ $p,0\,05$ as compared to static control at that timepoint (A), $^{\sigma\sigma}$ $p<0\,005$ as compared to static control (B) (student's t test)

### 5 2 4 Effect of the Notch Signalling Pathway on Cyclic Strain-induced RVSMC Apoptosis *in vitro*

This study has previously demonstrated that over-expression of Notch 1 IC and Notch 3 IC attenuates serum deprivation-induced apoptosis (Figure 4 6 - Figure 4 11), and that inhibition of CBF-1-dependent Notch signalling promotes apoptosis in VSMC (Figure 4 1 - Figure 4 4) In addition, this study has also revealed that cyclic strain decreases Notch signalling pathway component expression in RVSMC, whilst concomitantly increasing apoptosis Therefore, the effect of Notch 3 IC over-expression on cyclic strain-induced apoptosis was investigated, in an attempt to counteract the functional repercussions of cyclic strain on VSMC apoptosis RVSMC were transfected with either an empty vector (mock transfected) or Notch 3 IC, and co-transfected with the puromycin resistance plasmid Following overnight recovery from transfection, cells were exposed to puromycin-containmg media, to ensure a high degree of plasmid expression in the cells subsequently exposed to cyclic strain (10%, 24 h) Apoptosis was examined using histochemical analysis, caspase 3 activity assays, and measurement of both *bax* and *bcl-x*$_L$ expression levels

The effect of Notch 3 IC on cyclic strain-induced apoptosis was initially examined using the acridine orange/ethidium bromide dual stain (Figure 5 15) Representative histochemical analysis are shown in Figure 5 15A, and cumulative data on the percentage of apoptotic cells are also presented (Figure 5 15B) Cyclic strain resulted in a significant increase in apoptotic cells The percentage of apoptotic cells present in the mock transfected static control sample was 8 25±1 25%, which was increased to 30 75±8 98% following exposure of the cells to cyclic strain Over-expression of Notch 3 IC significantly attenuated this increase in percentage apoptotic cells by 50 15±8 4%

The anti-apoptotic effect of Notch 3 IC was further confirmed following analysis of caspase 3 activity (Figure 5 16) Cyclic strain resulted in a 30 0±1 0% increase in caspase 3 activity in strained cells, as compared to mock transfected static control cells Over expression of Notch 3 IC resulted in a significant decrease in caspase 3 activity in static cells of 34 0±4 0%, as compared to mock transfected static control cells Cyclic strain also resulted in an increase in capsase 3 activity of 33 0±4 0% in Notch 3 IC transfected cells Therefore, the level of caspase 3 activity in Notch 3 IC transfected cells exposed to cyclic strain is equivalent to that of mock transfected static control

cells, suggesting comparable levels of apoptosis in these cells, and functional recovery against cyclic strain-induced apoptosis due to Notch 3 IC

The effect of Notch 3 IC over-expression on cyclic strain-induced changes in *bcl-2* family mRNA levels was also investigated using quantitative real time PCR analysis (Figure 5 17) Exposure of RVSMC to cyclic strain resulted in a decrease in *bcl-$x_l$* expression by 29 0±17 0%, as compared to static mock transfected control cells (Figure 5 17A) Over expression of Notch 3 IC in static cells resulted in a significant increase in *bcl-$x_L$* expression by 60 0±21 0% as compared to static mock transfected control This was attenuated by exposure of the Notch 3 IC transfected cells to cyclic strain, such that the level of *bcl-$x_L$* expression in Notch 3 IC transfected cells exposed to cyclic strain is similar to that in mock transfected static control cells Expression of *bcl-$x_L$* in Notch 3 IC transfected strained cells is increased by only 12 0±3 0% over static mock transfected control cells, showing recovery of the cyclic strain-induced decrease in *bcl-$x_L$* expression

The effect of Notch 3 IC over-expression on cyclic strain-induced changes in *bax* was also examined Cyclic strain increased *bax* expression in RVSMC by 30 0±8 0% over static mock transfected control cells, whereas the level of *bax* expression in Notch 3 IC transfected static cells was significantly decreased by 34 0±1 0% over mock transfected static control cells Notch 3 IC transfected cells exposed to cyclic strain, however, exhibited an increase in *bax* expression to 149 0±120 0% over mock transfected static control cells

### 5 2 5 Effect of Notch Over-expression on *hrt-2* mRNA levels in RVSMC exposed to Cyclic Strain

Notch 3 IC over-expression protects against cyclic strain-induced apoptosis Notch 3 IC also increases Notch target gene mRNA levels in RVSMC (Figure 3 12B) Therefore, it was decided to investigate the mRNA levels of a Notch target gene in mock and Notch 3 IC transfected cells exposed to cyclic strain Exposure to cyclic strain resulted in a significant decrease of 33 0±7 0% in *hrt-2* mRNA expression levels, as compared to static control in mock transfected cells (Figure 5 18) Notch 3 IC over-expression resulted in a significant fold increase (2 81±0 4) in *hrt-2* mRNA expression levels, and exposure of these cells to cyclic strain reduced this fold increase to 1 99±0 46 over mock transfected static control

**Figure 5.15 Effect of Notch Over-expression on Cyclic Strain-induced Apoptosis, as measured by Acridine Orange/Ethdidum Bromide Dual Stain**

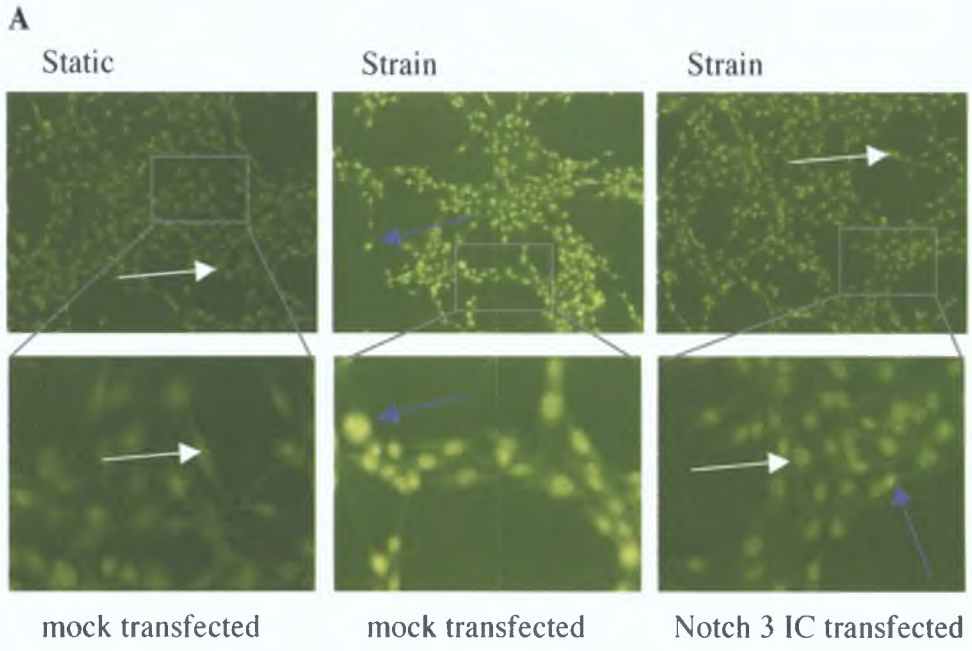



**Fig 5.15 Over-expression of Notch 3 decreases cyclic strain-induced apoptosis in RVSMC.** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later. Following overnight recovery, cells were incubated in puromycin containing growth medium (0.8 μg/ml, 48 h), and subsequently exposed to cyclic strain (10%, 24 h) as indicated. Cells were stained with the Acridine Orange/ Ethidium Bromide dual stain and viewed under a fluorescent microscope. A) Representative images; Viable cell, white arrow; Apoptotic cell, blue arrow. B) Graph showing percentage change in the number of apoptotic cells, n=2. $^{\beta}$ $p<0.05$ as compared to static control, $^{\$}$ $p<0.05$ as compared to mock-transfected strained cells (student's t test).

**Figure 5.16 Effect of Notch Over-expression on Cyclic Strain-induced Apoptosis, as measured by Caspase 3 Activity**

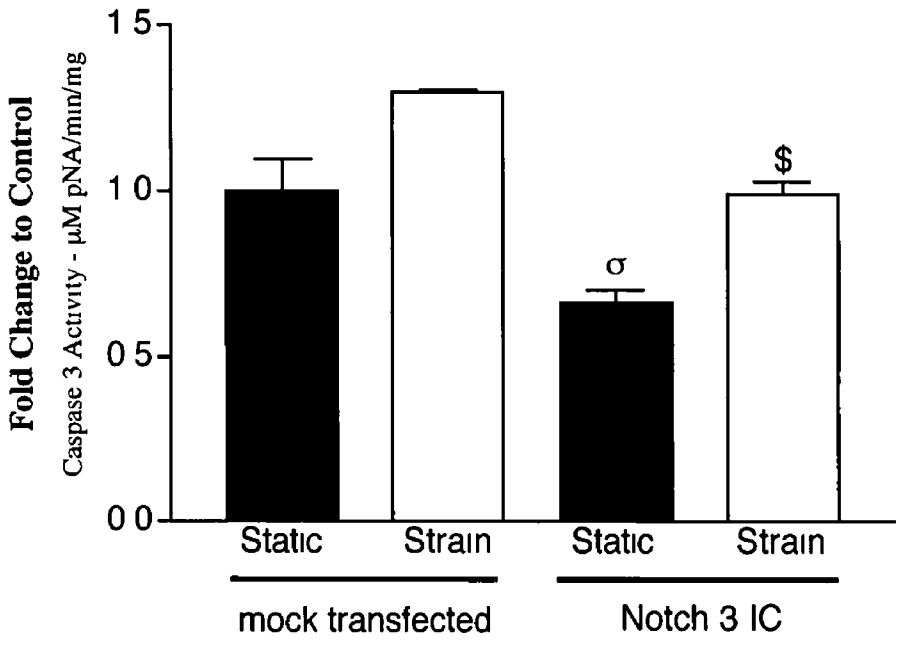
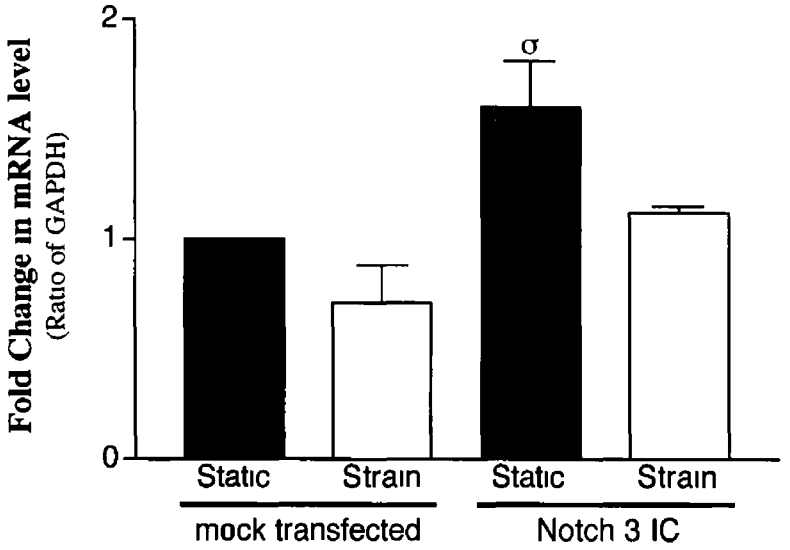



**Fig 5 16 Over-expression of Notch 3 decreases cyclic strain-induced apoptosis in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 µg/ml, 48 h), and subsequently exposed to cyclic strain (10%, 24 h) Protein was isolated and assayed for caspase 3 activity Caspase assays were normalized to protein levels, and expressed as fold increase over control (= the value obtained with static cells arbitrarily assigned a value of 1), representative experiment performed in triplicate $^{\sigma}$ $p<0$ 05 as compared to static control, $^{\$}$ $p<0$ 05 as compared to Notch 3 IC transfected static cells (student's t test)

**Figure 5.17 Effect of Notch Over-expression on Cyclic Strain-induced changes in Bcl-2 Family mRNA levels in RVSMC**

**A· *bcl-$x_L$* mRNA Expression**




**B: *bax* mRNA Expression**

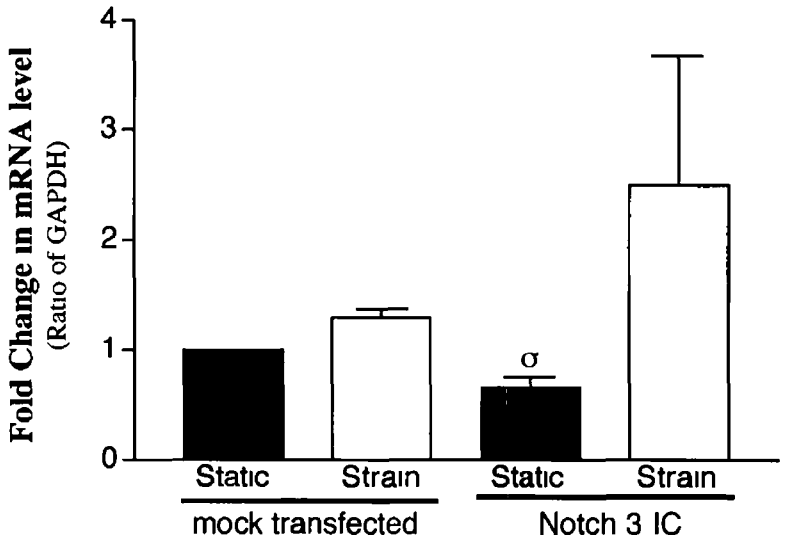



**Fig 5 17 Effect of cyclic strain and Notch 3 over-expression on *bax* and *bcl-$x_L$* expression in RVSMC** RVSMC were seeded onto Flexercell™ plates at 1 x $10^5$ cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 μg/ml, 48 h), and subsequently exposed to cyclic strain (10%, 24 h) mRNA levels 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 $^{\sigma}$ $p<0$ 05 as compared to static control (student's t test)

**Figure 5.18 Effect of Notch Over-expression on *hrt-2* mRNA Expression in RVSMC exposed to Cyclic Strain**

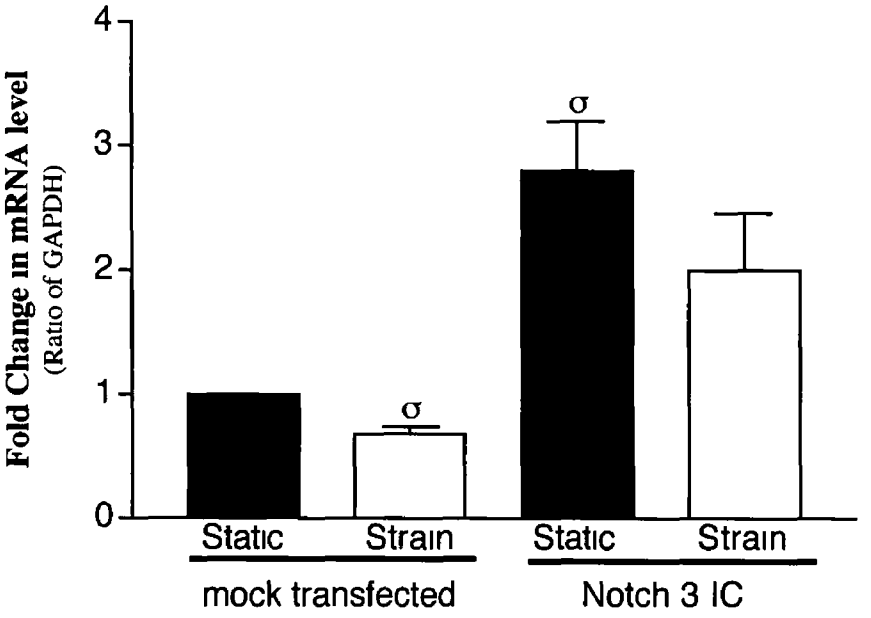



**Fig 5 18 Effect of Notch over expression on *hrt-2* mRNA expression in RVSMC exposed to cyclic strain** RVSMC were seeded onto Flexercell$^{TM}$ plates at 1 x $10^5$ cells/well, and were transiently transfected with the puromycin resistance plasmid, pGK3puro, and co-transfected with the empty vector p7pCMV or Notch 3 IC 2 days later Following overnight recovery, cells were incubated in puromycin containing growth medium (0 8 μg/ml, 48 h), and subsequently exposed to cyclic strain (10%, 24 h) mRNA levels 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, $^{\sigma}$ $p<0$ 05 as compared to static control (student's t test)

## 5 3 Discussion

This study clearly establishes that cyclic strain induces apoptosis in VSMC This concurs with other studies, which report increased VSMC apoptosis both *in vivo* and *in vitro* The level of cyclic strain-induced apoptosis observed in this study is comparable to that reported in other *in vitro* studies, which describe a 2- to 4-fold increase in apoptosis due to increased cyclic strain (Mayr *et al*, 2002, Wernig *et al*, 2003) The fact that cyclic strain-induced apoptosis is, at least in part, caspase 3-dependent is unsurprising, as activation of caspase 3 has been observed in atherosclerotic plaques at sites of VSMC apoptosis (Mallat *et al*, 1997) In addition, caspase 3 activation was observed in human umbilical arteries, which are exposed to dramatic increases in haemodynamic forces during delivery (Kim *et al*, 2000)

The importance of the Bcl-2 family in the regulation of apoptosis in a variety of cells is well established, and changes in Bcl-2 family expression are often observed during the pathogenesis of vascular disease (Bai *et al*, 1999, Cook *et al*, 1999) Therefore, this study investigated the effect of cyclic strain on the pro-apoptotic genes, *bad* and *bax*, and the anti-apoptotic genes, *bcl-2* and *bcl-x*$_L$ This study shows that cyclic strain changes the *bcl-2* family profile in favour of apoptosis, with an increase in both *bad* and *bax*, and a concomitant decrease in *bcl-x*$_l$ expression However, an increase in the anti-apoptotic *bcl-2* gene expression was also observed This is somewhat surprising as we, and others, have clearly demonstrated that cyclic strain induces apoptosis in VSMC *in vitro* However, this finding concurs with a recent study by Mayr *et al*, (2002) which demonstrates an increase in Bcl-2 following cyclic strain The anti-apoptotic activity of Bcl-2, however, may be cell type or context specific, as Bcl-2 has been shown to have variable anti-apoptotic effects in different cell types (Yeh 1997) In addition, despite a 1 8-fold increase in Bcl-2 expression observed in failing hearts, the normal anti-apoptotic action of Bcl-2 in these cells is insufficient to overcome the pro-apoptotic stimuli, such as mechanical stretch, that is present in the failing heart (Hetts 1998) Therefore, the up-regulation of *bcl-2* in VSMC exposed to cyclic strain may be a compensatory up-regulation in response to the increased pro-apoptotic *bcl-2* family gene expression, but is ineffective in protecting against apoptosis

The 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 and Pollman 2000, Kim *et al*, 2000)

The stimulus for the observed increase in *bax* gene expression due to cyclic strain is likely due to increased p53 activity as *bax* is a direct transcriptional target for p53, and two recent studies have shown increases in both p53 activity and Bax expression due to cyclic strain (Mayr *et al*, 2002, Wernig *et al*, 2003) Bax expression is up-regulated in the rat heart following coronary occlusion (Liu *et al*, 1998), and its over-expression in the ventricles of spontaneously hypertensive rats is said to contribute to myocyte apoptosis (Fortuno *et al*, 1998) In addition, an increased level of Bax expression was observed in the human umbilical vessel during delivery, which coincided with an increase in apoptosis in the vessel (Kim *et al*, 2000) The umbilical vessel is exposed to increased haemodynamic forces during delivery, therefore it is possible that the observed Bax up-regulation is a result of increased mechanical forces Similarly, Bax-associated apoptosis was also observed at other sites undergoing dramatic haemodynamic changes during the perinatal period, such as the ductus arteriosus and the branching point of large arteries (Kim *et al*, 2000) These observations indicate that Bax may play a key role in both neonatal and pathological vascular remodelling, and that the stimulus for Bax up-regulation may be exposure to increased haemodynamic forces

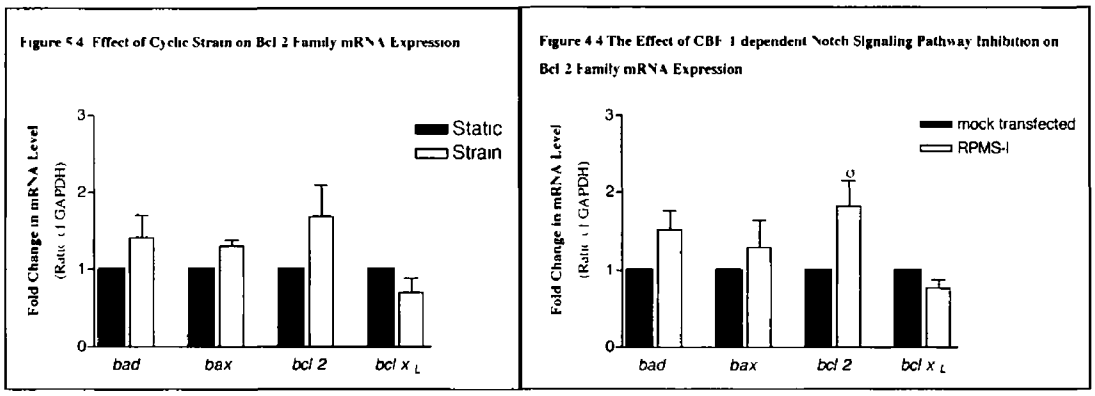
The observed decrease in *bcl-$x_L$* expression in this study is in contradiction with Mayr *et al*, (2002), who reports an increase in Bcl-$x_L$ following cyclic strain This discrepancy could perhaps be attributed to differences in VSMC phenotype, as this has previously been shown to determine the growth response of VSMC to mechanical forces *in vitro* (Cappadona *et al*, 1999) However, the decrease in *bcl-$x_L$* observed in this study following cyclic strain is consistent with the observed increase in cyclic strain-induced VSMC apoptosis In addition, a down-regulation of *bcl-$x_L$ in vivo* is repeatedly associated with VSMC apoptosis (Yeh 1997, Bai *et al*, 1999) Bcl-$x_L$ protein expression is dramatically decreased in medial VSMC following balloon injury (Yeh 1997), presumably as a result of the distention injury to the SMC due to balloon inflation This is also associated with an increase in medial VSMC apoptosis, and acute medial cell loss As we have previously established a relationship between the Notch signalling pathway and *bcl-$x_L$* expression, it is interesting to note that Wang *et al*, (2002a) report a decrease in expression of Notch signalling pathway components in medial SMC following balloon injury Therefore, it is tempting to speculate that Notch regulates the cyclic strain-induced decrease in *bcl-$x_L$* expression, further suggesting that cyclic strain may affect expression of Notch signalling pathway components In contrast to medial cells, neointimal cells are relatively resistant to apoptosis, and exhibit

an increased expression of Bcl-$x_l$ Vascular lesion formation is associated with an up-regulation of Bcl-$x_L$ within intimal VSMC in both animal models and human specimens of vascular disease (Bai *et al*, 1999) In addition, down-regulation of Bcl-$x_L$ using anti-sense oligonucleotides induces VSMC apoptosis and regression of vascular disease (Pollman *et al*, 1998) These findings indicate that increased Bcl-$x_L$ expression is a critical determinant of intimal VSMC viability and vascular lesion formation, and could be an attractive target for future therapeutic interventions

This study has clearly established that cyclic strain induces apoptosis in VMSC In addition, a functional coupling between the Notch signalling pathway and VSMC apoptosis was also established, in that the Notch signalling pathway exerts a protective effect against serum deprivation-induced apoptosis, and CBF-1-dependent inhibition of this pathway results in increased apoptosis in VSMC Therefore it was postulated that changes in Notch signalling pathway activity could contribute to cyclic strain-induced apoptosis in VSMC Cyclic strain has previously been shown to affect the level of expression of a number of VSMC components (Feng *et al*, 1999), therefore, the effect of cyclic strain on the Notch signalling pathway was investigated Additionally, the effect of Notch over-expression on cyclic strain-induced apoptosis was also analyzed to determine whether Notch could protect against cyclic strain-induced apoptosis in VSMC

This study presents previously unreported evidence of a cyclic strain-induced decrease in Notch signalling pathway expression in VSMC Exposure to increased cyclic strain results in a similar decrease in expression of all Notch signalling pathway components analyzed Moreover, this study provides evidence that this decrease in expression due to cyclic strain occurs in and time- and force-dependent manner Therefore, this study adds components of the Notch signalling pathway to the list of genes regulated by changes in cyclic strain *in vitro* As expression of the active forms of Notch receptors and CBF-1-dependent Notch target genes are decreased following exposure to cyclic strain, it is unsurprising that this study has additionally shown a cyclic strain-induced decrease in Notch signalling pathway activity in VSMC Inhibition of CBF-1-dependent Notch signalling following exposure to cyclic strain is further confirmed by comparing the expression profile of the Bcl-2 family of genes following cyclic strain and CBF-1-dependent signalling inhibition with RPMS-I, which is virtually identical, as illustrated in Figure 26 below

**Figure 26 Bcl-2 family gene expression following cyclic strain and CBF-1-dependent signalling inhibition**




While this is the first study of its kind, the findings we present concur with the *in vivo* findings presented by Wang *et al*, (2002a), who report a decrease in Notch receptor and Notch target gene expression following balloon injury and subsequent cellular distention, with the most dramatic decrease evident for the Notch 3 receptor and the HRT-1 target gene

This study has previously shown that the Notch signalling pathway confers a level of protection against serum deprivation-induced apoptosis in VSMC Therefore, we also investigated whether this anti-apoptotic effect of Notch was conserved against cyclic strain-induced apoptosis in VSMC This was analyzed through over-expression studies using Notch 3 IC, which was utilized as *in vivo* deformation of VSMC causes a greater down-regulation of Notch 3 as compared to other Notch receptors (Wang *et al*, 2002a) Over-expression of Notch 3 IC partially inhibited cyclic strain-induced apoptosis, as measured by the annexin V/propidium iodide dual stain One explanation for this could be that other signalling pathways co-operate with the Notch signalling pathway to regulate apoptosis in VSMC exposed to cyclic strain This is likely, as multiple signalling pathways are known to regulate apoptosis in most cell types studied, including VSMC (Gupta 2003) In addition, it is interesting to note that Notch 3 IC over-expression in VSMC exposed to cyclic strain resulted in a similar level of caspase 3 activity to mock transfected un-strained cells This suggests that the Notch signalling pathway regulates cyclic strain-induced apoptosis in a caspase 3-dependent manner

We have previously shown that the pro-apoptotic *bax* gene, and the anti-apoptotic *bcl-x*$_l$ gene are both regulated by the Notch signalling pathway in VSMC In addition, we have presented evidence showing a down-regulation of *bcl-x*$_L$ and a

concomitant up-regulation of *bax* in response to cyclic strain Further to this, we investigated whether Notch 3 over-expression affects *bcl-$x_L$* or *bax* expression under conditions of cyclic strain, in an attempt to elucidate the mechanism of the anti-apoptotic effect exerted by Notch 3 over-expression in response to cyclic strain-induced apoptosis Similar to the activity pattern in evidence for caspase 3, Notch 3 over-expression in VSMC exposed to cyclic strain resulted in a similar level of *bcl-$x_L$* expression to that seen in mock transfected un-strained cells Therefore, reversal of the cyclic strain-induced decrease in the Notch signalling pathway abolished the cyclic strain-induced decrease in *bcl-$x_L$* expression This suggests that Notch may mediate its anti-apoptotic effect against cyclic strain-induced VSMC apoptosis in a *bcl-$x_L$*-dependent manner, and down-regulation of the Notch signalling pathway in response to increased cyclic strain results in a decrease in *bcl-$x_L$* expression Bcl-$x_L$ acts to prevent against apoptosis in part by binding to the pro-apoptotic protein Bax, thus inhibiting the ability of Bax to form homodimers This inhibits the ability of Bax to increase mitochondrial membrane potential and cause activation of the caspase cascade Therefore, down-regulation of *bcl-$x_L$* could increase the formation of Bax homodimers and caspase 3-activation A contributing factor to the increase in Bax homodimer formation would also be the increase in *bax* gene expression in VSMC following cyclic strain However, over-expression of Notch 3 m VSMC exposed to cyclic strain did not result in the expected attenuation of the cyclic strain-induced increase in *bax* expression, but rather potentiated the response This suggests, although *bax* expression is regulated by the Notch signalling pathway in static VSMC, that additional factors affected by cyclic strain act to regulate and potentiate *bax* expression in VSMC

Whilst it is now well established that increased mechanical stress induces apoptosis in VSMC, the molecular mechanisms regulating this response are not yet fully elucidated As with regulation of apoptosis by other stimuli, it is likely that several mechanisms may mediate the apoptotic response of VSMC to increased cyclic strain Wernig *et al* , (2003) have recently shown that cyclic strain-induced apoptosis in VSMC is mediated by the $\beta_1$-integrin-p38-p53 signalling pathways Whilst we have conclusively shown that the Notch signalling pathway also regulates cyclic strain-induced VSMC apoptosis, it is also possible that the Notch signalling pathway interacts with one or more components of the $\beta_1$-integrin-p38-p53 signalling pathway Several reports provide evidence for these interactions, and will be reviewed in Chapter 7 In addition, (Li and Xu 2000) suggest that G proteins may be a primary mechanosensor in VSMC, as treatment of the cells with pertussis toxin (PTX), a $G_i$ inhibitor, resulted in

the blocking of cyclic strain-induced p38MAPK activation In concurrence with this report, this study has identified a role for G proteins in mediating the cyclic strain-induced increase in caspase 3 activity in VSMC Preliminary data suggests that that treatment of VSMC with PTX also abolishes the cyclic strain-induced increase in caspase 3 activity Cyclic strain causes a 66 0±15 0% increase in VSMC (Figure 5 3), however, treatment of cells with PTX attenuates this increase, bringing caspase 3 activity to within 5 0±15% of that observed in un-strained cells *(n=3, data not shown)* As we have shown that G protein inhibition and over-expression of Notch 3 both result in the same abolition of the cyclic strain-induced increase in caspase 3 activity, we suggest that an interaction between G proteins and the Notch signalling pathway could exist in VSMC Although further exploration of this was beyond the scope of this project, we propose further examination of this in future studies within the laboratory, and provide additional evidence from the literature of the links between G proteins and the Notch signalling pathway, which will be discussed in more detail in Chapter 7

## 5 4 Conclusion

In summary, we have conclusively shown that cyclic strain induces apoptosis in VSMC Cyclic strain-induced apoptosis is attributable, at least in part, to a cyclic strain-induced decrease in expression and activity of Notch signalling pathway components In addition, we propose that over-expression of Notch 3 inhibits cyclic strain-induced apoptosis in a *bcl-$x_L$*- caspase 3-dependent manner However, we acknowledge that the artery is a mechanically complex three-dimensional structure, therefore it is likely that *in vitro* cellular deformations cannot completely simulate *in vivo* mechanical stimuli Therefore, this study also investigates the effect of altered mechanical stress on Notch signalling pathway expression *in vivo*, which will be discussed in Chapter 6

# Chapter 6

# Results

# Effect of Alterations in Biomechanical Stress on the Notch Signalling Pathway and Apoptosis *in vivo*

## 6 1 Introduction

As Notch signalling is relevant in vascular disease (Lindner *et al*, 2001, Wang *et al*, 2002) this study examines the relative expression of Notch receptors and target genes, in addition to apoptosis *in vivo*, using two models of vascular injury This study has previously established that increased mechanical stretch both increases apoptosis and decreases Notch signalling pathway expression in VSMC *in vitro* To determine whether this effect of altered biomechanical forces extrapolates to an *in vivo* situation, we investigated Notch signalling pathway expression and levels of apoptosis in animal models subjected to either carotid artery or portal vein ligation (PVL) Such models provide a template for the study of altered haemodynamic forces and resultant vascular remodelling

Mechanical forces associated with blood flow have been implicated with the regulation of vascular cell fate, both as a fundamental feature in development, and in the pathogenesis of vascular disease Apoptosis has, for example, been demonstrated following changes in blood flow after birth, in intimal arterial thickening following balloon catheter injury, and in both animal and human atherosclerotic plaques (Sho *et al*, 2001) Apoptosis is virtually absent in normal adult vessels, but is a prominent feature of pathological conditions involving vascular remodelling (Xu 2000) Changes in VSMC fate decisions, including apoptosis, results in vascular remodelling, however, the molecular mechanisms of apoptosis, and its regulation, following alterations in biomechanical stress remain to be fully elucidated

Vascular remodelling is the structural re-organization of a vessel, and is considered both a physiological and pathological process within the vasculature Physiological vascular remodelling is evident after birth, for example, during the closure of the ductus arteriosus (Slomp *et al*, 1997) Inappropriate or pathological vascular remodelling is a common feature of many vascular disease states, and contributes to the progression of vascular disease Remodelling of an arterial wall occurs, for example, after chronic changes in blood pressure and blood flow in response to vessel injury, and plays a critical role in the degree of vessel narrowing during cardiovascular disease progression Vascular remodelling can result in both arterial narrowing or arterial enlargement, termed inward and outward remodelling respectively (Mulvany 1999)

The models of altered haemodynamic forces, and subsequent vascular remodelling, analyzed in this study include the rat carotid artery and portal vein ligation

models.

The carotid artery ligation model is a particularly powerful research tool, as it provides a model of bilateral carotid remodelling in a single animal. The simultaneous reduction in blood flow in the left (ligated) carotid artery and the increase in blood flow in the right carotid artery allows examination of the effects of altered haemodynamics on expression of vascular genes and apoptosis in a single animal. Indeed, many groups utilize this model in an attempt to elucidate the molecular mechanisms of vessel wall remodelling (Bryant *et al.,* 1999; Kraemer 2002; Sullivan and Hoying 2002; Korshunov and Berk 2003). However, this model can be further sub-divided into two groups, the "partial ligation" model, utilized in this study, and the "flow-cessation" model. Korshunov and Berk (2003), show that the partial ligation of the left carotid artery results in a dramatic decrease (90%) in blood flow in the left carotid artery, with the maintenance of an intact endothelium and no thrombosis. A concomitant increase (70%) in blood flow was also observed in the right carotid artery. This study describes outward remodelling or vessel enlargement in both the left and right carotid arteries, which was maximal at 7 days post-ligation. Remodelling was greater in the right carotid artery, with a predominantly increased lumen, and little increase in the medial or adventitial layer, whereas the left carotid artery displayed a dramatic increase in both the medial and adventitial layers.

**Figure 27: Example of Carotid Remodelling due to Left Carotid Ligation**

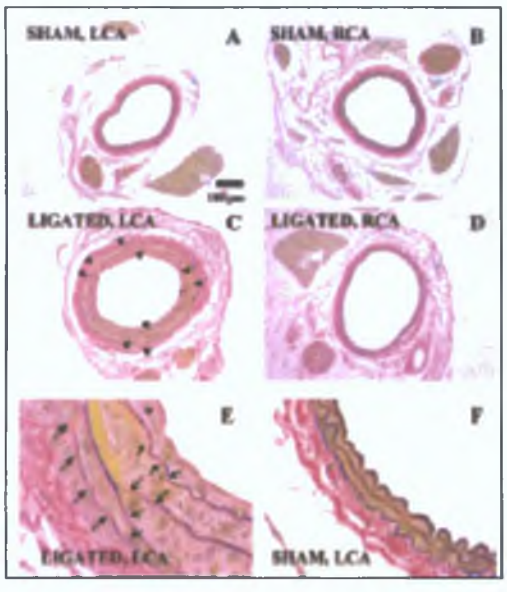



**Figure 27: Carotid remodelling.** Verhoeff Van Giesson staining of sham, right carotid artery (RCA) and left carotid artery (LCA) 14 days post-ligation. Sham (A, B, F); Ligated (C, D, E). Elastic fibres, black; nuclei, brown; collagen, red; other tissue structure, yellow. Partial ligation resulted 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).

The "flow-cessation" model of carotid ligation is achieved by total ligation of the carotid artery, resulting in complete cessation or interruption of blood flow in this

vessel Bryant *et al*, (1999), utilize this model and have recorded a decrease in blood flow from 0 58±0 05 ml/min in normal vessels to zero in ligated vessels In contrast to the previous model, the ligated carotid artery displays inward vascular remodelling or arterial narrowing, and also endothelial denudation and thrombus formation, which lessens the physiological relevance of this model to study human cardiovascular disease (Korshunov and Berk 2003) In addition, Korshunov and Berk argue that the "partial ligation" model is more physiologically relevant in the study of cardiovascular disease, as although decreased blood flow is associated with human vascular disease, the no-flow phenomenon that occurs with the "flow cessation" model is not generally seen in atherosclerotic/restenotic arteries Therefore, this study utilizes a physiologically relevant model of altered haemodynamic forces to determine the effect, if any, on Notch signalling pathway expression and apoptosis in the vascular wall

In addition, we also utilize a model of partial portal vein ligation (PVL) as a model of altered haemodynamic forces, and compare this to a sham-operated control model PVL induces pre-hepatic portal hypertension, which is characterized by increased cardiac output and increased plasma volume (Yokoyama *et al*, 2001) Blood pressure, and therefore, biomechanical forces, are increased in the ligated animals, as compared to the sham-operated control Yokoyama *et al*, (2001), observed that portal pressure was increased in PVL rats to 13 2±0 9 mm Hg, compared to sham-operated control rats, who exhibited a portal pressure of 5 41±0 6 mm Hg It must be noted that whilst the expression of the Notch signalling pathway is described as being restricted to the arterial vessels, a number of exceptions to this pattern of expression exist, and components of the Notch signalling pathway have previously been identified in the portal vein (Villa *et al*, 2001)

Whilst the importance of apoptosis in vessel remodelling and vascular disease is well established, the molecular mechanisms regulating this process remain to be fully elucidated This study has clearly demonstrated that over-expression of the Notch signalling pathway protects against cyclic strain-induced VSMC apoptosis Similarly, inhibition of this pathway, or decrease in Notch signalling pathway expression also results in VSMC apoptosis Therefore, this study also investigates whether alterations in mechanical forces affect Notch signalling pathway expression and apoptosis in two *in vivo* models Although two previous reports have examined Notch signalling pathway expression in rat carotid arteries following balloon injury, none have examined the effect of a sustained increase in biomechanical force to date Linder *et al*, (2001) report that components of the Notch signalling pathway are up-regulated in rat carotid

arteries in response to injury, whereas Wang *et al*, (2002a) report a decrease in Notch signalling pathway expression in the carotid artery following injury Although these reports initially appear to contradict one another, a number of factors must be considered which may account for the discrepancies between the studies Firstly, the findings of Lindner *et al*, were limited to the inner face of the artery, as they used *en face* preparations for *in situ* hybridization They observed an increase in Notch signalling pathway component expression in both EC and SMC following injury However, the increase in SMC expression was observed following endothelial denudation, which does not occur normally within the vasculature, and may, in itself, affect gene expression and function of the underlying SMC Indeed, Langille *et al*, (1993) have shown that the endothelium is a critical mediator of the flow-dependent remodelling response In addition, increases in gene expression in the innermost SMC is not necessarily representative of changes in gene expression in the medial SMC layer as a whole In contrast, Wang *et al*, who report a co-ordinate down regulation of Notch receptors and HRT target genes in response to vascular injury, analyzed arteries following removal of the intimal and adventitial layers Therefore the observed changes in gene expression are representative of the medial SMC layer Whilst this study uses RNA and protein extracted from total vessels, we believe that the results are representative of changes in gene expression in VSMC, as these constitute the majority of the cells analyzed

Although it would be tempting to analyze the effect of Notch signalling pathway manipulation on vessel apoptosis and vascular remodelling *in vivo*, the models utilized in this study do not permit this One possible method of such analysis would be generation of a transgenic animal model However, as both Notch over-expression and absence result in embryonic lethality, generation of such a model has proved elusive, until recently Liu and Lobe (2003) recently presented reports of a viable Notch 1 transgenic mouse model, in which activation of Notch 1 IC is achieved post-natally to ensure viability Possible collaboration with this group, or future generation of a similar model could prove to be an exciting research tool, further validating the *in vitro* findings previously obtained within the laboratory

## 6 2 Results

The following section presents data on the relative expression of Notch receptors and target genes, and the level of apoptosis using two *in vivo* models of vascular injury and remodelling

### 6 2 1 Expression of Notch Signalling Pathway Components Post-Carotid Ligation

The effect of altered mechanical forces on the Notch signalling pathway *in vivo* was initially studied using a rat carotid artery ligation model Notch signalling pathway 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 Flow rates were significantly altered in both the right and left carotid arteries of ligated animals, compared to that of the sham-operated animals (Figure 6 1) Measurements were taken at 0, 3 and 28 days post-carotid ligation, and expressed as fold change over sham-operated control (arbitrarily assigned a value of 100%) Flow rates in sham-operated animals remained constant over the time period examined, however the flow rates observed in the right carotid artery of the ligated animal ("right ligated") were significantly increased at both 3 days and 28 days post-ligation, to 165±13% and 175±8% over sham-operated control The flow rates in the left ligated carotid artery were concomitantly decreased to 5±0 8% and 3±0 4% at 3 days and 28 days post-ligation respectively with respect to sham-operated controls

#### 6 2 1 1 Three Days Post-Carotid Ligation

Altered mechanical forces in both the right and left carotid arteries of ligated animals resulted in increased protein expression of Notch receptors and target genes, as compared to sham-operated control Equal protein loading on the gels was verified using a Ponceau S stain, as described in section 2 3 7 4 Notch 1 protein expression exhibited a slight increase in the right and left carotid arteries of the ligated animal over sham-operated control (arbitrarily assigned a value of 1) An increase of 1 19±0 04 was evident in the right carotid artery compared to control, and a slight but significant increase in the left carotid artery (1 21±0 02) of the ligated animal was also evident

(Figure 6 2) Similarly, Notch 3 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 (Figure 6 3)

Significant increases in Notch target gene protein expression were also observed in the right and left carotid arteries of ligated animals, as compared to sham-operated controls (Figure 6 4) 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 Similar fold increases were also evident in the right and left carotid arteries for HRT-2 and HRT-3 protein expression 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 This pattern of protein up-regulation was also evident for Hes-1 and Hes-5 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

## Fig 6 1 Blood Flow in Young Rats following Carotid Ligation

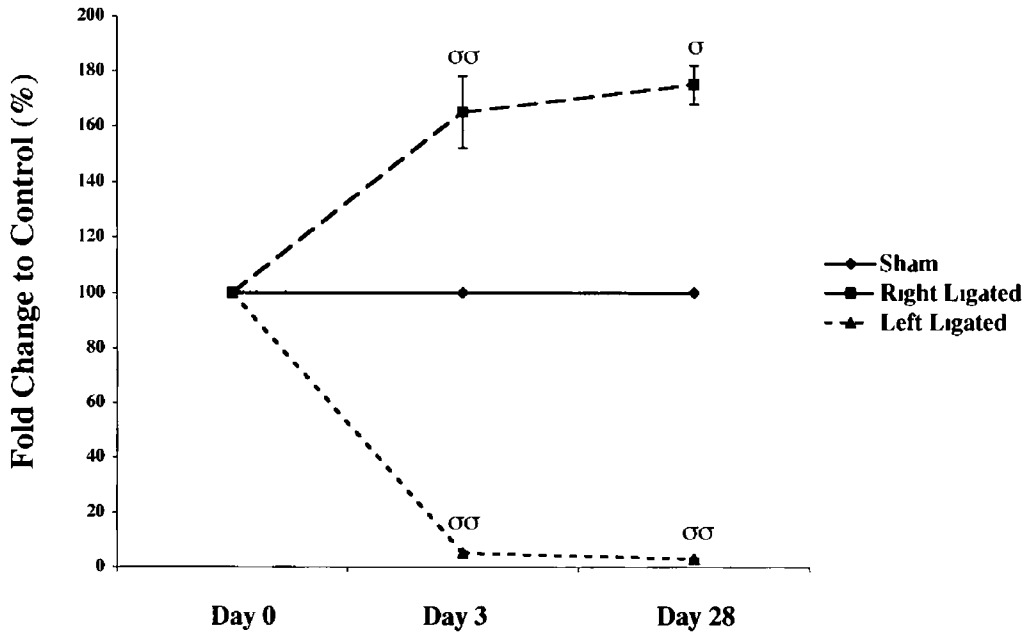



**Fig 6 1 Graphs showing blood flow rates in young rats post-carotid ligation compared to sham control animals** The left carotid artery of young rats was ligated, and the blood flow rates measured in the left and right carotid arteries of both ligated and sham-operated animals using a Transonic flowprobe(g/ml/min) 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% $^{\sigma}$ $p<0.05$, $^{\sigma\sigma}$ $p<0.005$ as compared to sham-operated control (student's t test)

**Fig 6 2 Changes in Notch 1 Receptor Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation**

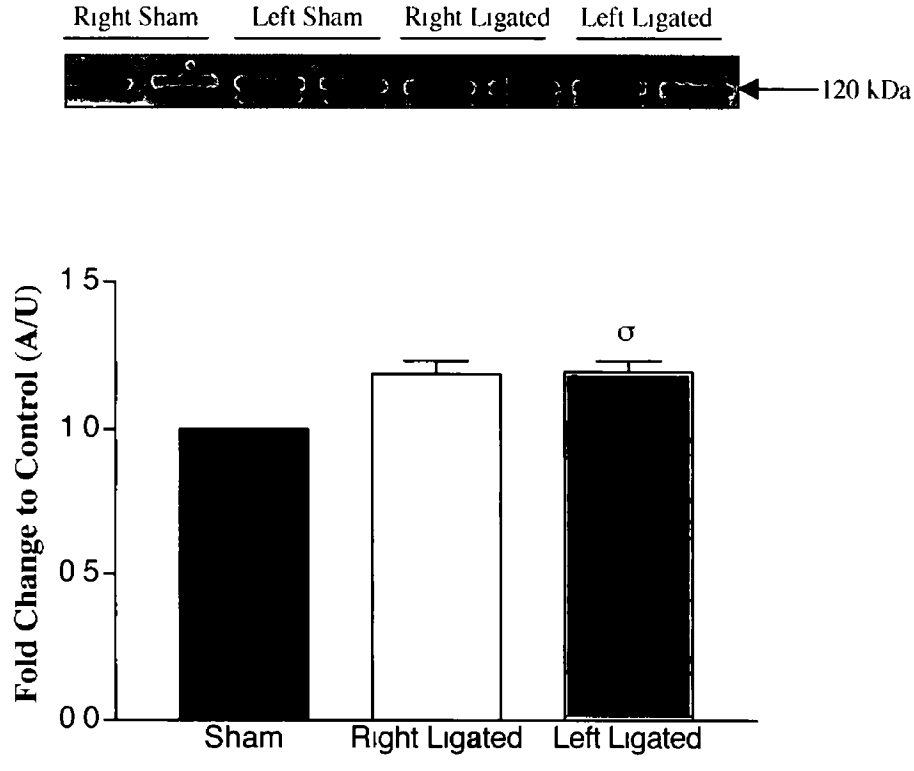



**Fig 6 2 Changes in Notch 1 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls** The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 3 days post 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 $^{\sigma}$ $p<0.05$ as compared to sham-operated control (student's t test)

**Fig 6 3 Changes in Notch 3 Receptor Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation**

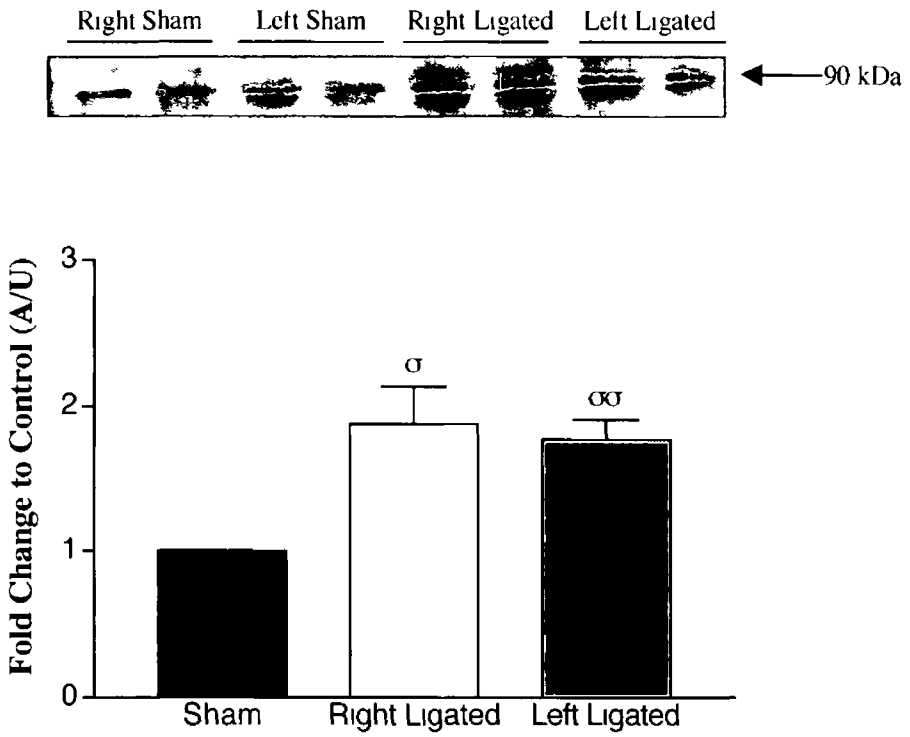
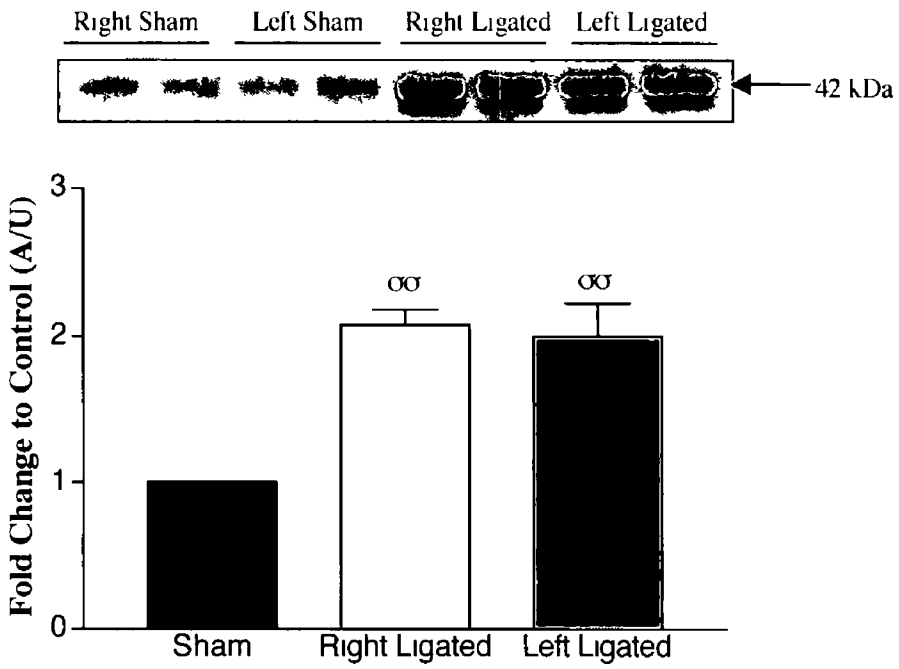


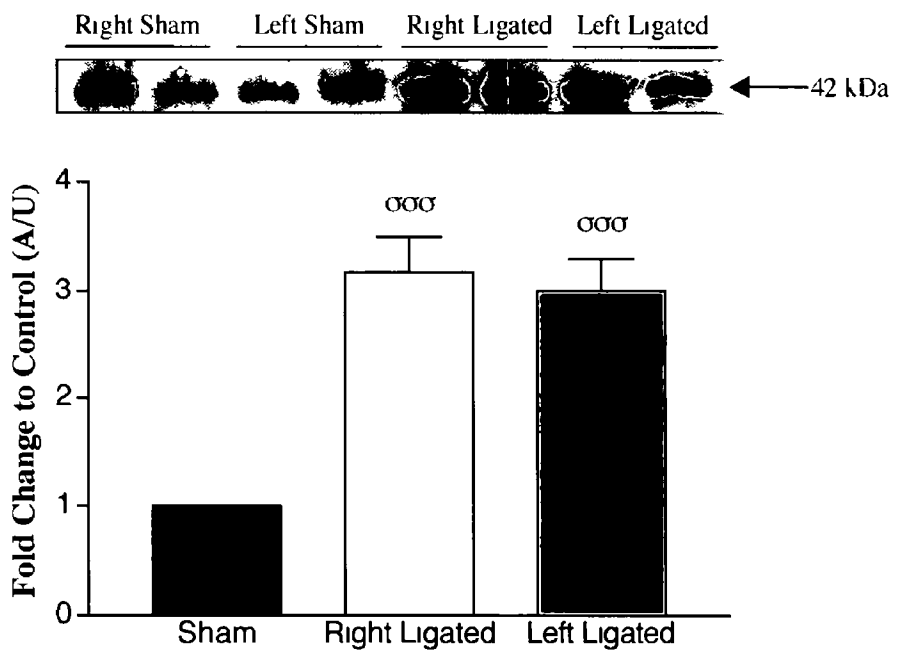

**Fig 6 3 Changes in Notch 3 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls** The left carotid artery of young rats was ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation 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 $^{\sigma}$ $p<0.05$, $^{\sigma\sigma}$ $p<0.005$ as compared to sham-operated control (student s t test)

**Fig 6 4 Changes in Notch Target Gene Expression in Ligated versus Sham-operated Animals 3 Days Post-Carotid Ligation**

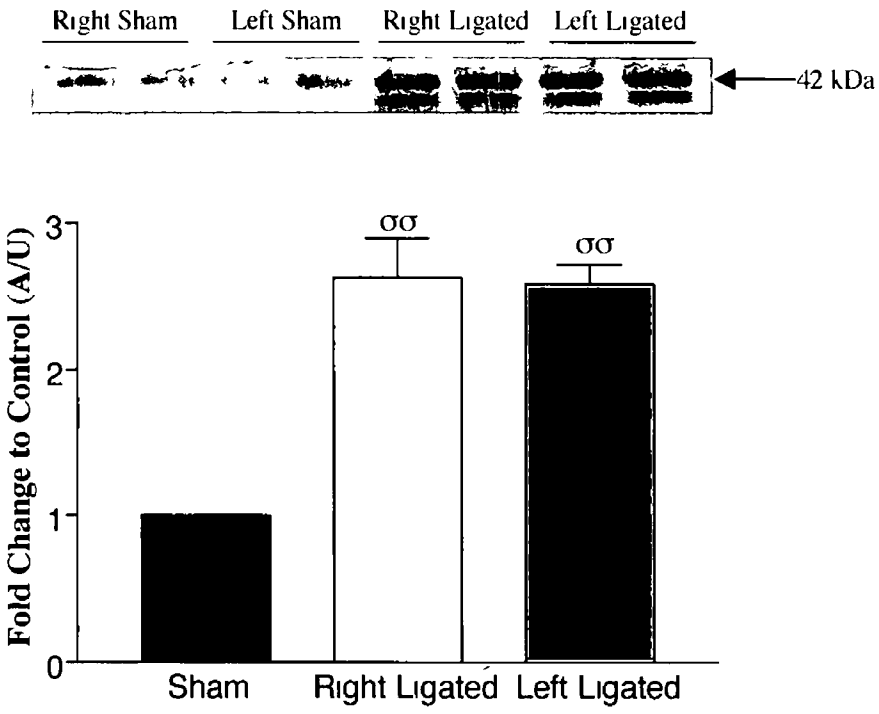
**A HRT-1**



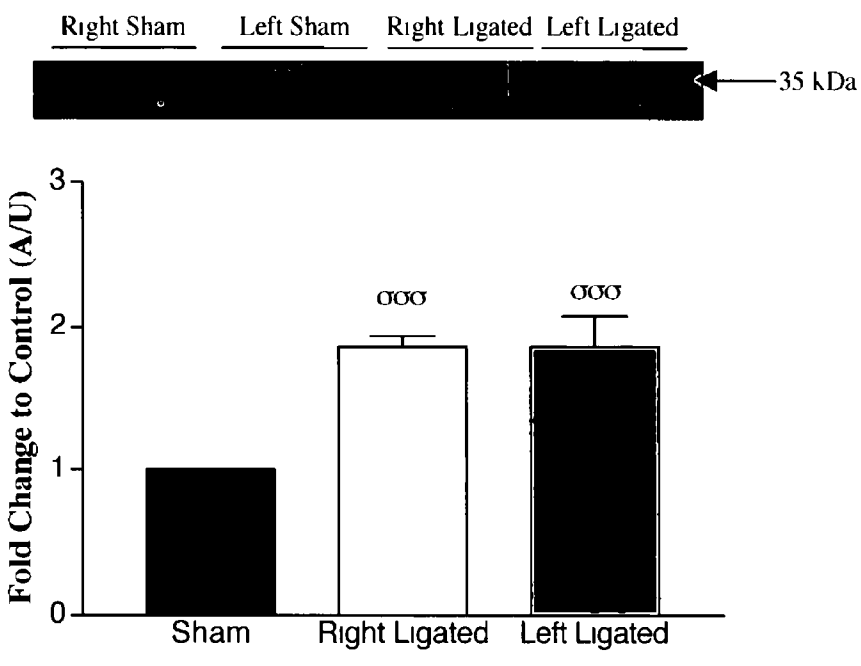

**B HRT-2**



C HRT-3


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

E Hes-5

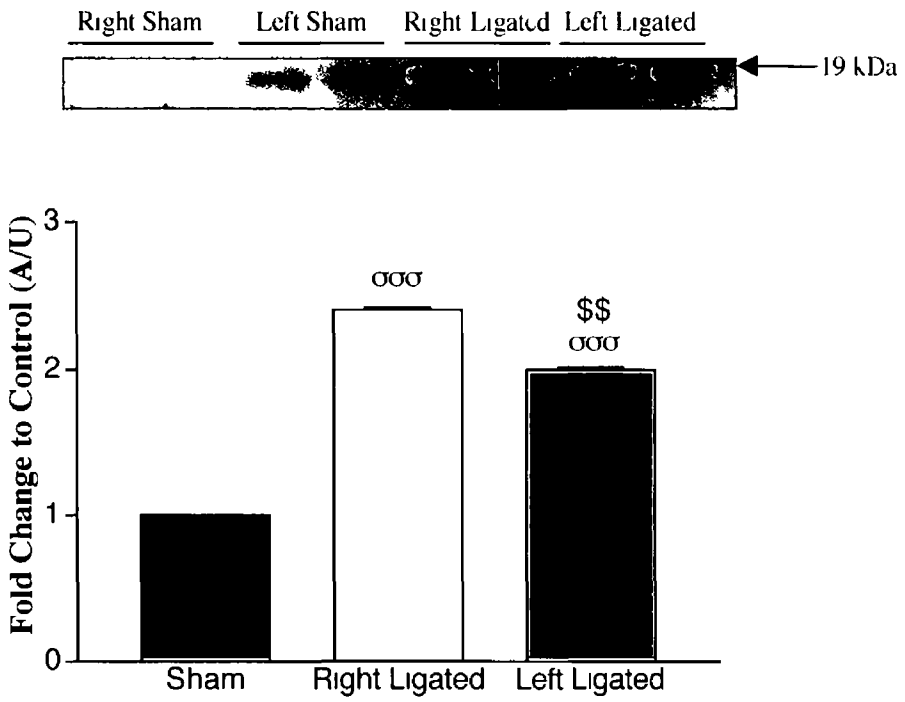



**Fig 6 4 Changes in Notch Target Gene expression in the right and left carotid arteries of ligated animals compared to sham-operated controls** The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 3 days post ligation Protein extracted from these vessels was assayed for HR1-1 (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 $^{\sigma\sigma}$ $p<0.005$, $^{\sigma\sigma\sigma}$ $p<0.0005$ as compared to sham-operated control, $^{\$\$}$ $p<0.005$ as compared to right artery of ligated animal (student s t test)

### 6 2 1 2 Twenty Eight Days Post-Carotid Ligation

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 levels (arbitrarily assigned a value of 1)

Notch 1 IC protein levels were decreased in both the right and left carotid arteries of ligated animals (Figure 6 5) 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 (Figure 6 6) 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 (Figure 6 7) 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 (Figure 6 7A) 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 (Figure 6 7B) 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 In addition, the level of HRT-3 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 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 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 6 5 Changes in Notch 1 Receptor Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation**

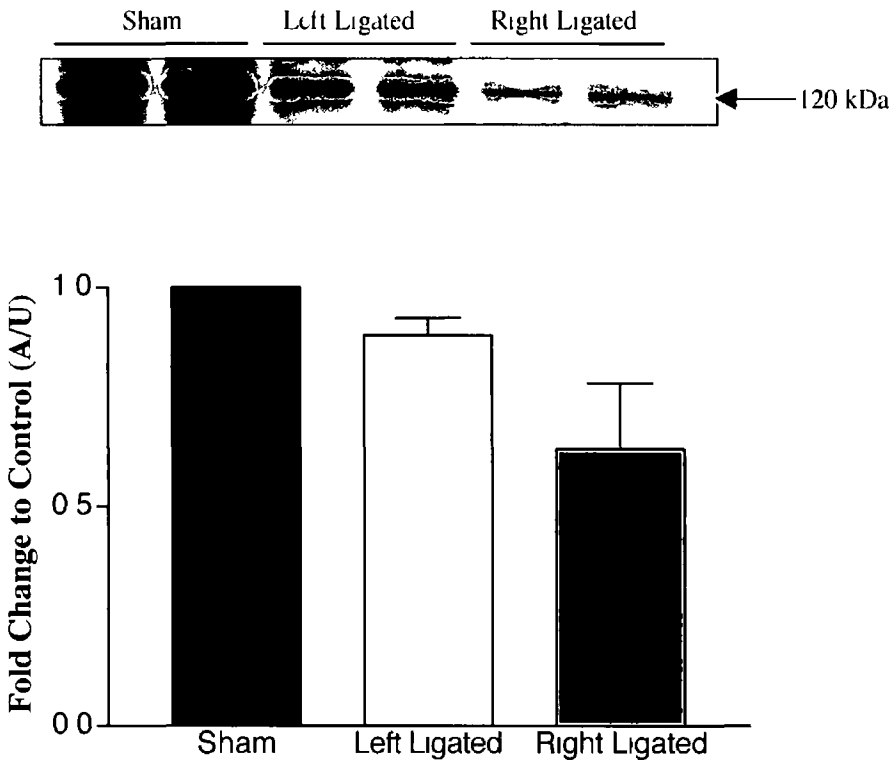



**Fig 6 5 Changes in Notch 1 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls** The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 28 days post 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 6 6 Changes in Notch 3 Receptor Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation**

**Notch 3**

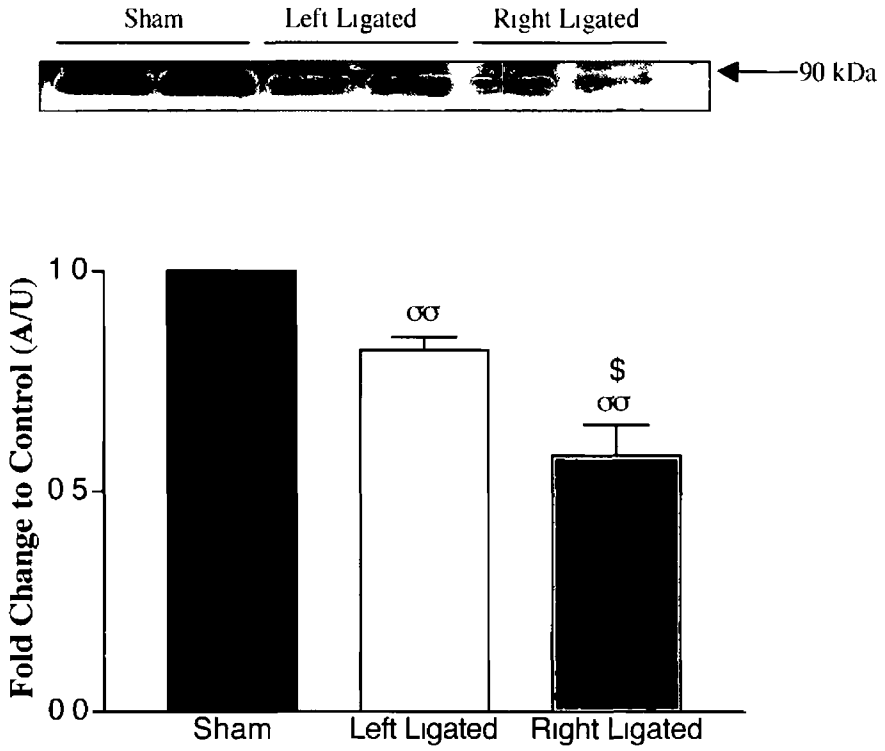
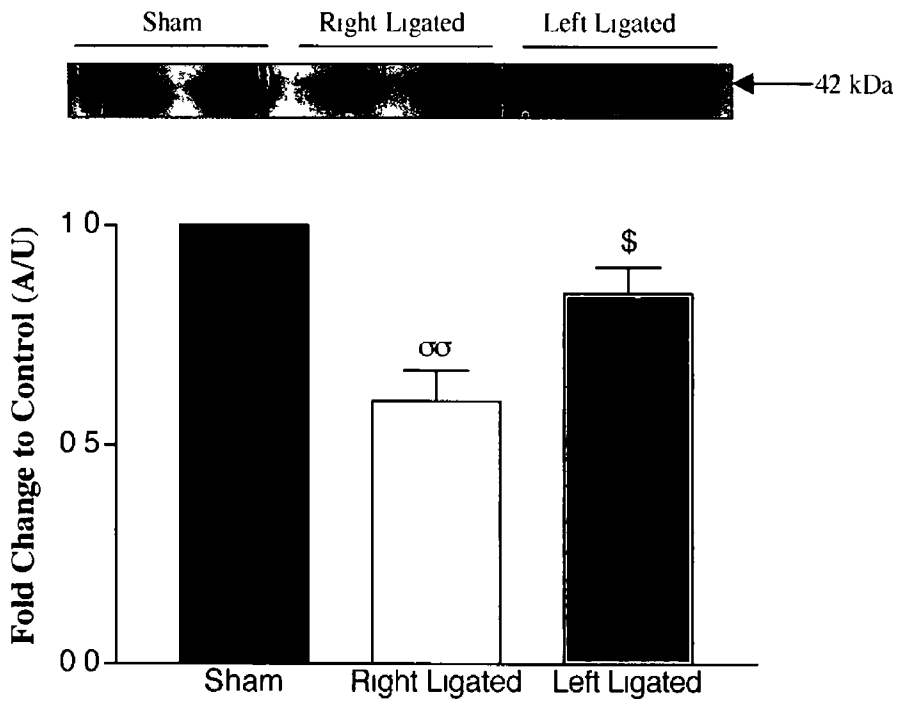


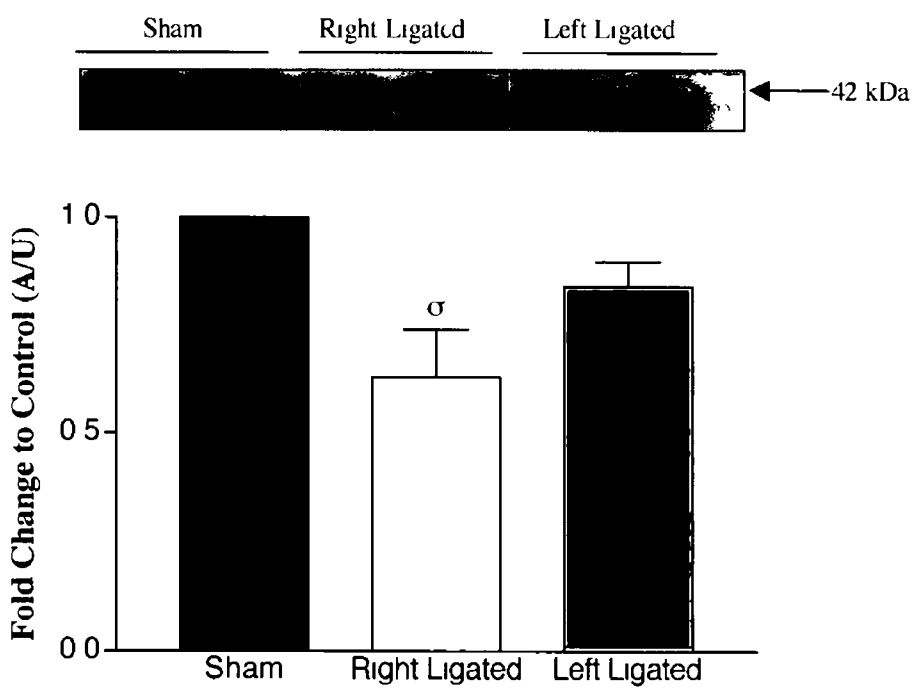

**Fig 6 6 Changes in Notch 3 receptor expression in the right and left carotid arteries of ligated animals compared to sham-operated controls** The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 28 days post 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 $^{\text{ϞϞ}}$ $p<0.005$ as compared to sham-operated control, $^{\$}$ $p<0.05$ as compared to left ligated artery (student's t test)

**Fig 6 7 Changes in Notch Target Gene Expression in Ligated versus Sham-operated Animals 28 Days Post-Carotid Ligation**

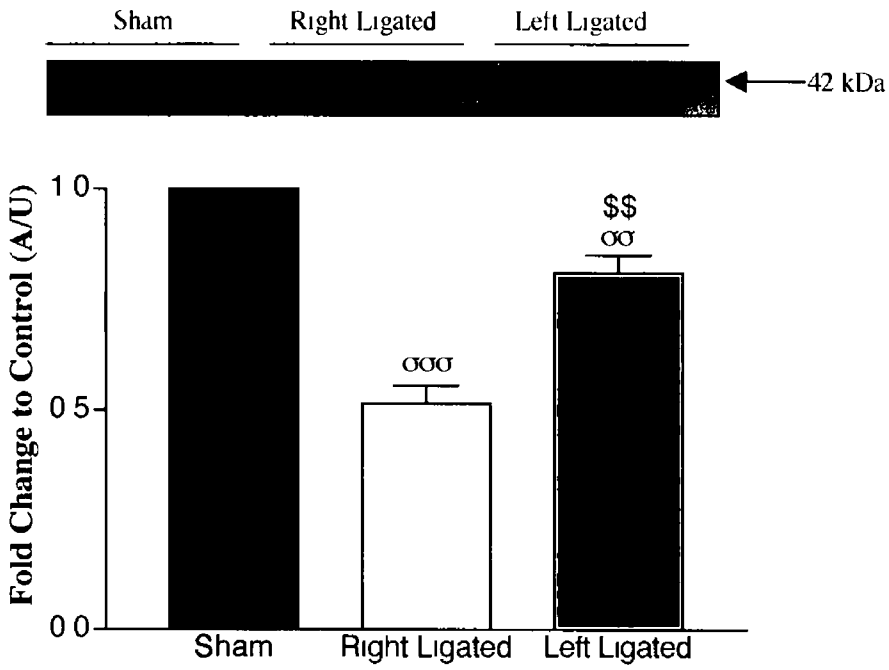
**A HRT-1**



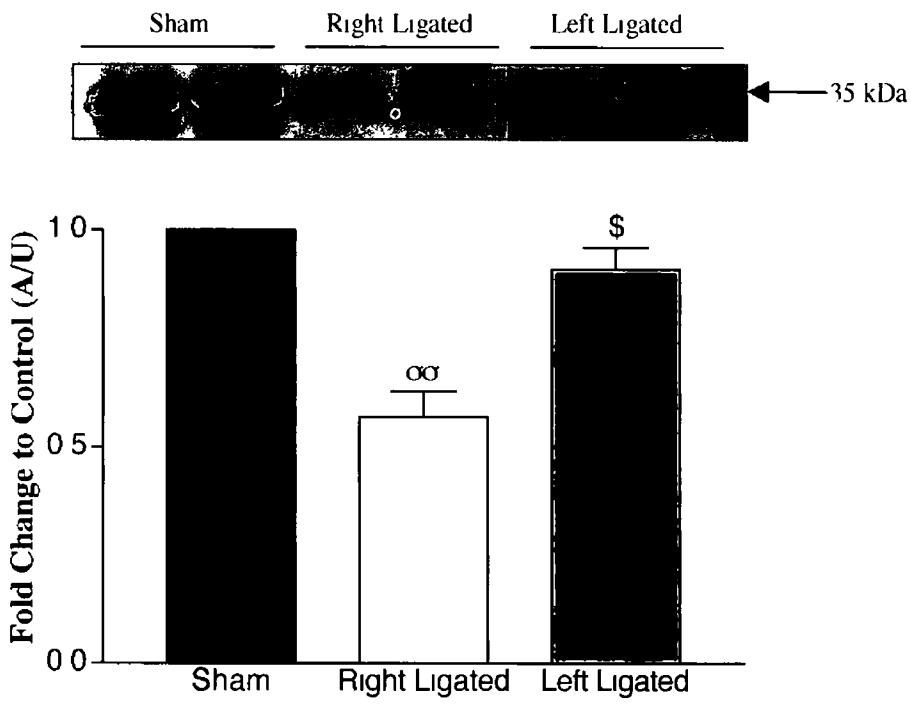

**B HRT-2**



**C HRT-3**


Sham
Right Ligated
Left Ligated
42 kDa
Fold Change to Control (A/U)
1 0
0 5
0 0
$$
σσ
σσσ
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
$
σσ
Sham
Right Ligated
Left Ligated

**E Hes-5**

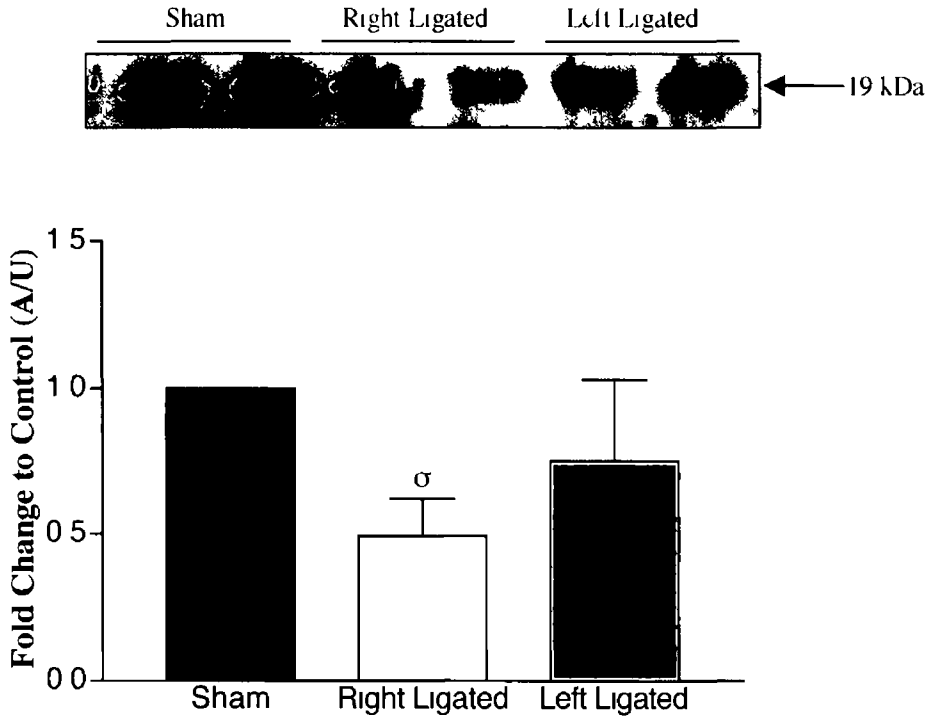



**Fig 6 7 Changes in Notch Target Gene expression in the right and left carotid arteries of ligated animals compared to sham-operated controls** The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 28 days post 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 $^{\sigma}$ p<0 05, $^{\sigma\sigma}$ p<0 005, $^{\sigma\sigma\sigma}$ 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)

### 6 2 2 Portal Vein Ligation

The 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 Blood flow was measured 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 (Figure 6 8A) 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 (Figure 6 8B) 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

Expression levels of Notch receptors, ligand and target genes in the ligated models were analyzed, 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 The Notch signalling pathway generally appears to exhibit a triphasic pattern of expression in response to portal vein ligation over the time period studied Expression of components of the signalling pathway was generally decreased initially, at 2 days post-ligation Expression of Notch signalling 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 (Figure 6 9A) A considerable fold increase in Notch 1 IC protein expression was evident however 4 days post-ligation, with the level of Notch 1 IC protein increased in the ligated animal by 60% over sham-operated control However, 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 Figure 6 9B 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 also observed for Notch 3 IC protein (Figure 6 10A) Notch 3 IC protein expression was initially decreased 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 The 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 (Figure 6 10B) 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 (Figure 6 11A) 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 (Figure 6 11B) 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, analysis of Notch target gene protein expression levels revealed a similar pattern of expression to that of the Notch receptors (Figure 6 12 - Figure 6 16) HRT-1 expression analysis revealed a considerable decrease of 40% in expression 2 days post-ligation, as compared to sham-operated control (Figure 6 12A) 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 Figure 6 12B 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 (Figure 6 13A) Analysis of HRT-2 protein expression 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 Figure 6 13B, 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 (Figure 6 14A) 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 (Figure 6 14B)

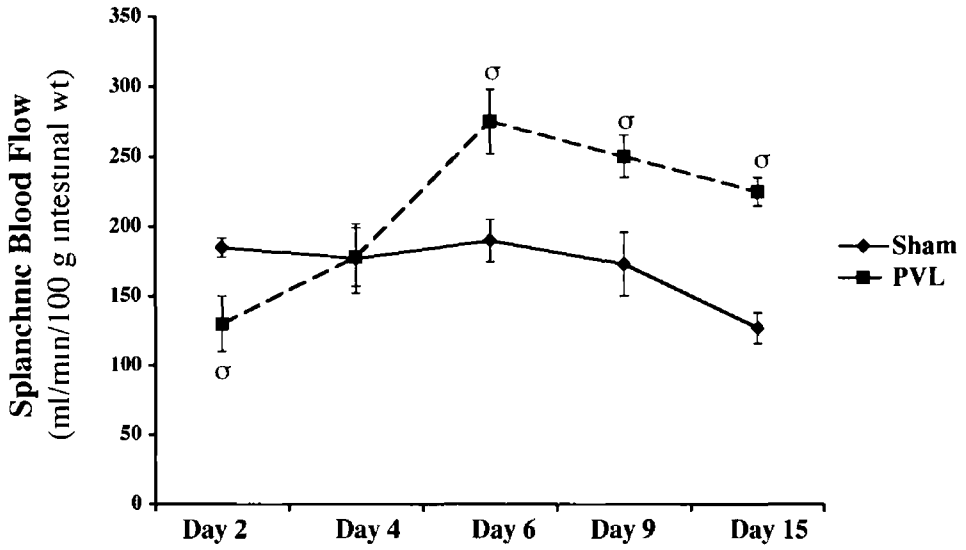
Unlike the Notch receptors, or the HRT target genes studied, the Hes target genes exhibited an increase in protein expression of 33% and 70% over sham-operated control 2 days post-ligation respectively (Figures 6 15A and 6 16A) The subsequent expression pattern of these target genes, however, mirrors that of the other components of the Notch signalling pathway studied Hes-1 protein expression was increased 4 days post-ligation by 54% fold over sham-operated control (Figure 6 15A) 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 (Figure 6 16A) 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 signalling 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 (Figure 6 15B), 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 (Figure 6 16B)

This data clearly shows altered expression of Notch signalling pathway components both following control surgery and PVL Therefore, analysis of the effect of PVL on protein expression requires that the expression in the ligated animal is compared to the appropriate sham-operated animal at each timepoint However, the presentation of the absolute protein expression values reveal that expression of Notch signalling pathway components is decreased at days 6, 9 and 15 post-ligation, as compared to sham-operated controls

**Fig 6 8 Splanchnic Blood Flow and Splenic Pressure following Portal Vein Ligation**

**A Blood Flow**




**B Splenic Pressure**

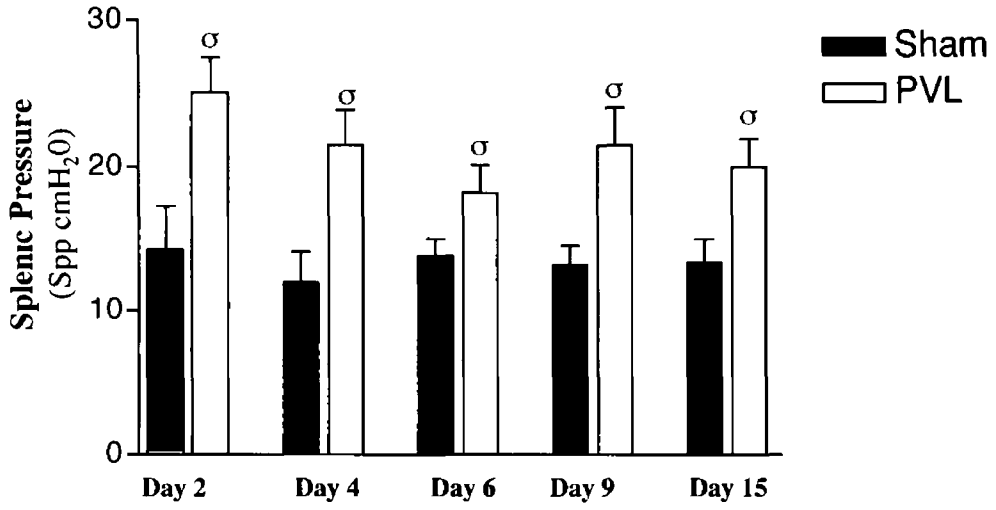



**Fig 6 8 Graphs showing splanchnic blood flow and splenic pulse pressure in young rats post-PVL compared to sham-operated control animals** A) A Transonic flowprobe was used to measure splanchnic blood flow in both ligated and sham-operated animals at at 2, 4, 6, 9 and 15 days post-ligation B) Portal venous pressure was measured indirectly with direct splenic puncture (splenic pulse pressure using a Doppler flow probe) n=3 $^{\sigma}p<0.05$ as compared to sham-operated control (student's t test)

**Fig 6 9 Changes in Notch 1 IC Receptor Expression in Portal Vein Ligated versus Sham-operated Animals**

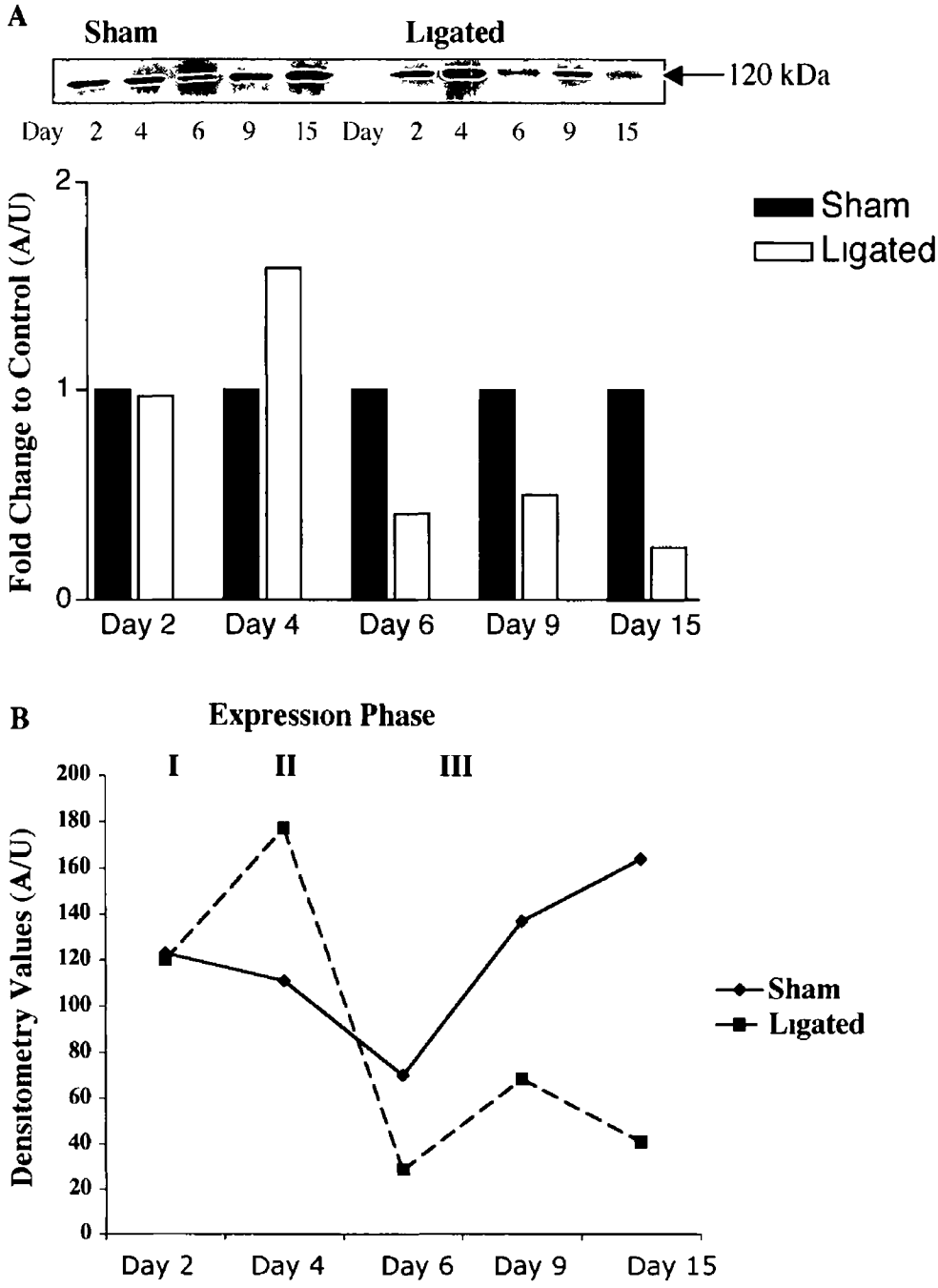



**Fig 6 9 Changes in Notch 1 IC expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these 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 6 10 Changes in Notch 3 IC Receptor Expression in Portal Vein Ligated versus Sham-operated Animals**

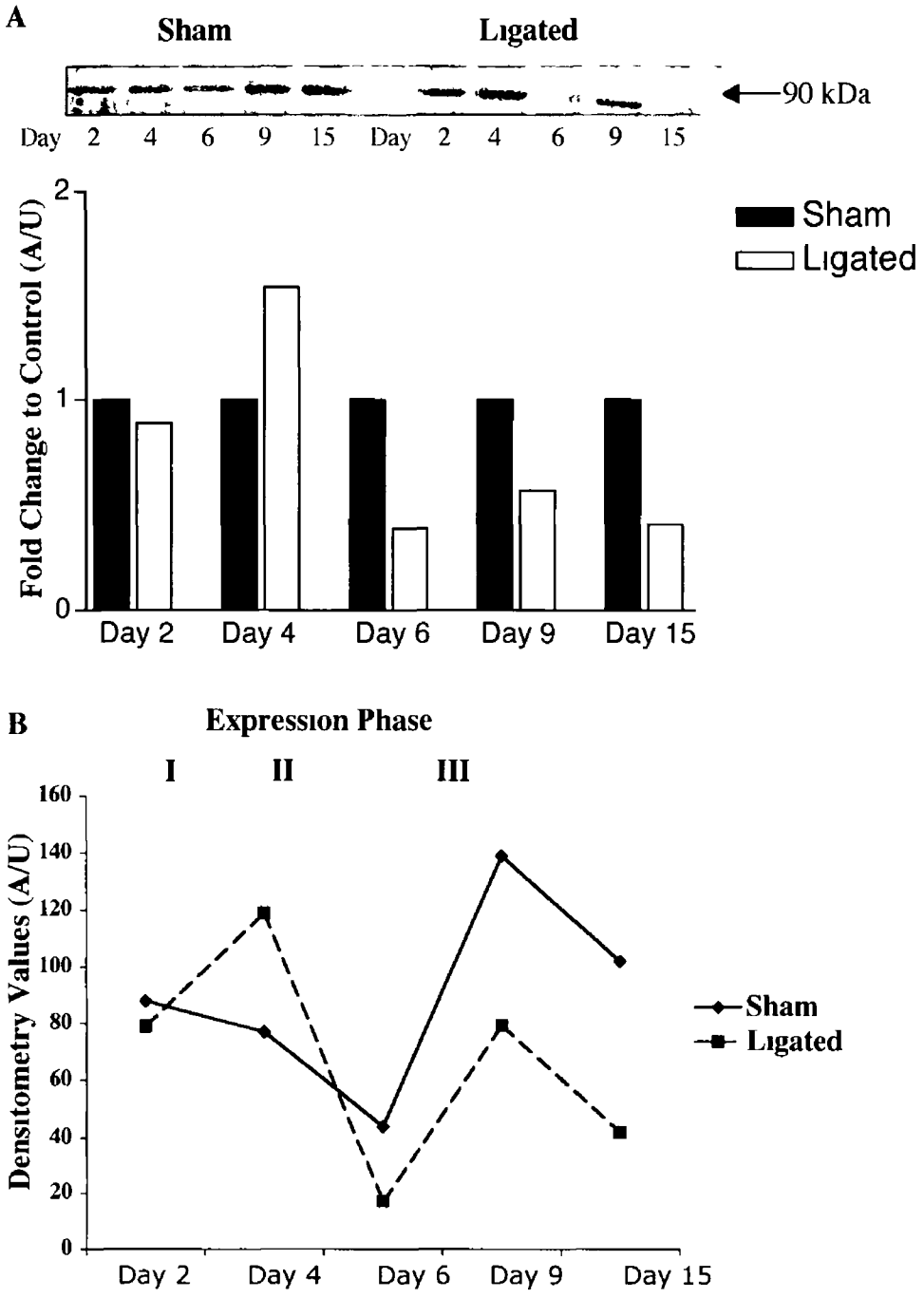



**Fig 6 10 Changes in Notch 3 IC expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these 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 6 11 Changes in Jagged Expression in Portal Vein Ligated versus Sham-operated Animals**

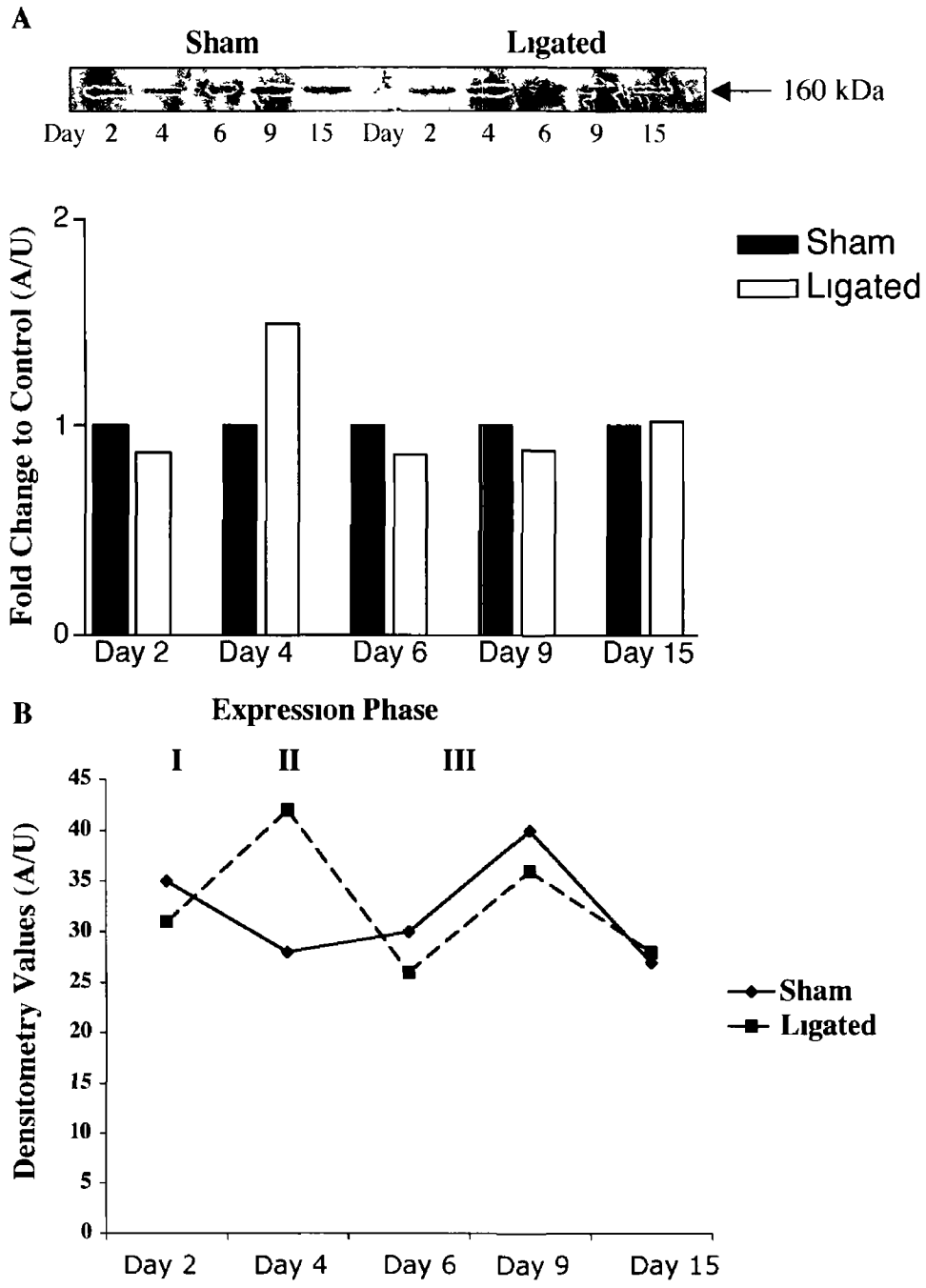



**Fig 6 11 Changes in Jagged expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these 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 6 12 Changes in HRT-1 Expression in Portal Vein Ligated versus Sham-operated Animals**

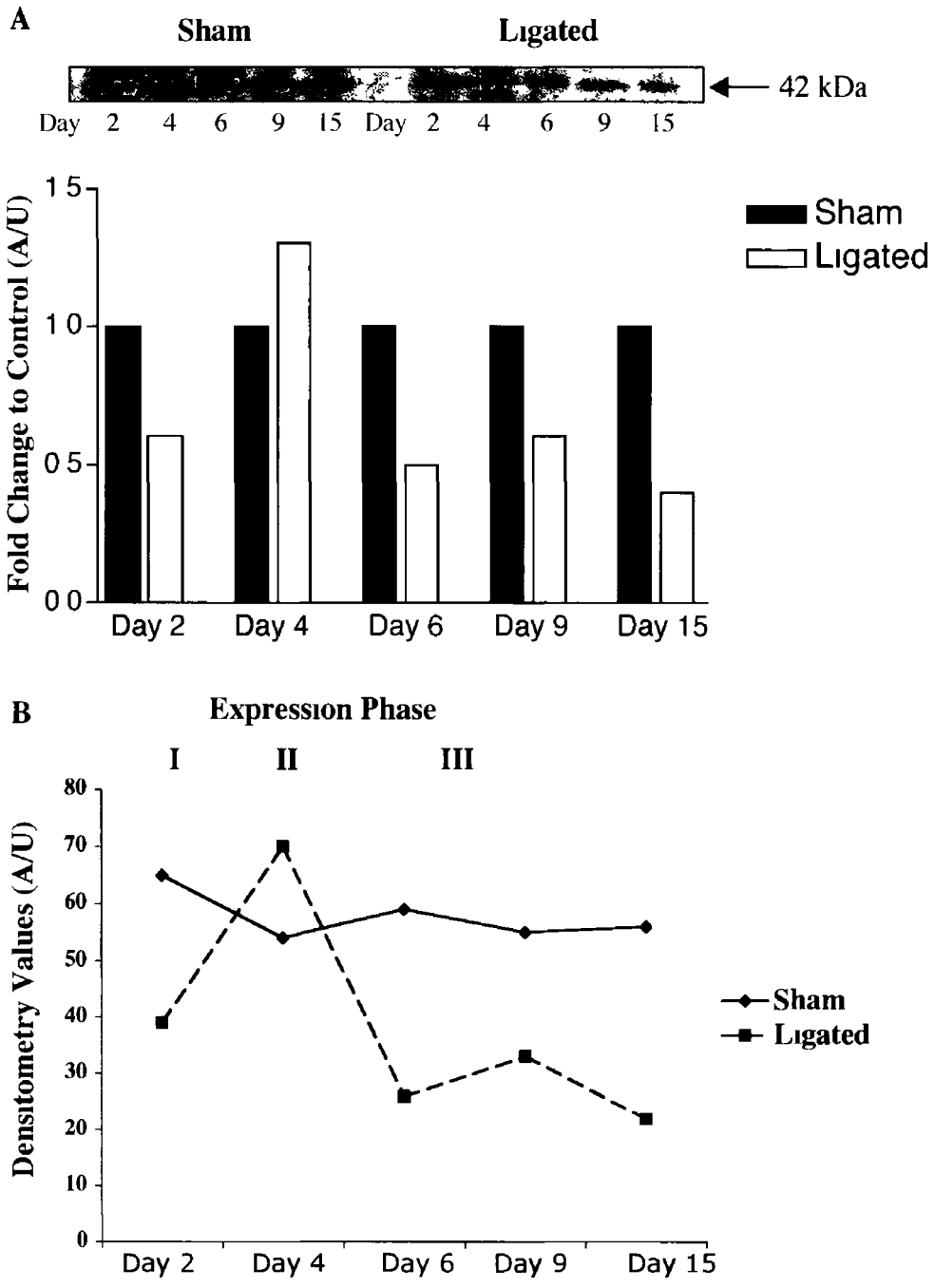



**Fig 6 12 Changes in HRT-1 expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these vessels 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 6 13 Changes in HRT-2 Expression in Portal Vein Ligated versus Sham-operated Animals**

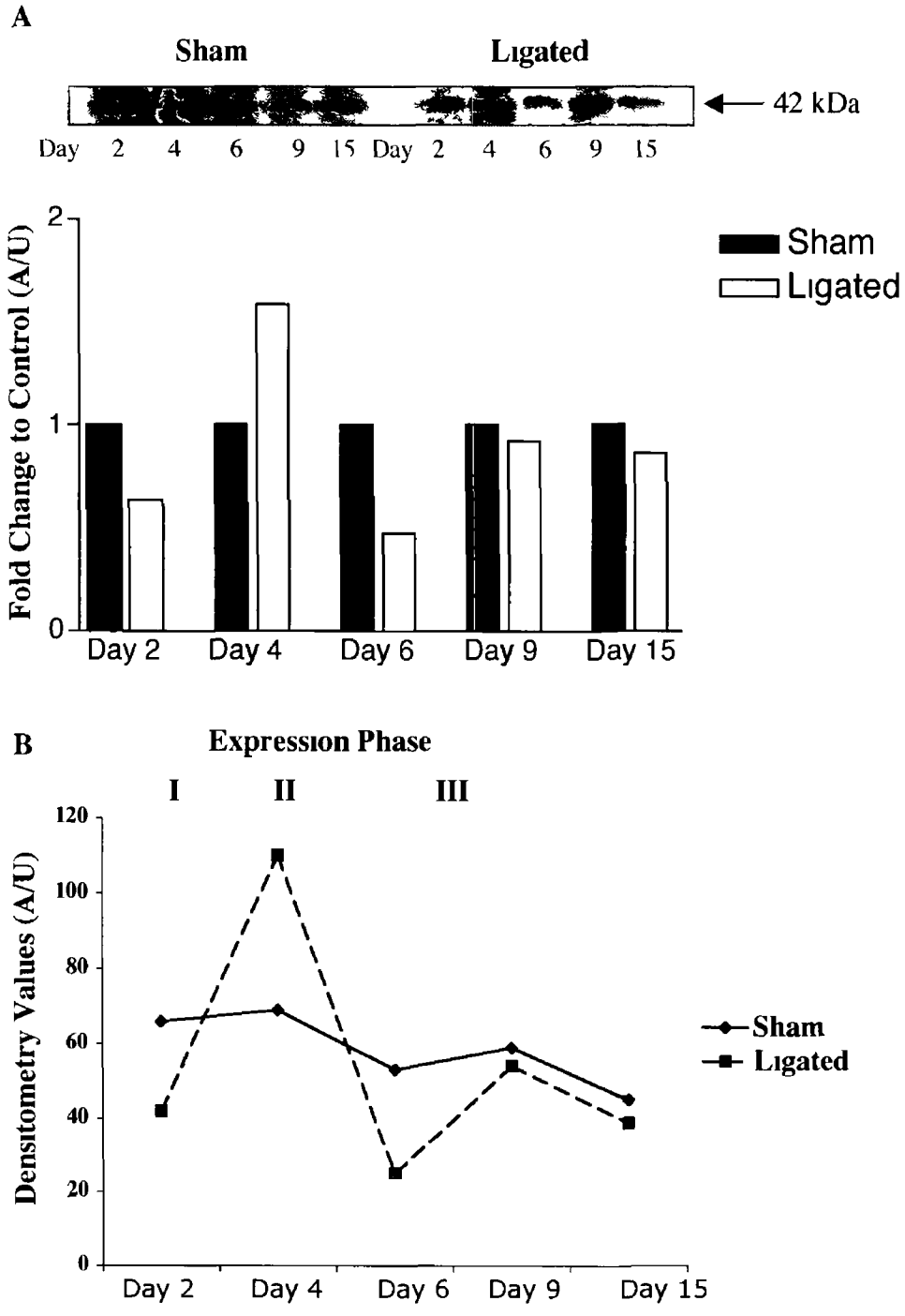



**Fig 6 13 Changes in HRT-2 expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these vessels 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 6 14 Changes in HRT-3 Expression in Portal Vein Ligated versus Sham-operated Animals**

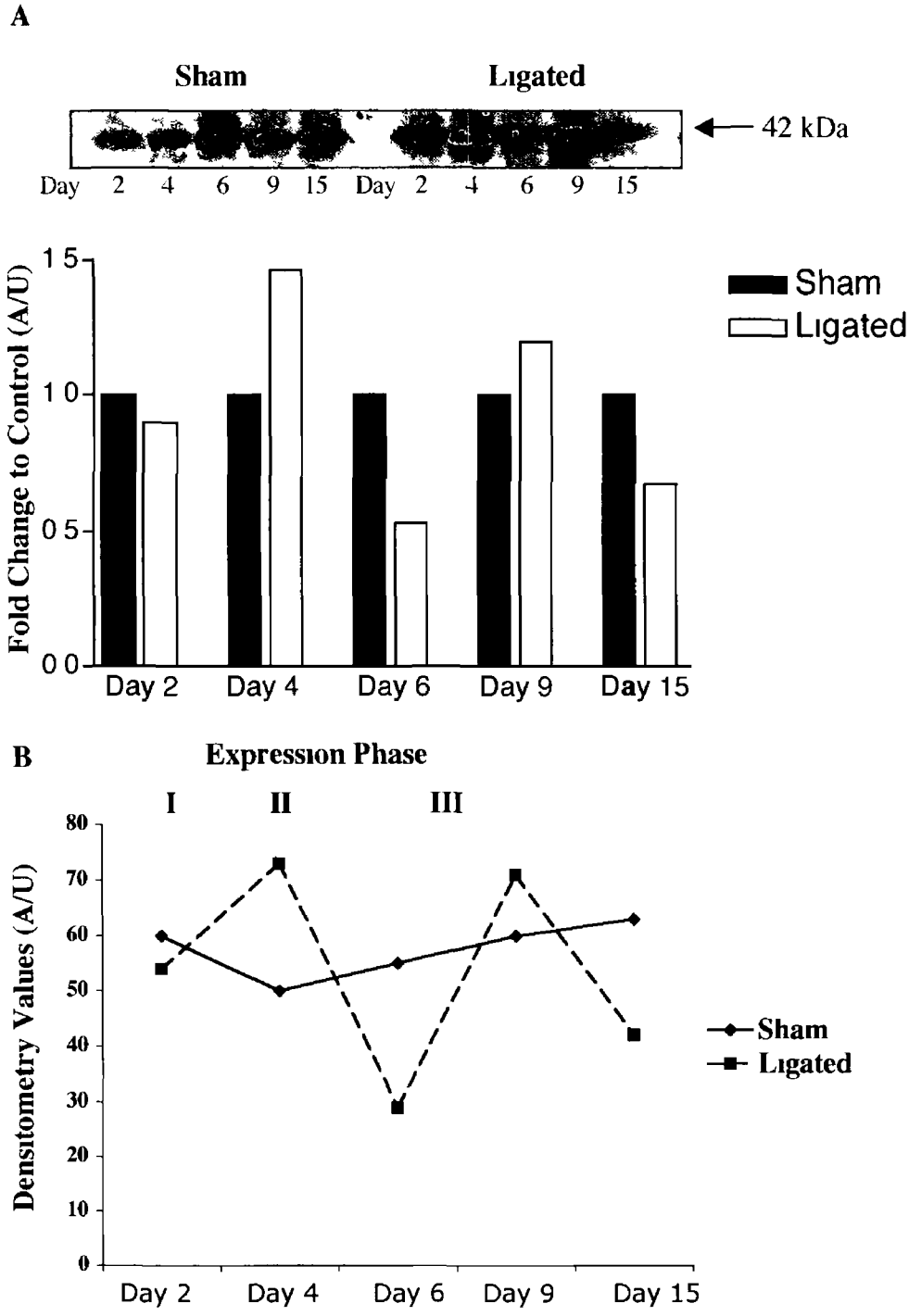



**Fig 6 14 Changes in HRT-3 expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these vessels 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 6 15 Changes in Hes-1 Expression in Portal Vein Ligated versus Sham-operated Animals**

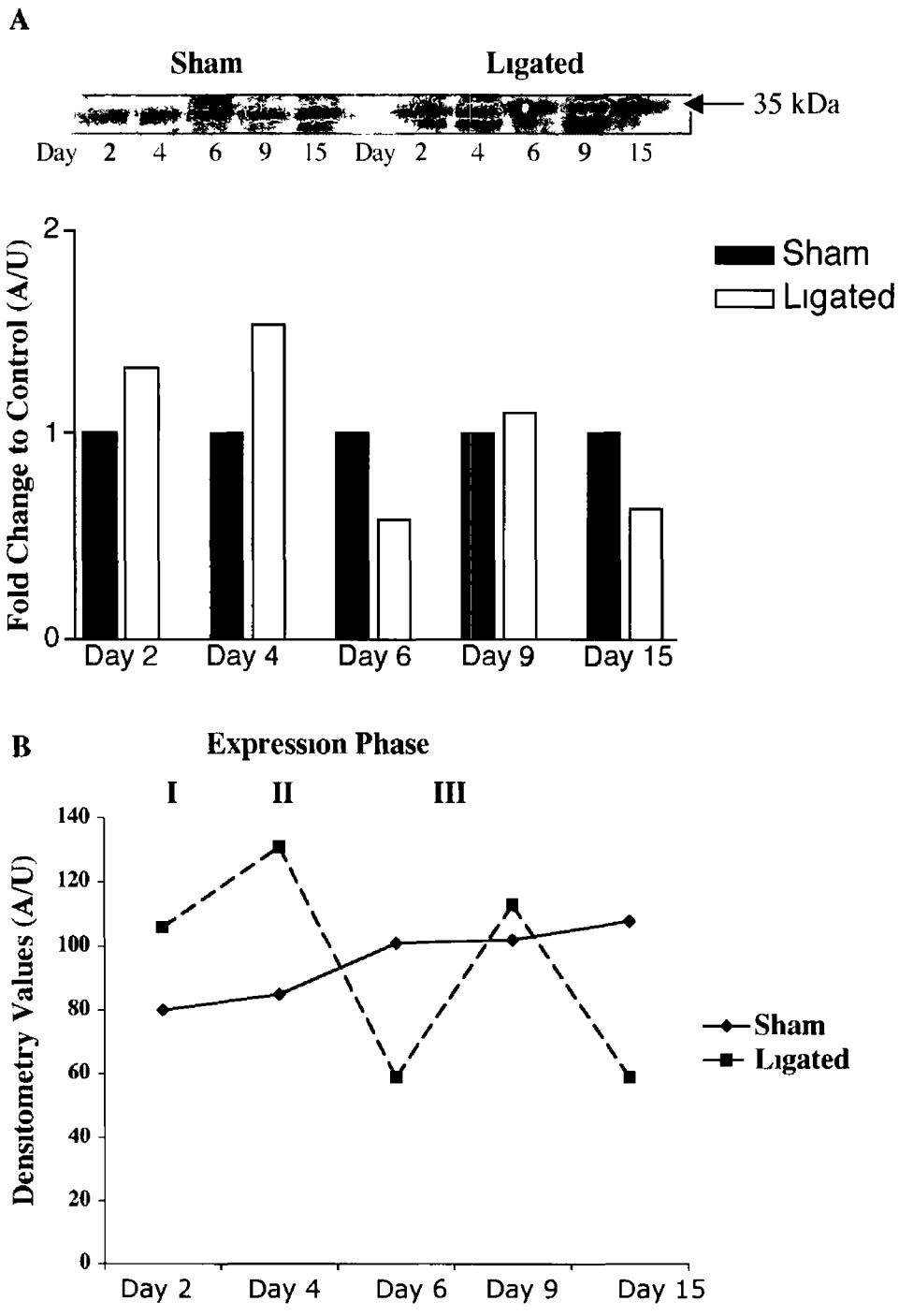



**Fig 6 15 Changes in Hes-1 expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted from these vessels 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 6 16 Changes in Hes-5 Expression in Portal Vein Ligated versus Sham-operated Animals**

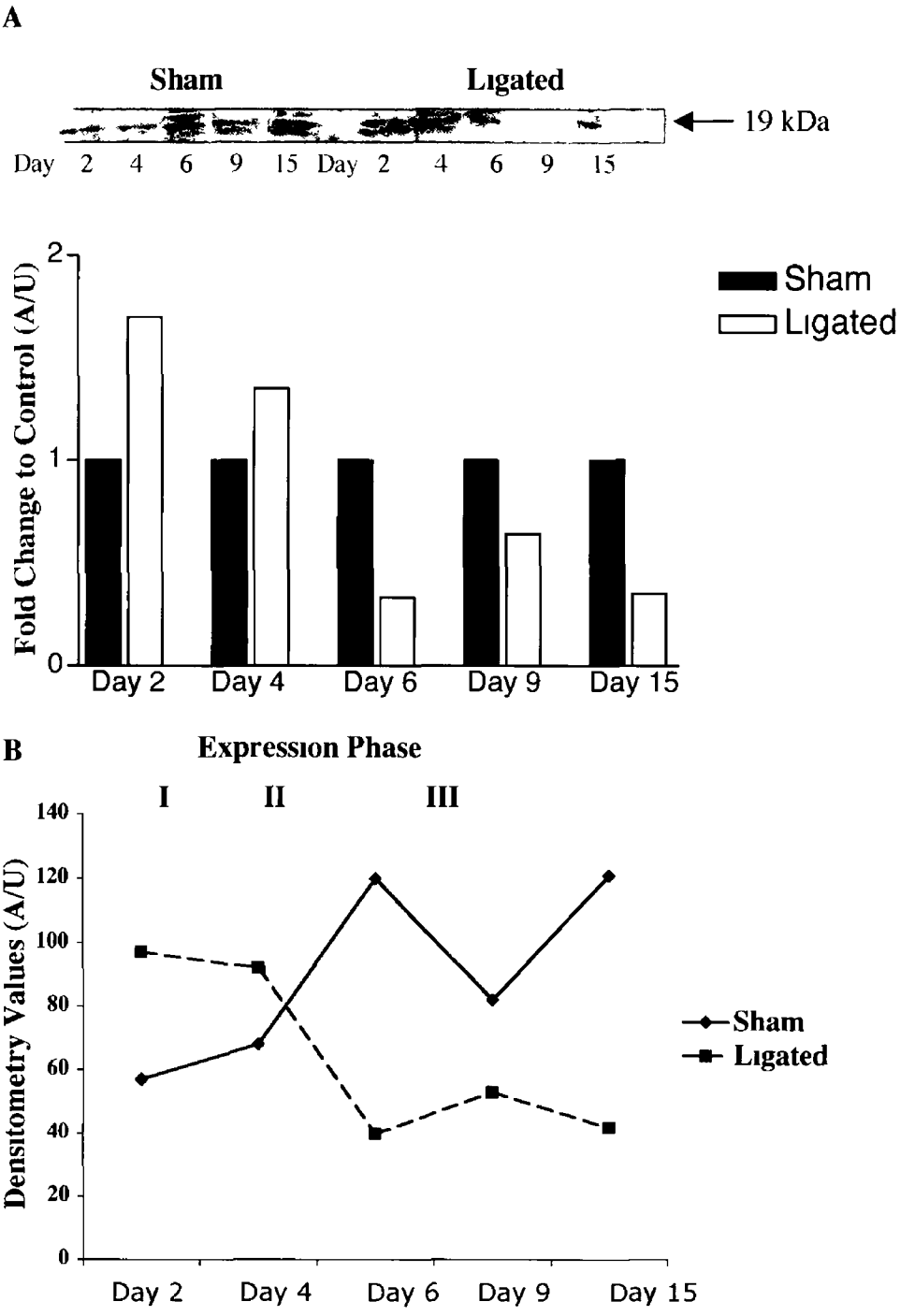



**Fig 6 16 Changes in Hes-5 expression in portal vein ligated animals compared to sham-operated controls** The portal veins of young rats were ligated at day 0 The portal veins of both sham-operated and ligated animals were harvested 2, 4 6, 9 and 15 days post ligation Protein extracted from these vessels 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

### 6 2 3 The Effect of Altered Mechanical Forces on Apoptosis *in vivo*

The effect of altered mechanical forces on apoptosis *in vivo* was studied using both the carotid ligation and portal vein ligation models Apoptosis was measured using caspase 3 activity assays and western blotting analysis for Bcl-2 on ligated and sham-operated samples

Caspase 3 activity in vessels 3 days post-carotid ligation was significantly decreased in the right carotid arteries of ligated animals as compared to sham-operated control (Figure 6 17A) Caspase 3 activity was reduced to 0 79±0 05 and 0 95±0 02 as compared to sham-operated control in the right and left carotid arteries respectively, which corresponds to decreases of 21 0±5 0% and 5 0±2 0% Conversely, caspase 3 activity was increased in both the right and left arteries of ligated animals 28 days post carotid ligation, as compared to sham-operated control (Figure 6 17B) The increase in caspase 3 activity for the right and left carotid arteries of ligated animals was 1 46±0 1 and 1 26±0 02 respectively over sham-operated control

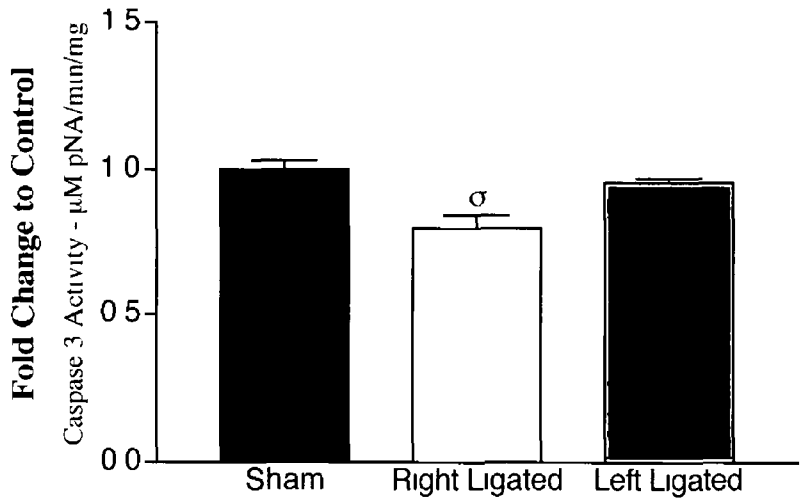
Analysis of caspase 3 activity following portal vein ligation revealed significant fold increases in caspase 3 activity of 3 99±0 08, 2 75±0 1 and 1 49±0 04 at 2, 6 and 15 days post-ligation (Figure 6 17C) In contrast, caspase 3 activity remained unchanged as compared to static control at 4 days (1 06±0 03) and 9 days (1 09±0 06) post-ligation Caspase 3 activity is altered in PVL sham-operated animals (Figure 6 17D) Increases in caspase 3 activity are seen from days 2 - 4 and days 6 - 9 post-surgery, whereas caspase 3 activity is decreased from days 4 - 6 and days 9 - 15 post-surgery

Western blot analysis revealed significant up-regulation of Bcl-2 protein in both the right and left carotid arteries 3 days post carotid ligation (Figure 6 18A) Bcl-2 expression was increased in the right carotid by 31 0±2 0%, and in the left carotid by 25 01 0%, as compared to sham-operated control Bcl-2 protein expression was decreased in the right carotid artery by 49 0±13 0% 28 days post ligation, as compared to sham-operated control (Figure 6 18B) However, expression of Bcl-2 at this timepoint in the left ligated carotid artery was similar to sham-operated control, decreased only by 5 0±7 0%, and was significantly increased over the expression level observed in the right carotid artery The level of Bcl-2 expression following portal vein ligation was also analyzed Bcl-2 expression was considerably increased 2 days post ligation by 134% fold over sham-operated control, and lesser increases were also evident at 4 days (23%) and 9 days (27%) post ligation Conversely, decreases in Bcl-2 expression of 47% and 65% as compared to sham-operated control were apparent 6 and

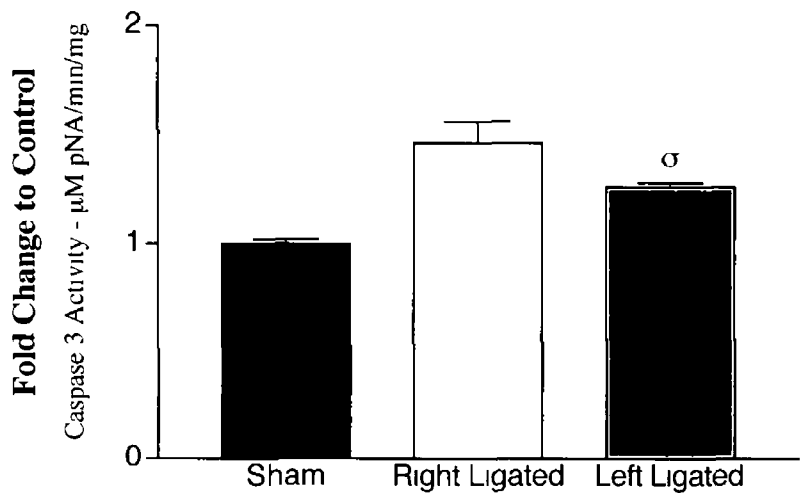
15 days post-ligation respectively Changes in Bcl-2 expression were also noted in sham-operated animals post-surgical intervention Bcl-2 expression increases from day 2 - day 6 post-surgery, decreases at day 9 and increases at day 15, thus exhibiting a tri-phasic pattern of expression post-surgical intervention (Figure 6 18D)

**Fig 6 17 Changes in Caspase 3 Activation in Ligated versus Sham-operated Animals**

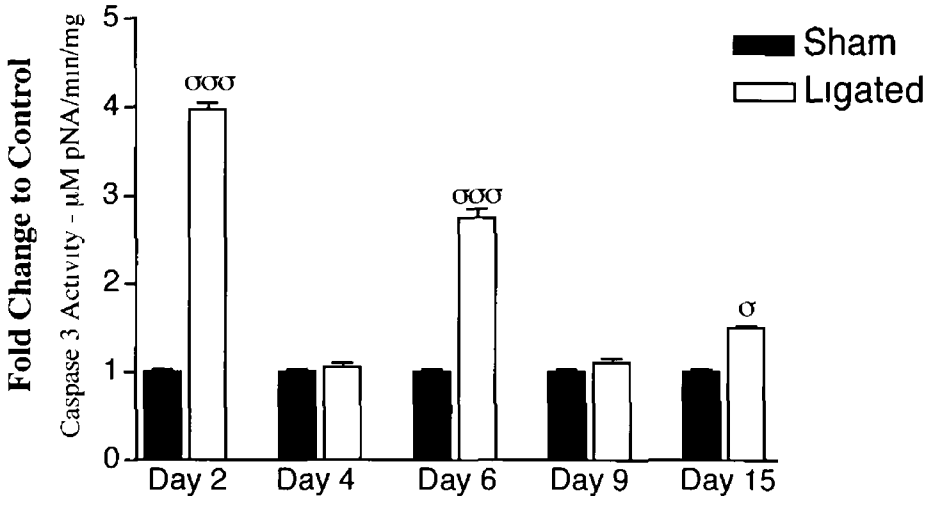
**A 3 Days Post-Carotid Ligation**




**B 28 Days Post-Carotid Ligation**



C Caspase 3 Activity Post Portal Vein Ligation




D Caspase 3 Activity Post Portal Vein Ligation

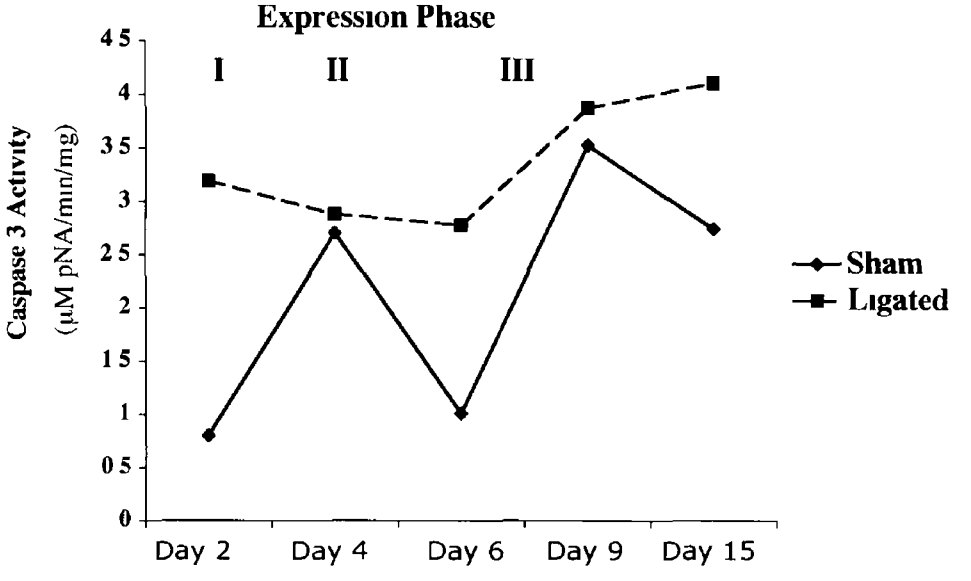
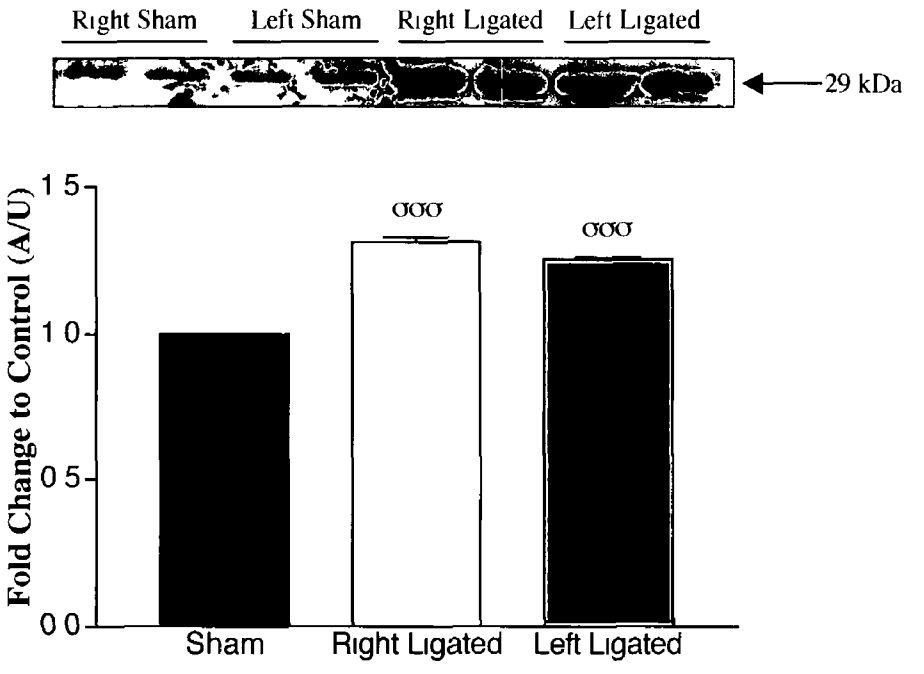


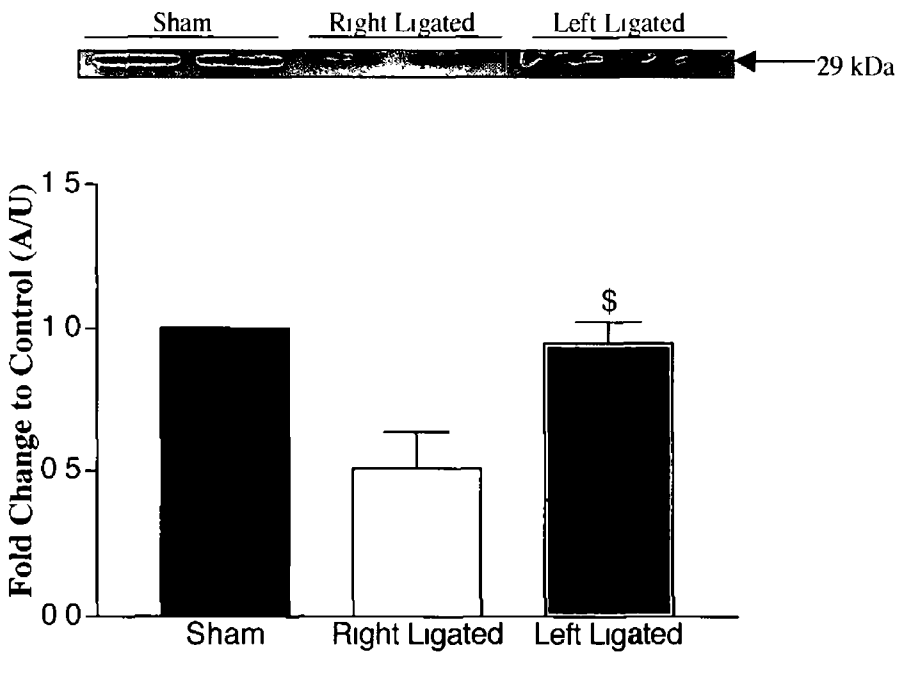

**Fig 6 17 Changes in caspase 3 activation in ligated versus sham-operated animals**
Changes in capase 3 activity the right and left carotid arteries of ligated animals compared to sham-operated controls at 3 days (A) and 28 days (B) post ligation The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 3 and 28 days post ligation as indicated (C + D) Changes in caspase 3 activity 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 vessels was assayed for caspase 3 activity (C) Values are expressed as fold increase over sham operated animals (arbitrarily assigned a value of 1), n=1, two vessels were pooled for each protein preparation $^{\sigma}$ $p<0.05$, $^{\sigma\sigma\sigma}$ $p<0.0005$ as compared to sham-operated control (student's t test (D) Values showing changes in caspase 3 activity in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation

**Fig 6 18 Changes in Bcl-2 Expression in Ligated versus Sham-operated Animals**

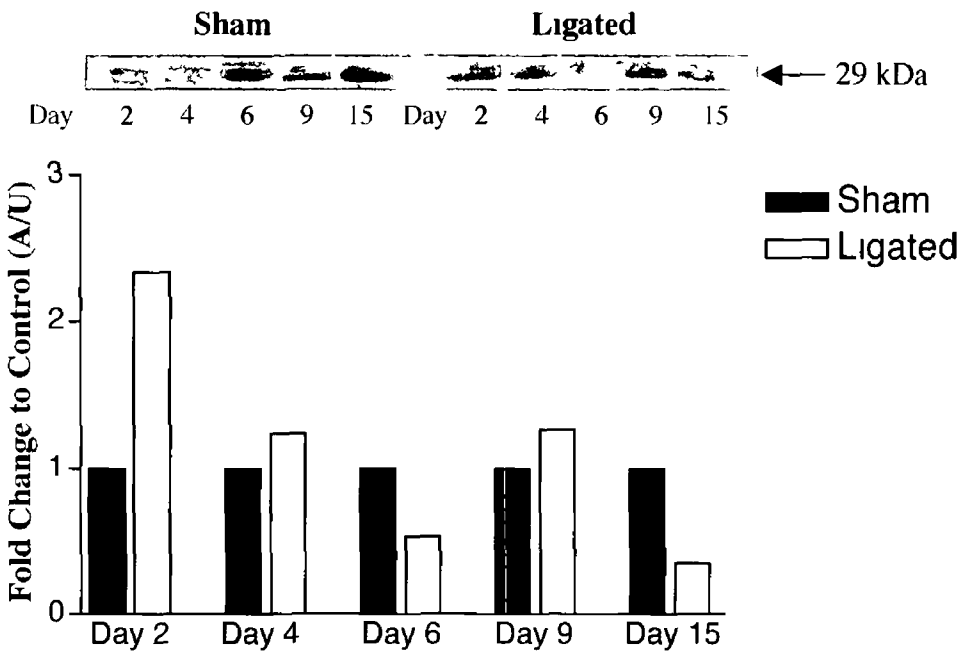
**A 3 Days Post-Carotid Ligation**




**B 28 Days Post-Carotid Ligation**



C Bcl-2 Expression Post-Portal Vein Ligation




D Bcl-2 Expression Post-Portal Vein Ligation

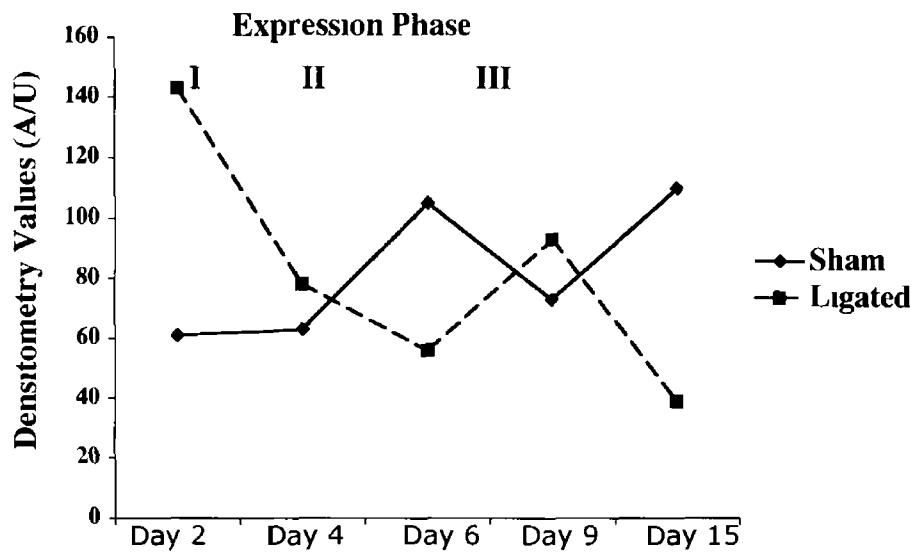



**Fig 6 18 Changes in Bcl-2 expression levels in ligated versus sham-operated animals** Changes in Bcl-2 expression in the right and left carotid arteries of ligated animals compared to sham-operated controls at 3 days (A) and 28 days (B) post carotid ligation The left carotid artery of young rats were ligated at day 0 The left and right carotids of both sham-operated and ligated animals were harvested 3 and 28 days post ligation as indicated n=2, $^{\alpha\alpha\alpha}$ p<0 0005 as compared to sham-operated control, $^{\$}$ p<0 05 as compared to right artery of ligated animal (student's t test) (C + D) Changes in Bcl-2 expression in portal vein ligated animals compared to sham-operated controls The portal veins of both sham-operated and ligated animals were harvested 2, 4, 6, 9 and 15 days post ligation Protein extracted these vessels was assayed for Bcl-2 using western blot analysis (C)Values are expressed as fold increase over sham-operated animals (arbitrarily assigned a value of 1) n=1, two vessels were pooled for each protein preparation (D) Absolute densitometry values showing changes in Bcl-2 expression in both sham-operated and ligated animals, n=1, two vessels were pooled for each protein preparation

## 6 3 Discussion

This study has clearly established the co-ordinate regulation of Notch signalling pathway component expression and apoptosis *in vivo*, using two models of vascular disease associated with vascular remodelling This complements our previous data showing the cyclic strain-mediated down-regulation of Notch signalling pathway expression and activity, in addition to the anti-apoptotic effect of this pathway on cyclic strain-induced apoptosis *in vitro*

Partial ligation of the left carotid artery in the rat results in a significant increase in blood flow in the contralateral right carotid artery, at both 3 and 28 days post-ligation In contrast, the left ligated carotid artery exhibits a significant decrease in blood flow 3 days post-ligation, an effect which is also sustained at 28 days post-ligation The level of blood flow alteration observed in our model is consistent with previous reports using the same model (Sullivan and Hoying 2002, Korshunov and Berk 2003) These studies report a 40-70% increase in blood flow in the right carotid artery, and a concomitant 80-90% decrease in blood flow in the left carotid artery Korshunov and Berk 2003 also show that systolic blood pressure remains equal in sham-operated and ligated animals at both 3 and 28 days post-carotid ligation This suggests that a vascular remodelling event occurs in both the right and left carotid of the ligated animal to compensate for altered flow rates in these vessels As changes in cell fate decisions are associated with vascular remodelling, it is likely that changes in Notch signalling pathway expression is associated with vascular remodelling in both the right and left carotid arteries *in vivo*

Notch signalling pathway component expression was examined in this study in the rat carotid model at 3 and 28 days post-ligation, and in the PVL model over a time-course of 2 - 15 days post-ligation In both cases, a bi-phasic pattern of Notch signalling pathway component expression emerged when compared to sham-operated control expression levels

The rat carotid ligation model exhibits an up-regulation of Notch signalling pathway component expression 3 days post-ligation, with respect to sham-operated control Whilst all receptors and target genes are significantly upregulated, the most dramatic increase in receptor and target gene expression is evident with Notch 3 and HRT-2 It is interesting to note that a similar level of up-regulation is observed in both the right and left carotid arteries of the ligated animal, suggesting that up-regulation of the Notch signalling pathway is an acute response to alterations in blood flow and

biomechanical stress, and is associated with an acute remodelling event in both the right and left carotid arteries *in vivo* In contrast, analysis of Notch signalling pathway expression 28 days post-carotid ligation reveals a decrease in Notch signalling pathway component expression in both the left and right carotid arteries of ligated animals, as compared to sham-operated control Interestingly, the level of Notch signalling pathway expression in the high flow right carotid artery is decreased, oftentimes significantly, compared to that in the low flow left carotid Therefore, in light of our previous results, we propose that the increased level of cyclic strain experienced in the right carotid artery may account for the decrease in Notch signalling pathway expression, as compared to that in the left carotid artery It is interesting to note that expression of Notch signalling pathway components is decreased under both low and high flow conditions as compared to sham-operated control This may initially appear surprising due to the increased and decreased flow rates, and subsequent biomechanical stress, in the right and left carotid arteries respectively However, despite this, a recent study has shown that both the right and left carotid arteries undergo outward vascular remodelling, with the level of remodelling increased in the right carotid artery as compared to its contralateral left counterpart Therefore, it is unsurprising that differing biomechanical stimuli that result in the same type of vascular remodelling affects Notch signalling pathway expression in a similar manner

An initial decrease in splanchnic blood flow 2 days post-PVL was observed in the ligated animal, as compared to the sham-operated control However, the subsequent increase in splanchnic blood flow compared to control evident at days 6 - 15 post-ligation, despite the continued obstruction to portal inflow caused by ligation, is suggestive of vascular remodelling in the ligated vessels at these timepoints Analysis of splenic pressure, and hence portal venous pressure, revealed significant increases in pressure in the ligated animals at all timepoints post-ligation versus sham-operated control, which is suggestive of increased vascular remodelling in the ligated vessel

Notch signalling pathway expression in the PVL model of portal hypertension reveals a bi-phasic pattern of expression, as compared to sham-operated control, similar to that observed following carotid artery ligation The general pattern exhibited was an up-regulation of Notch signalling pathway expression 4 days post-ligation, and a subsequent down-regulation of expression 6 days post-ligation, which was generally sustained at 9 and 15 days post-ligation A tri-phasic pattern for both HRT-3 and Hes-1 expression was also observed, in that increases in expression of these proteins was evident 9 days post-ligation, as compared to sham-operated controls Interestingly,

although the bi-phasic pattern of expression was conserved with the Notch ligand Jagged, the fold change in expression was not as pronounced as with any of the other Notch signalling pathway components examined This suggests that, in addition to changes in Notch receptor activation, other factors, such as those affecting the level of Notch turnover within the cell, may also affect the level of Notch receptor and target gene expression Whilst all the Notch target genes exhibited a similar fold changes in expression, it is interesting to note that only the Hes target genes exhibited an increase in expression 2 days post-ligation, thus highlighting the oftentimes differential regulation of the Hes and HRT target genes, or suggesting that factors, other than the Notch signalling pathway, may be regulating Hes expression at this stage

The observed bi-phasic pattern of Notch signalling pathway expression following both the carotid artery ligation and PVL models is somewhat unsurprising, as a group from the Morehouse School of Medicine, Atlanta (Campos *et al*, 2002, Wang *et al*, 2002a) claim to have observed a bi-phasic pattern of Notch signalling pathway expression following balloon catheter injury to the rat carotid artery However, the pattern of expression that they observe conflicts with our report They observed an initial down-regulation of Jagged, Notch 3 and HRT-1 3 days following balloon injury (Wang *et al*, 2002a), and an up-regulation of both Jagged and Notch 3 expression 28 days post injury, whilst no increase was observed in HRT-1 expression levels (Campos *et al*, 2002) However, whilst these changes in Notch signalling pathway expression levels are no-doubt correct, the validity of their claim of bi-phasic expression must be questioned, as Wang *et al*, measured expression in medial SMC, whereas Campos *et al*, measured same in intimal SMC, and different SMC phenotypes often respond differently to the same stimulus (Cappadona *et al*, 1999) Therefore, whilst this study does not tally with the bi-phasic expression observed in the two previous reports, we believe that we provide a more consistent model to claim bi-phasic regulation of the Notch signalling pathway in the vessel wall Also, unlike the aforementioned studies, which utilize a model of vascular intervention (balloon catheter injury), our study utilizes a model representative of vascular disease states, in which cells are exposed to consistently increased/decreased blood flow and biomechanical forces, and compared to sham-operated control In addition, another obvious difference between our study and those of Wang *et al*, and Campos *et al*, is the fact that we analyzed Notch signalling pathway expression in the vessel wall as a whole Future studies within the laboratory could additionally adopt the approach of Wang *et al*, and analyze the medial SMC layer only, however, we believe that it is also important to determine the changes in Notch

signalling expression in the entire vessel wall

The importance of apoptosis in vascular remodelling following injury is well recognized (Bennett 1999), however, the regulation of VSMC apoptosis is incompletely understood This study has, however, clearly shown that the Notch signalling pathway, at least in part, regulates VSMC apoptosis Therefore, using the rat carotid ligation and PVL models, we investigated whether alterations in biomechanical forces result in changes in vessel wall apoptosis *in vivo* This was achieved by analyzing caspase 3 activity and anti-apoptotic Bcl-2 protein expression within the vessel, as activation of caspase 3 has been observed in atherosclerotic plaques at sites of VSMC apoptosis (Mallat *et al* , 1997), and changes in Bcl-2 family expression are often observed during the pathogenesis of vascular disease (Bai *et al* , 1999, Cook *et al* , 1999)

Using the rat carotid ligation model, we observed what appears to be a bi-phasic pattern of apotosis following ligation Caspase 3 activity is decreased in the right, and to a lesser extent, in the left carotid artery 3 days post-ligation, compared to sham-operated control In addition, a concomitant increase in Bcl-2 protein expression was observed in the right and left carotid arteries of ligated animals This apparent decrease in apoptosis as an acute response to alterations in biomechanical forces within the vessel tallies with the observed increase in Notch signalling pathway component expression, and subsequent increased anti-apoptotic effect of Notch at this timepoint Analysis of this model 28 days post-carotid ligation revealed an increase in caspase 3 activity in both the right, and to a lesser extent, the left carotid arteries This was coupled with a significant decrease in Bcl-2 expression in the right, but not the left, carotid artery This data suggests that increased apoptosis could be experienced in the right carotid artery, as compared to the left carotid artery at this timepoint This data, therefore, correlates with the suggestion that whilst both the right and left carotid arteries of ligated animals undergo outward vascular remodelling, increased vascular remodelling is observed in the right carotid artery (Korshunov and Berk 2003) This could, in part, be attributable to the fact that Notch signalling pathway expression, and subsequent anti-apoptotic effects, are lower in the right than in the left carotid artery Whilst a bi-phasic pattern of apoptosis following carotid ligation has previously been reported (Sullivan and Hoying 2002), the pattern of apoptosis we present differs from that in the literature Sullivan and Hoying, 2002 report an increase in apoptosis 4 days post-ligation, followed by a decrease in apoptosis 28 days post-ligation However, other studies claim that following arterial injury, where the long-term response to injury is also vascular remodelling, that VSMC apoptosis is detectable in two phases The first phase is a

rapid increase in medial SMC apoptosis occurring within 30 min of vascular injury, whereas apoptosis of both intimal and medial SMC also occurs as a later response, at 8-21 days post-injury (Han *et al*, 1995, Perlman *et al*, 1997)

In addition, we also analyzed the levels of apoptosis in the PVL model over a time-course of 2-15 days post-ligation A tri-phasic increase in caspase 3 activity was clearly evident in the PVL model at 2, 6 and 15 days post-ligation Analysis of Bcl-2 protein expression at the same time-points revealed an unsurprising down-regulation of expression at 6 and 15 days post-ligation However, Bcl-2 expression was increased 2 days post-ligation, despite the significant increase in caspase 3 activity and subsequent apoptosis This suggests that other regulators of apoptosis, such as other members of the Bcl-2 family, may play a part in regulating apoptosis in response to changes in biomechanical stress As we have established *bax* and *bcl-x*$_L$ as downstream mediators of the Notch signalling pathway, and *bcl-x*$_L$ as a factor in Notch signalling pathway protection against cyclic strain-induced apoptosis, analysis of these components in *in vivo* models could prove interesting The similar levels of caspase 3 activity in the sham-operated and ligated vessels 4 days post-ligation was surprising due to the consistent increases observed in Notch signalling pathway component expression at this timepoint Similar blood flow rates, but increased pressure are observed in the ligated versus the sham-operated control vessel at this timepoint, conditions which often precipitate inward vascular remodelling, and associated decreased apoptosis Therefore it is possible that caspase 3-independent apoptosis could occur at this timepoint The apparent increase in apoptosis at 6-15 days post-ligation tallies with the observed decrease in Notch signalling pathway expression at these time-points In addition, a significant increase in apoptosis was observed 2 days post-ligation with a concomitant decrease in HRT and increase in Hes target gene expression This suggests that the HRT target genes may mediate the anti-apoptotic effect of the Notch signalling pathway within the vasculature This is likely, as experiments within the laboratory have shown that inhibition of HRT target genes, using siRNA knockdown technology, increases expression of the pro-apoptotic *bax* gene and decreases anti-apoptotic *bcl-x*$_L$ expression, whereas Hes target gene inhibition achieves the opposite, but to a lesser extent *(D Morrow personal communication)*

## 6 4 Conclusion

This study provides previously unreported evidence of alterations in Notch

signalling pathway expression due to altered biomechanical forces *in vivo*, further validating the cyclic strain-induced decrease in Notch signalling pathway expression observed in this study *in vitro* We have also previously shown that cyclic strain-induced apoptosis is attributable, at least in part, to a decrease in Notch signalling pathway expression and activity Therefore, we propose that the observed alterations in levels of apoptosis in the *in vivo* models could also be partly attributed to changes in Notch signalling pathway expression Whilst we acknowledge that this is not conclusively proven in this study, due to limitations of the experimental models used, we feel that this study provides important preliminary evidence of *in vivo* regulation of Notch signalling pathway expression that could provide a basis for future investigations A model to pursue further studies has been proposed in the form of the Notch 1 transgenic mouse model (Liu and Lobe, 2003) In addition, factors mediating the cyclic strain-induced regulation of Notch signalling pathway expression remain undefined, and should be further analyzed Possible candidates as mediators of Notch signalling pathway expression will be discussed in detail in Chapter 7

# Chapter 7

# Discussion

## Discussion

This study has succeeded in its specific aims, clearly establishing the presence and activity of the Notch signalling pathway in adult VSMC, and providing further insight into the regulation of apoptosis in these cells, both *in vivo* and *in vitro* Increased understanding of VSMC apoptosis may contribute to future therapies against cardiovascular disease, as aberrant regulation of apoptosis is a common response to vascular injury, and a feature of many vascular disease states (Bai *et al*, 1999, Pollman *et al*, 1999) A concise summary of results will be presented here, but for more expansive explanations of results please refer to individual chapter discussions

This study comprehensively documents the presence of many components of the Notch signalling pathway in RVSMC, some of which had previously remained undetected in these cells This study utilized antibodies directed against the IC portion of the Notch receptors, and as such detects both the uncleaved and cleaved (IC) portion of the receptors However, the appropriate molecular weight corresponding to Notch IC was unequivocally determined using antibodies from different commercial sources, appropriate blocking peptides, coupled with analysis of Notch IC over-expression with both Notch- and HA-directed antibodies, which confirmed the molecular weight of Notch IC This ensured that analysis of Notch IC protein expression was indicative of the levels of Notch signalling pathway activity within the cell We also present the novel finding that both Notch 1 IC and Notch 3 IC signal via a CBF-1-dependent pathway in VSMC, with both receptors exerting equivalent levels of pathway activation This complements data presented by Wang *et al*, (2002b), claiming that Notch 3 signals in a CBF-1-independent manner in RVSMC Therefore, we can conclude that the Notch signalling pathway acts via both CBF-1-dependent and -independent manners in RVSMC, as has previously been shown for Notch signalling in EC (MacKenzie *et al*, 2004) In addition, this study has clearly shown that Notch signalling pathway activation increases expression and activity of Notch target genes in a CBF-1-dependent manner, and has established HRT-1 and Hes-5, but not Hes-1, as among the primary targets of the Notch signalling pathway in RVSMC This data was obtained using artificial stimulation of the Notch signalling pathway through transient transfection To determine whether this data was relevant to a less artificial situation, and to begin to determine the factors regulating the Notch signalling pathway, we investigated the effect of serum on Notch pathway expression and activity in VSMC We provide previously unreported evidence that serum stimulation temporally promotes Notch

signalling and target gene expression in a CBF-1-dependent manner This was achieved due to the observed up-regulation of Notch 1 IC and Notch 3 IC expression following serum stimulation In addition, we also found that endogenous ligand presentation significantly stimulated CBF-1-dependent Notch signalling in RVSMC, therefore we believe that the results obtained due to over-expression and serum stimulation of the Notch signalling pathway are relevant under conditions of physiological Notch signalling

The Notch signalling pathway is a highly conserved method of cell-cell communication, that controls cell fate decisions in many cell types, a function that is conserved from *Drosophila* to humans (Miele and Osborne 1999) As such, we postulated that the Notch signalling pathway might also regulate cell fate decisions in VSMC This study has clearly established that the Notch signalling pathway exerts an anti-apoptotic effect in VSMC, both endogenously within the cell, and in response to serum deprivation As it is unlikely that the Notch signalling pathway acts to control only one cell fate decision in VSMC, in particular due to the fact that the pathways controlling apoptosis and proliferation are closely linked, the regulation of other cell fate decisions by the Notch signalling pathway was also investigated within the laboratory Therefore, studies within the laboratory have also established additional roles for the Notch signalling pathway in regulating VSMC proliferation, migration and differentiation The Notch signalling pathway promotes VSMC proliferation, whilst concomitantly inhibiting migration and differentiation, in addition to apoptosis Whilst we have conclusively shown that that Notch signalling pathway acts to inhibit apoptosis in a CBF-1-dependent manner, we also provide evidence that Notch 3 IC additionally exerts a CBF-1-independent anti-apoptotic effect This is unsurprising as Notch has recently been shown to prevent apoptosis in EC in both a CBF-1-dependent and -independent manner (MacKenzie *et al*, 2004) In addition, Wang *et al*, (2002b) have shown that Notch 3 inhibits apoptosis in VSMC in a CBF-1-independent manner through up-regulation of c-FLIP, a primary inhibitor of the pro-apoptotic FasL signalling pathway The mechanisms through which serum deprivation induces apoptosis in VSMC appear to be complex and, to date, poorly defined This study proposes that serum deprivation-induced apoptosis may, in part, be due to a serum deprivation-induced decrease in the level of Notch signalling pathway expression and activity, thus decreasing the endogenous anti-apoptotic effect of this pathway on the cell

Numerous molecular pathways are involved in the regulation of apoptosis in VSMC (Hengartner 2000) In an attempt to elucidate the mechanism through which the Notch signalling pathway exerts its anti-apoptotic effect on VSMC, Notch interaction with, and regulation of, a number of known regulators of apoptosis, including members of the Bcl-2 and NFβB families was examined This list is by no means exhaustive, indeed, it is likely that the Notch signalling pathway exerts its anti-apoptotic effect on VSMC through interaction with multiple regulators of apoptosis This study provides previously unreported evidence that the Notch signalling pathway regulates the level of expression of the pro-apoptotic *bax*, and anti-apoptotic *bcl-x*$_L$, members of the Bcl-2 family of apoptotic genes In addition, it was established that neither Notch 1 IC nor Notch 3 IC activates the *bfl-1* promoter, suggesting that the anti-apoptotic effect of the Notch signalling pathway is not mediated through Bfl-1 in VSMC This study also provides initial evidence of interaction of the Notch signalling pathway with member of the NFβB family of transcriptional regulators in VSMC, which will provide a platform for further research within the laboratory

As this study has clearly established the anti-apoptotic properties of the Notch signalling pathway against serum deprivation-induced apoptosis in adult VSMC, the effect of this pathway on cyclic strain-induced apoptosis in VSMC was investigated As a result of these investigations, this study proposes that cyclic strain-induced apoptosis is attributable, at least in part, to a strain-induced decrease in Notch signalling pathway expression and activity in VSMC

Under conditions of cardiovascular disease, such as hypertension, or cardiovascular injury, such as that caused by balloon angioplasty, VSMC are often exposed to increased levels of cyclic strain (DeBakey *et al*, 1985, Thubrikar and Robicsek 1995) This study, and others (Mayr et al, 2000, Wernig et al, 2003), has shown that increased cyclic strain induces apoptosis in VSMC However, this study presents the novel findings that cyclic strain acts to decrease Notch signalling pathway expression and activity in VSMC This was additionally confirmed through comparison of Notch target gene expression both following Notch inhibition and exposure of the cells to cyclic strain Both cyclic strain and CBF-1-dependent Notch signalling pathway inhibition result in a decrease in HRT and Hes target gene expression In addition, the effect of RPMS-I and cyclic strain on the expression profile of the Bcl-2 family is virtually identical, further proving that cyclic strain causes decreased CBF-1-dependent Notch activity in VSMC It was also clearly established that over-expression of Notch 3 IC, at least in part, protects cells from cyclic strain-induced apoptosis, in a *bcl-*

$x_L$-caspase 3-dependent manner This study additionally provides previously unreported evidence of biomechanical regulation of the Notch signalling pathway and apoptosis *in vivo*, using both the carotid ligation and PVL models Notch signalling pathway expression was decreased at 3 days and increased at 28 days post-carotid ligation, which was accompanied by a concomitant increase and decrease in apoptosis respectively This pattern of an inverse relationship between Notch signalling pathway expression and apoptosis was generally maintained following PVL This validates the *in vitro* results obtained in this study, and proves that our *in vitro* model is representative of an *in vivo* situation

Whilst this study has provided evidence of the expression and activity of the Notch signalling pathway in VSMC, and has contributed to the understanding of the regulation of VSMC apoptosis, it also, as any body of research should, poses a considerable number of questions Specifically, whilst we have established that both serum and cyclic strain regulate Notch signalling pathway expression, the mechanisms of this remain undefined In addition, whilst we have established a number of possible mechanisms whereby the Notch signalling pathway regulates apoptosis, it is likely that these only constitute a small part of what could prove to be a considerable list, but as yet, remain undefined The final aim of this study is, therefore, to postulate as to how these questions could be addressed, in the hope of providing a platform for what could prove to be exciting future investigations within the laboratory

The Notch signalling pathway is clearly activated by serum, however, the exact mechanism of this serum-mediated Notch activation is currently undefined One of the obvious candidates for Notch activation would be the presence of a Notch ligand in serum Although Notch ligands are primarily transmembrane proteins, secreted forms of these proteins have been identified (Klueg *et al*, 1998, Qi *et al*, 1999), and could potentially be present in serum as endogenous activators of the Notch signalling pathway This study has shown that serum stimulates increases in Notch signalling pathway component activity and CBF-1-dependent activity Serum deprivation conversely decreases expression and CBF-1-dependent activity of this pathway Serum deprivation inhibits Jagged expression in VSMC, therefore, it is possible that additional factors that regulate Notch signalling pathway activity in VSMC may act through regulating of Jagged expression One of the potential mechanisms of Notch signalling pathway regulation is via the presence of growth factors within serum Recent studies have shown that both VEGF and PDGF alter expression of Notch signalling pathway components in both vascular and other cell types (Ho and Scott 2002, Lawson *et al*,

2002, Wang *et al*, 2002)

The VEGF family comprises of six known ligand and three known receptor sub-types The ligands include VEGF, placenta growth factor, and VEGF-B - E, whilst the VEGF-receptor (VEGF-R) sub-family includes VEGF-R1 (Flt-1), VEGF-R2 (KDR/Flk-1) and VEGF-R3 (Flt-4) The most prominent ligand of the VEGF family is VEGF itself, which binds to VEGF-R1 and -R2 (Veikkola and Alitalo 1999)

VEGF is a major regulator of both physiological and pathological neo-vascularization (Ferrara and Davis-Smyth 1997), a role that is conserved from development Gene knockout studies have revealed the critical role of VEGF in developmental vasculogenesis and angiogenesis, in that mice with disputed VEGF-R1 expression die embryomcally due to vascular defects (255-52) This is highly reminiscent of the effect of Notch signalling pathway disruption, which also results in embryonic lethality due to defects in vasculogenesis and angiogenesis (Xue et al , 1999, Smith et al , 2000) Consistent with the findings that VEGF induces Notch signalling pathway activity (Lawson *et al*, 2002), and Notch acts to inhibit apoptosis in EC (MacKenzie *et al*, 2004) VEGF has been shown to exert an anti-apoptotic effect on EC (Gerber *et al*, 1998) It is possible, therefore, that any VEGF-mediated regulation of VSMC fate decisions could, at least in part, be mediated by the Notch signalling pathway The Notch and VEGF signalling pathways have a number of other similar functions, including regulation of arterial-venous differentiation in development, further highlighting the interaction between these two pathways Lawson *et al*, (2002) has recently shown that VEGF acts upstream of the Notch signalling pathway to determine arterial cell fate, and furthermore that Notch signalling is required to mediate VEGF-induced arterial differentiation (Lawson *et al*, 2002) Additional studies have revealed that VEGF-mediated activation of the Notch signalling pathway appears to be self-limiting, as over-expression of components of the Notch signalling pathway decreases VEGF-R levels Henderson *et al*, (2001) have shown that over-expression of HRT-1 in EC down-regulates VEGF-R2 mRNA expression levels, and inhibits VEGF-meditated cell fate decisions in these cells *in vitro* Similarly, zebrafish *notch 5* has been shown to repress VEGF-R3 expression in venous EC *in vivo* (Lawson *et al*, 2001) Therefore the presence of VEGF in serum could, at least in part, account for the serum-mediated increase in Notch signalling pathway expression and activity The fact that VEGF-mediated increase in Notch signalling appears to be self-limiting could account for the return of Notch signalling pathway component expression and activity towards baseline levels at 24 h post-serum stimulation

As VEGF is an important factor in Notch signalling pathway regulation, we must also consider factors that mediate VEGF expression, as these will also affect expression of Notch signalling pathway components Transcription of VEGF mRNA is induced by a variety of growth factors and cytokines, many of which are present in serum, including PDGF-BB, EGF, TNF-β, TGF-β1 and interleukin-1-β In addition, two groups have recently shown that Sonic hedgehog (Shh), a member of the Hedgehog signalling family, acts upstream of VEGF, and therefore upstream of the Notch signalling pathway, increasing VEGF expression and therefore regulating its function (Pola *et al*, 2001, Lawson *et al*, 2002)

The Hedgehog signalling family was first identified in *Drosophila*, and was so named as haploinsufficiencies of this family of genes produces a phenotype resembling a hedgehog (Nusslein-Volhard and Wieschaus 1980) As with the Notch signalling pathway, the Hedgehog signalling pathway shows a high degree of evolutionary conservation, from *Drosophila* to humans

Components of the Hedgehog signalling pathway include three highly conserved ligands, Shh, Indian hedgehog (Ihh) and Desert hedgehog (Dhh), the hedgehog receptor, Patched (Ptc), the transmembrane protein Smoothened (Smo) and the transcription factor Gli, which is the principal mediator of the Hedgehog signalling pathway (Villavicencio *et al*, 2000, Pola *et al*, 2001)

The hedgehog family of molecules are secreted proteins that undergo several post-translational modifications to gain full activity The hedgehog precursor protein is autolytically cleaved to generate an N-terminal active polypeptide, and a C-terminal fragment that appears to have no function other than catalyzing the autolytic cleavage During this reaction a cholesterol moiety is attached to the C-terminal end of the N-terminal hedgehog fragment This cholesterol moiety is thought to mediate hedgehog binding to cell membranes A further modification of the hedgehog protein subsequently occurs, with the addition of a palmitoyl group to the N-terminus of the mature active protein (Weed *et al*, 1997, Ho and Scott 2002, Nybakken and Perrimon 2002) These processes are mediated by proteins known as Skinny hedgehog, Rasp and Central missing, and are believed to regulate hedgehog activity, diffusion and potency Hedgehog proteins are subsequently secreted in a process that is mediated by the transmembrane protein Dispatched in *Drosophila*, although no vertebrate homologue has been identified to date Secreted hedgehog proteins can act at sites proximal or distal to the site of secretion Post-translational modifications of hedgehog facilitate this, as following secretion hedgehog molecules form a multi-dimeric complex with the

hydrophobic regions aggregating at the centre of the complex, whilst the exposed hedgehog molecules interact with proteoglycans that facilitate their transport (Nybakken and Perrimon 2002).

**Figure 28: Hedgehog Modification and Secretion in a Hedgehog Producing Cell.**

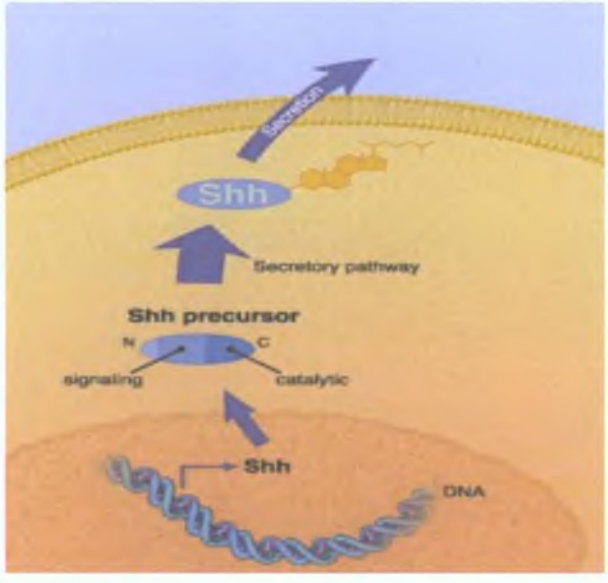



**Figure 28:** Representation of post-translational modification and secretion of hedgehog protein in a hedgehog-producing cell.

The hedgehog receptor Ptc consists of twelve TM domains with two large ExC loops. In the absence of hedgehog binding within the cell, the Ptc receptor acts to repress the Hedgehog signalling pathway. This is achieved through Ptc-mediated inhibition of Smo, a seven TM protein that is required for hedgehog signal transduction in both vertebrates and invertebrates. The method of Ptc-mediated inhibition of Smo is unknown, but is most likely indirect as Ptc-Smo interaction is not required, as was originally thought (Bijlsma *et al.,* 2004). In the absence of hedgehog signalling, Ptc is principally found on the surface of the cells, whereas Smo is mainly located intra-cellularly. Signalling downstream of Smo remains somewhat obscure. However, it is known that, in *Drosophila* (accepted as the standard model of hedgehog signalling), Smo signals to a complex containing the proteins Fused/Costal 2/Cubitus interruptus (Fu/Cos2/Ci). Ci is the *Drosophila* homologue of the mammalian transcription factor Gli. To date, three Gli homologues have been identified in humans, designated Gli-1, -2 and -3. These transcription factors, similar to CBF-1 in the Notch signalling pathway, are multifunctional as they can act as transcriptional repressors or activators (Villavicencio *et al.,* 2000). In the absence of hedgehog signalling the Fu/Cos2/Ci acts to sequester the transcription factor Ci through binding to microtubules, which is aided

by an antagonist of the hedgehog pathway, Suppressor of Fused (Su(Fu)). The full length Ci molecule is phosphorylated by a number of kinases, and subsequently cleaved to form a 75 kDa product, which translocates to the nucleus, and acts as a suppressor of hedgehog target genes.

In response to hedgehog binding, the Ptc receptor is internalized which results, indirectly, in the movement of Smo to the cell surface, perhaps due to the association of Smo with lipid rafts (Karpen *et al.*, 2001). Positioning of Smo on the cell surface results in hyperphosphorlyation of Smo, perhaps due to its proximity with other activator molecules (Ho and Scott 2002). Smo signals, through unknown mechanisms, to the Fu/Cos2/Ci complex, resulting in the phosphorylation of Fu and Cos2 causing the complex to partially detach from the microtubules. This results in stabilization of full length Ci, as it is no longer subject to phosphorylation, which can then translocate to the nucleus and function as a transcriptional activator of the Hedgehog signalling pathway (Ho and Scott 2002; Nybakken and Perrimon 2002; Bijlsma *et al.*, 2004).

**Figure 29: Proposed Mechanism of Hedgehog Signal Transduction Pathway**

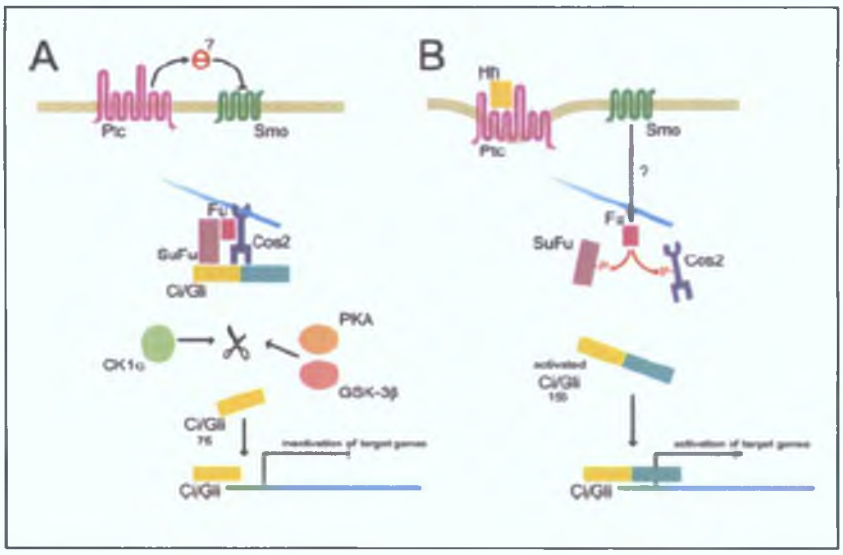



**Figure 29: Proposed mechanism of hedgehog signal transduction.** A: absence of hedgehog signal, B: presence of hedgehog, refer to text (Bijlsma *et al.*, 2004).

It is interesting to note that, like the Notch signalling pathway, the hedgehog pathway can induce activation of a Gli-independent pathway, involving the activation of the orphan nuclear receptor, COUPTF-II (Pola *et al.*, 2001; Pola *et al.*, 2003).

Downstream target genes of the Gli-dependent Hedgehog signalling pathway include Ptc and Gli itself, which are therefore both components and target genes of the Hedgehog signalling pathway. Other target genes of the hedgehog family include bone

morphogenic protein-4, and members of the TGF-β and Hox gene families (Roberts *et al.,* 1995). Activation of the Hedgehog signalling pathway also affects the expression of a number of other growth factors. Hedgehog, for example, down-regulates expression of basic fibroblast growth factor (bFGF) in fibroblasts in a Gli-dependent manner (Pepicelli *et al.,* 1998). In addition, Pola *et al.*, (2001) describes Shh as an indirect angiogenic agent, as it acts to up-regulate expression members of two families of angiogenic growth factors, including VEGF and Angiopoietin 1 and 2. As no Gli response elements are present on either VEGF or Angiopoietin 1/2, it is likely that hedgehog signalling up-regulates these components in a Gli-independent manner. Subsequent studies confirmed the fact that Shh acts upstream of both the VEGF and Notch signalling pathways (Lawson *et al.,* 2002; Pola *et al.,* 2003). The VEGF and Notch signalling pathways are required for arterial EC differentiation. Lawson *et al.*, (2002) however, demonstrated that cells lacking Shh failed to undergo arterial differentiation, and that this was rescued with over-expression of *Shh* mRNA. Furthermore, this lack of arterial differentiation in Shh negative cells is also rescued with the over-expression of VEGF (in the presence of Notch), or over-expression of Notch itself. In addition, Pola *et al.*, (2003) demonstrated that Shh-responding cells up-regulate VEGF expression and angiogenesis in response to skeletal muscle ischaemia, and that this is blocked with Shh-blocking antibody treatment. It has recently emerged that the Notch signalling pathway also influences expression of Shh (Lopez *et al.,* 2003). Members of the Notch signalling pathway, specifically Notch IC and presenilin, act to up-regulate Shh expression in *Xenopus* embryos, and as regulation of these pathways are highly conserved, it is likely that this is the case in other species. However, we know that Shh causes up-regulation of both VEGF and Notch expression, and Notch causes up-regulation of Shh and down-regulation of VEGF expression (Henderson *et al.,* 2001; Lawson *et al.,* 2001). This suggests that the Notch signalling pathway also influences VEGF expression in a Shh-independent manner, which remains undefined.

As with Notch, the importance of the Hedgehog signalling pathway within the vasculature is increasingly recognized. Ptc-1 is expressed throughout the vasculature in both juvenile and adult mice, suggesting that adult cardiovascular tissues may contain several cell populations responsive to hedgehog signalling (Pola *et al.,* 2001). The Hedgehog pathway also plays a pivotal role in development, regulating many of the same functions as the Notch signalling pathway, such as limb and neural tube development, further highlighting the functional interactions between these pathways

(Artavanis-Tsakonas *et al*, 1995, Weed *et al*, 1997, Artavanis-Tsakonas *et al*, 1999) Hedgehog signalling also appears to be important in proper development of the vascular system Similar to the Notch signalling pathway the correct level of Hedgehog signalling appears to be important, as both up- and down-regulation of hedgehog proteins results in vascular defects (Sullivan and Bicknell 2003), mutations of the Hedgehog signalling pathway, for example, results in lack of proper vascularization in the developing mouse lung As with the Notch signalling pathway, the Hedgehog pathway is implicated in regulating cell fate decisions, Shh regulates both proliferation and survival of oligodendrite precursors, and acts to promote proliferation and inhibit differentiation in both neuronal and non-neuronal cell types (Ho and Scott 2002) Dysregulation of both the Hedgehog and Notch pathways lead to dysregulated cell growth and abnormal cellular accumulations, contributing to many types of cancers (Miele and Osborne 1999, Villavicencio *et al*, 2000) Therefore, although it is likely, whether the Hedgehog signalling pathway regulates cell fate decisions through the Notch signalling pathway remains to be fully established

Additional studies within the laboratory have established the presence of components of the Hedgehog signalling pathway within RVSMC, and have shown that inhibition of this pathway decreases Notch 3 expression in these cells In addition, both Notch over-expression and serum stimulation increase Hedgehog signalling pathway component expression and/or activity levels This appears to occur, at least in part, in a CBF-1-dependent manner *(D Morrow personal communication)* Therefore, we can conclude that serum stimulation of the Notch signalling pathway involves the activation of both the VEGF and Hedgehog signalling pathways, however, the precise regulation of this remains to be fully determined

My work has clearly established the presence of one developmental pathway, the Notch signalling pathway, within VSMC Complementary studies within the laboratory have identified the presence of an additional developmental pathway, the Hedgehog signalling pathway in these cells Moreover, combined analysis of the regulation of these pathways suggests that they may be functionally coupled This study, and others within the laboratory have revealed that mechanical forces exert similar effects on expression of both the Hedgehog and Notch signalling pathways Cyclic strain down-regulates expression of both Hedgehog and Notch signalling pathway components, both *in vivo* and *in vitro* However, several other studies have indicated that cyclic strain increases VEGF expression in bovine VSMC and cardiomyocytes (Smith *et al*, 2001, Zheng *et al*, 2001) This suggests that the cyclic

strain-mediated down-regulation of the Notch signalling pathway is not regulated in a VEGF-dependent manner, but is affected by other components, or by mechanical strain directly, which in turn causes down-regulation of the Hedgehog signalling pathway Possible candidates involved in cyclic strain-mediated regulation of the Notch signalling pathway include PDGF, Angiotensin II (AngII) and endothelin-1 (ET-1)

The PDGF family is composed of ligands and receptors that are widely implicated in regulating cell fate decisions The biologically active form of the PDGF ligand is a homo- or heterodimer of A and B polypeptide chains, which determines the biological activity of the ligand The PDGF-receptor (PDGF-R), a member of the receptor tyrosine kinase family, is also a dimer of β or β chains The PDGF-R dimer is only formed after ligand binding, so that the ββ constituent of the receptor is influenced by the form of PDGF present PDGF-R-β binds all forms of PDGF, whilst PDGF-R-β binds PDGF-BB and -AB only (Xu 2000) Ligand binding results in tyrosine phosphorylation of the receptor, and triggers sequential activation of MAPK cascades, which play a pivotal role in regulating cell fate decisions (Cano and Mahadevan 1995)

PDGF are important mediators of vascular remodelling and lesion formation, and several groups have shown that PDGF-R and PDGF-B expression are up-regulated in response to mechanical stress in VSMC both *in vivo* and *in vitro* (Bryant *et al*, 1999, Ma *et al*, 1999) Similarly, the level of PDGF-R-β phosphorylation is increased in VSMC in response to cyclic strain (Li and Xu 2000) Wang *et al*, (2002a) have recently shown that PDGF decreases Notch receptor and HRT target gene expression in VSMC via an ERK-dependent pathway Therefore, the cyclic strain-induced increase in PDGF expression and activity could, at least in part, account for the cyclic strain-mediated decrease in Notch signalling pathway expression observed in this study Wang *et al*, report a time- and dose-dependent decrease in Notch signalling pathway expression following PDGF administration, which may account for the time- and strength-dependent decrease in Notch expression observed in this study following exposure to cyclic strain Interestingly, both PDGF and vascular injury cause the greatest down-regulation in Notch 3 expression, which further suggests that PDGF is responsible for injury-induced decrease in Notch signalling pathway expression

Another possible candidate for regulating Notch signalling pathway expression in response to cyclic strain is Ang II Ang II is part of the Renin-Angiotensin system, which plays an important role in regulating blood volume, arterial pressure and cardiac and vascular function Ang II is the active vasopressor derived from activation of this system, and is generated by enzymatic cleavage of its precursor, Ang I Cyclic

strain has also been shown to increase Ang II levels in VSMC (Li *et al*, 1997) In addition, Ang II affects Notch signalling pathway component expression, in a similar manner to PDGF Campos *et al*, (2002) have shown that Ang II markedly down-regulates both Notch 3 and Jagged 1 expression in an ERK-dependent manner in VSMC *in vitro* Therefore, cyclic strain-induced increase in Ang II expression may contribute to the strain-induced decrease in Notch signalling pathway expression levels In addition, Ang II causes a time-dependent decrease in Notch expression, which may contribute to the time-dependent decrease in Notch signalling pathway component expression in response to cyclic strain observed in this study

It is possible that cyclic strain exerts its effects on the Notch signalling pathway through a concerted effort of these, and other, molecules It is likely that many VSMC components interact with, and regulate expression of the Notch signalling pathway, however as the study of this pathway in VSMC is in its infancy, it is possible that these interactions have not yet been determined One possible additional candidate for regulating Notch signalling in response to cyclic strain could be ET-1

ET-1 is a potent vasoconstrictor produced in many cells, including EC and SMC ET-1 has a variety of functions, including the regulation of cell fate decisions, and has been shown to inhibit apoptosis in VSMC (Wu-Wong *et al*, 1997) As cyclic strain decreases ET-1 expression in VSMC, it is possible that cyclic strain-mediated apoptosis is regulated, at least in part, in an ET-1-dependent manner The link between ET-1 and the Notch signalling pathway is a tenuous one, but could provide the basis for further clarification of Notch signalling pathway interactions in VSMC Embryonic ET-1 and Jag-1 mutations produce the same phenotype in the form of cardiac outflow tract defects (Srivastava and Olson 2000), suggesting that these pathways may interact with one another in the vasculature, whether this is true in adult cells remains to be determined

This study has established interactions between the Notch signalling pathway and both the NFβB signaling pathway, and the Bcl-2 family in VSMC However, it is likely Notch interacts with multiple signalling pathways in VSMC to regulate apoptosis, which could provide the basis for further investigation into the mechanism of Notch-mediated cell survival in VSMC

This study reveals that serum deprivation-induced apoptosis is attenuated by the over-expression of both Notch 1 IC and Notch 3 IC in VSMC Serum deprivation-induced apoptosis in VSMC is also attenuated by TGF-β1, and the extent of the attenuation is similar to that seen with Notch over-expression (Pollman *et al*, 1999)

TGF-β and related proteins bind two different types of serine/threonine kinase receptors, and transmit intracellular signals through Smad proteins Receptor-regulated Smads are directly activated by the receptor and function as components of transcription factor complexes in the nucleus The TGF-β signalling pathway has been shown to control cell fate decisions in many cell types, and mutations of the receptors and Smad proteins have been implicated in many types of cancers, including colorectal and pancreatic (Miyazono *et al*, 2003) A number of studies indicate an interaction between the Notch and TGF-β signalling pathways, and the fact that they exert similar effects on serum deprivation-induced apoptosis in VSMC suggests a possible interaction in VSMC Activation of TGF-β signalling in myogenic cells up-regulates Hes-1 expression both *in vivo* and *in vitro* (Blokzijl *et al*, 2003), and TGF-β has been shown to induce both Jagged 1 and HRT-1 expression in development (Zavadil *et al*, 2004) In addition, both TGF-β and Notch 3 have been implicated in the regulation of the cell-cycle inhibitor, $p27^{kip}$ (MacDonald *et al*, 1996, Campos *et al*, 2002) TGF-β acts to up-regulate VEGF (Veikkola and Alitalo 1999), a known Notch signalling pathway regulator, therefore it is possible that TGF-β up-regulates Notch in a VEGF-dependent manner Whether the interaction between these two pathways is conserved in VSMC remains to be defined, but could promise to be an exciting new area of research

As activation of the Notch signalling pathway is coupled to the regulation of both apoptosis and proliferation (Miele and Osborne 1999), it is tempting to speculate that Notch may interact with other families, such as the Myc family, that also govern both apoptosis and proliferation The cellular-myc (c-myc) gene, part of the myc gene family, was discovered as the cellular homologue of the retroviral v-myc oncogene, and has since been shown to be elevated in many tumour types Burkitts lymphoma, for example, exhibits a point mutation in, and subsequent up-regulation of, its c-myc gene This protein plays a role in the initiation of apoptosis and transformation in many cell types, two mechanisms that are important for tumourigenesis (Dang 1999) Several lines of evidence point to the interaction of the Notch signalling pathway and *c-myc*, and suggest a functional link in the regulation of apoptosis Similar to the Notch signalling pathway, *c-myc* is highly conserved from *Drosophila* to humans, and targeted mutations in murine *c-myc* gene results in widespread embryonic lethality, suggesting that it is critical 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 have been observed *(Swiss Institute of Bioinformatics)* Class C bHLH proteins, such as Hes and HRT, are known to bind to some degree to the class B bHLH proteins, such as c-Myc (Iso *et al*, 2003) As described previously, HRT are known to bind to a number of E box motifs on their target genes (Nakagawa *et al*, 2000), therefore using bioinformatics, and the cloned *c-myc* promoter sequence (Ray and Miller 1991) this study analyzed whether any of the HRT binding sequences were present on the *c-myc* promoter It was determined that although the preferential CACGTG E box motif was not present, three other HRT E boxes were present on the *c-myc* promoter region, these being CAACTG, CACCTG and CACTTG, further highlighting the potential interaction between the Notch signalling pathway and c-myc In addition, c-Myc is a direct target of EBNA-2, which shares many functional similarities with Notch IC (Schlee *et al*, 2004) The effect of EBNA-2 on c-myc expression is unclear, as some studies report that *c-myc* is down-regulated by EBNA-2 (Strobl *et al*, 2000), whereas others maintain that EBNA-2 induces *c-myc* expression (Kaiser *et al*, 1999)

Serum deprivation-dependent apoptosis in VSMC is dependent on endogenous *c-myc* expression (Shichiri *et al*, 2000) This study has established that Notch 1 IC and Notch 3 IC attenuates serum deprivation-induced apoptosis in VSMC In addition, other studies have documented that Notch IC represses *c-myc* expression in B cells (Strobl *et al*, 2000) Therefore it is possible that one of the mechanisms of Notch-mediated repression of apoptosis is via *c-myc* down-regulation in VSMC

It is interesting to note that a recent paper describes the effect of TGF-β on c-myc in VSMC (Khanna 2004), in that TGF-β inhibition in these cells resulted in an increase in c-myc expression, however, the mechanism mediating the increase was not addressed As the literature has shown that TGF-β activates the Notch signalling pathway, and Notch acts to decrease c-myc expression in other cell types, we propose that TGF-β mediated regulation of c-myc could be regulated by the Notch signalling pathway in VSMC

VSMC apoptosis is regulated in both a p53-dependent and -independent manner However, VSMC apoptosis, mediated by *c-myc* expression following serum deprivation is dependent on, and mediated by p53 (Bennett *et al*, 1995) This, coupled with several recently reported links between the Notch signalling pathway and p53 family members, suggests that Notch could mediate VSMC apoptosis through interaction with the

p53 family

The first link between the Notch signalling pathway and p53 family members reported that p63 and p73 up-regulated expression of the Notch ligands Jagged 1 and Jagged 2 respectively (Sasaki *et al*, 2002) In the case of p63, up-regulation of Jagged 1 resulted in increased expression of the Notch target gene *hes-1* In addition, this study identified a *p63*-binding site in the second intron of the *jagged 1* gene, and direct interaction between *p63* and *jagged 1* was demonstrated p63 and p73 are members of the p53 family that play a critical role in development The interaction between these genes and the Notch signalling pathway suggests p63 and p73 may function in development through modulation of this pathway This study, and the fact that both the Notch signalling pathway and p53 are de-regulated in many cancers indicate an interaction between Notch and p53, which has subsequently been reported in some cell types

Notch 1 IC is reported to increase p53 expression and activity in both human hepatocellular carcinoma cells (Qi *et al*, 2003) and neural progenitor cells (Yang *et al*, 2004) Notch 1 IC also induces extensive apoptosis in these cells, in a p53-dependent manner Although these studies clearly provide a link between the Notch signalling pathway and p53, it is likely that this positive effect of the Notch signalling pathway on p53 is cell type specific Therefore, it is possible that a decrease in p53 expression and activation will be observed in cells in which Notch exerts an anti-apoptotic effect

The link between the Notch signalling pathway and p53 is further confirmed by the observation that activation of Notch 1 signalling prevents apoptosis induced by p53 over-expression in an immortalized human epithelial cell line (Nair *et al*, 2003) Additionally, the crosstalk between the Notch signalling pathway and p53 appears to be bi-directional, as p53 negatively regulates Notch 1 activation during T-cell development (Laws and Osborne 2004) Two possible mechanisms have been proposed for this regulation p53 negatively regulates PS1 expression, which is required for Notch IC cleavage, thus resulting in decreased Notch activation In addition, p53 associates with the transcriptional co-activatior CBP/p300 in the nucleus, therefore it is suggested that p53 decreases Notch activity through competition for CBP/p300 binding Both of these models suggest that p53 inhibits CBF-1-dependent Notch signalling pathway activation, and do not address whether p53 also inhibits CBF-1-independent Notch signalling Several observations point to the fact that p53 might also act to decrease Notch signalling pathway component expression in VSMC Cyclic strain increases p53

expression (Mayr *et al*, 2002) and decreases Notch signalling pathway expression in VSMC, suggesting that p53 could act to negatively regulate Notch component expression in these cells Therefore, p53 can also be added to the list of possible regulators of the cyclic strain-meditated alteration in Notch signalling pathway expression

The importance of p53 in VSMC apoptosis is increasingly recognized p53 accumulation has been observed in human atherosclerotic and restenotic lesions (Speir *et al*, 1994, Ihling *et al*, 1998), suggesting that p53 may play an important role in the pathogenesis of human vascular disease In addition, decreased apoptosis was observed in $p53^{-/-}$ lesions (Sata *et al*, 2003) VSMC apoptosis is a prominent feature of the vascular response to injury and subsequent formation of the neointima This is increasingly recognized as being due to up-regulation of pro-apoptotic, and down regulation of anti-apoptotic mediators (Morishita *et al*, 1993) As Notch signalling pathway expression is decreased following vascular injury (Wang *et al*, 2002), one of the precursors of vascular remodeling and lesion formation, and p53 is up-regulated in vascular lesions, it is tempting to speculate that Notch could mediate VSMC apoptosis through regulation of p53

Therefore, this study has established the presence of the Notch signalling pathway in VSMC both *in vivo* and *in vitro* and its importance in the regulation of apoptosis in these cells However, whilst the regulation of the Notch signalling pathway in VSMC remains undefined, we propose a number of potential molecules, including VEGF, Shh, PDGF, Ang II and perhaps ET-1, which may act synergistically or competitively with one another in the regulation of this pathway In addition, this study has elucidated potential mechanisms of Notch signalling regulation of apoptosis in VSMC Numerous molecular pathways are involved in the regulation of apoptosis in VSMC, and it is likely that Notch interact with many of these pathways to mediate apoptosis We propose that TGF-β, c-myc and p53 pathways are potential mechanisms through which the Notch signalling pathway may regulate apoptosis, which provides a basis for further investigation into the mechanism of Notch-mediated cell survival in VSMC The ultimate aim of any body of molecular health related research is to elucidate and define potential new pathways and to increase an expanding repertoire of knowledge on disease states The present problem of cardiovascular disease in Ireland, and the growing concern of public health and epidemiologic statistics in this area show a worrying trend The epidemic of obesity will accentuate these statistics The social costs and productivity issues surrounding these diseases directly and indirectly, indicate

the importance of research in this field Further knowledge on the mechanisms of vascular cell death and remodelling may give us crucial new insights into the molecular basis of atherosclerosis, arteriosclerosis, hypertensive disease and restenosis post-treatment The ability to alter these disease states via new potential therapeutic targets represents a vital and exciting challenge within the field of cardiovascular research

# Chapter 8

# Bibliography

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