

**NOTCH SIGNALING PATHWAY
IN HUMAN VASCULAR SMOOTH
MUSCLE CELL
DIFFERENTIATION**

A dissertation submitted for the degree of M.Sc.

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 Master of Science 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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DMEM - Dulbecco Modified Eagle's Media

DSL - Delta/Ser/lag2 domain

DTT - dithiothreitol

dTTP - 2-deoxythymidine 5-triphosphate

EC - endothelial cells

EDTA - Ethylenediaminetetra acetic acid

EGF - epidermal growth factor

EtOH - ethanol

FasL - Fas ligand

FCS - Foetal Calf Serum

FITC - isothiocyanate form of fluorescein

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GOM - granular osmiophilic material

HASMC - Human Aortic Smooth Muscle Cells

HAT - Histone Acetylase

HBSS - Hanks Balanced Salt Solution

HDAC - Histone Deacetylase

HECT - ubiquitin-protein ligases

HERP - HES related repressor protein

HES - Hairy-Enhanced Split

HRT - Hairy-related transcription factor

HVSMC - Human Vascular Smooth Muscle Cells

IgG – Immunoglobulin G

LB – Lauret Broth

LFA – Lipofectamine

LNG – lin12/Notch/Glp-1

MLV-RT - murine leukemia virus reverse transcriptase

N1 – Notch 1

N3 - Notch 3

NICD – Notch Intercellular Domain Notch IC

P7-CMV – mock transfected cells

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PCV – posterior cardinal vein

PDGF – platelet derived growth factor

PEST – proline-glutamine-serine-threonine-rich sequence

pH – log of the reciprocal of the hydrogen ion concentration

PMSF - Phenylmethylsulphonylfluoride

RAM –one of four domains found in the intracellular domain of Notch protein

RASMC - Rat Aortic Smooth Muscle Cells

RNase – Ribonuclease

rpm – Revolutions Per Minute

RPMS-1 – Epstein Barr virus encoded gene product

RT – Reverse transcriptase

SD – Spondylocostal Dysostosis

SDS – Sodium dodecyl sulphate

SKIP – SKI-related protein

SM-MHC – Smooth Muscle Myosin Heavy Chain

SMRT – Silencing Mediator of Retinoid and Thyroid Hormone Receptor

SSB – Sample Solubilisation Buffer

SuH – Suppressor of Hairless

TAD – transcription activator domain

TAE – Tris Acetone EDTA

Taq – Thermophilus Aquaticus

TE – Tris EDTA

TEMED – N, N, N, N' – tetramethyl ethylenediamine

TGF – transforming growth factor

Tris – Trizma base

VEGF – vascular epidermal growth factor

x g – Centrifugal force

UNITS AND PREFIXES

UNITS

A – Amp

Da – Dalton

°C – Degrees Celsius

g – Gram

L – Litre

M – Molar

Min – Minute

V – Volt

W – Watt

PREFIXES

c – centi (1×10^{-2})

m – milli (1×10^{-3})

μ – micro (1×10^{-6})

n – nano (1×10^{-9})

p – pico (1×10^{-12})

ABSTRACT

Notch receptor-ligand interactions are a highly conserved mechanism, originally described in developmental studies using *Drosophila*, that regulate inter-cell communication and dictate, in part, vascular smooth muscle cell (VSMC) fate in response to mechanical stimuli. VSMC differentiation is a crucial developmental process regulating angiogenesis and vasculogenesis, with phenotypic modulation of SMC a key factor in vascular pathology. It has been previously shown that cyclic strain decreases the proliferation of VSMC's *in vitro*. Thus, characterization of the mechanisms controlling the differentiation state of VSMC is of critical importance in determining the cell fate response of these cells to various mechanical stimuli. We investigated the role of Notch 1 and 3 receptor signaling in controlling vascular SMC differentiation *in vitro*, and established a role for cyclic strain induced changes in Notch in mediating this response. The expression of smooth muscle cell specific α -actin, calponin, myosin and smoothelin was examined by immunocytochemistry, Western blot analysis and quantitative real time PCR in human vascular SMC cultured under static conditions following over-expression of constitutively active Notch 1 and 3 receptors. The effect of equibiaxial cyclic strain (10% 24 h) on the expression of Notch receptors and SMC differentiation was subsequently determined using a Flexercell Tension Plus Unit. Over-expression of constitutively active Notch intracellular receptors (Notch 1 IC and 3 IC) resulted in a significant down-regulation of α -actin, calponin, myosin and smoothelin expression, an effect that was significantly attenuated following inhibition of Notch mediated CBF-1/RBP-Jk dependent signaling by co-expression of RPMS-1-I. Cells cultured under conditions of defined equibiaxial cyclic strain (10% strain, 60 cycles/min, 24 h) exhibited a significant reduction in Notch 1 and 3 IC expression, concomitant with a significant increase in smooth muscle cell α -actin, calponin, myosin and smoothelin expression. Moreover, this cyclic strain-induced increase in SMC differentiation marker expression was further enhanced following inhibition of CBF-1/RBP-Jk dependent signaling with RPMS-1. These findings suggest that Notch receptors modulate vascular SMC phenotype *in vitro* and cyclic strain enhances SMC differentiation in part through inhibition of Notch receptor expression. The Notch signaling pathway may therefore represent a novel mechanism for targeting vascular disorders in which SMC phenotypic diversity occurs *in vivo*.

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INTRODUCTION

Chapter 1. Angiogenesis and Vascular Development

Angiogenesis describes the formation of new blood vessels from pre-existing vessels. Although angiogenesis focuses mainly on the formation of capillaries, it includes the formation of small and large blood vessels. In contrast, vasculogenesis, the other recognised mechanism of vessel development, involves the differentiation of new vessels from embryonic structures known as blood islands. Development and 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, formation of blood vessels occurs from a population of angioblasts that are mesodermally derived endothelial cell (EC) precursors. Angioblasts first differentiate and gather into a reticulum of homogeneously sized primitive blood vessels, or the primary vascular plexus, in vasculogenesis [1, 2, 3, 4]. Angiogenesis that involves sprouting, bridging, and intussusceptions, remodels the primary vascular plexus to generate both the large and small vessels of the mature vascular system [1, 2, 3, 4]. During this process, endothelial channels are covered by multiple layers of smooth muscle

cells (SMCs) in large vessels and by single pericytes around small vessels to provide stability for the vascular walls.

1.1 Blood vessel structure

The normal blood vessel wall is composed of three layers; the intima layer, the media layer and the adventitial [Fig. 1]. The intima layer consists of tightly connected endothelial cells (EC), which line the lumen. Underneath EC, separating the intima and media, is an internal elastic lamella, rich in sulphated polysaccharides, hyaluronic acid, and contain intimal smooth muscle cells, which are tightly adhered to a media layer of vascular smooth muscle cells. The vessel is surrounded by an adventitial layer, consisting of connecting tissue and capillaries, fibroblasts and fat cells, which are separated from the outer media by an internal elastic lamina [5, 6]. The endothelial lining, located at the interface between the blood and the vessel wall, serve as a correspondent and transducer of signals within the local environment. Endothelial cells help to maintain the homeostatic balance of the vessel by producing factors, which regulate vessel tone, the state of coagulation, cell growth, death and leukocyte trafficking.

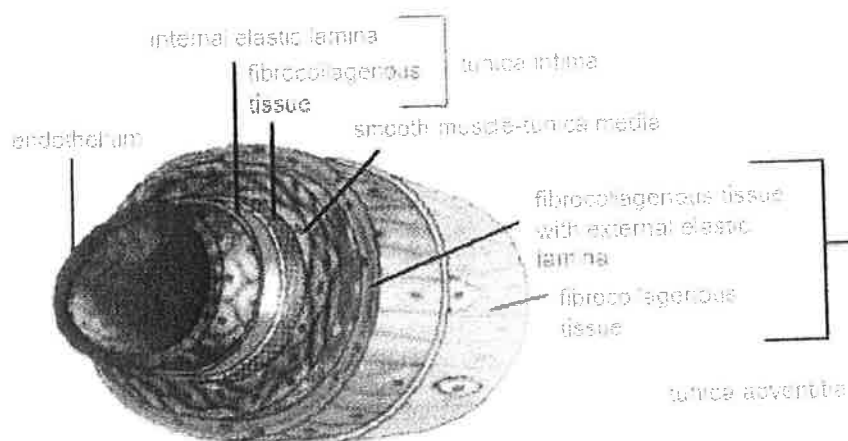


Fig. 1 Diagrammatic representation of a muscular artery [9].

1.2 Vascular smooth muscle cells in vessel development

The first step towards SMC's development is the coalescing of angioblasts into long tubes to form angioplastic cords consisting of a single layer of endothelial cells. Then, presumptive SMC's are recruited from mesenchyme to surround the endothelium, and subsequently differentiate into smooth muscle. The intimal endothelial tubes become surrounded by locally derived irregularly shaped mesenchymal cells that include the precursors of SMC's and adventitial fibroblasts [8].

In the early period of development, locally recruited mesenchymal cells, which lack SMC's features, move into the region surrounding small endothelial tubes. Subsequently, these cells assume the structural characteristic of SMC's and incorporate into the wall as cellular layers [9]. At this stage, the immature SMC's display a dynamic state of growth and differentiation characterized by

proliferation and migration, as well as changes in the pattern of gene expression.

The intimal layer appears relatively late in the vessel development. Intimal thickening begins with an accumulation of glycoaminoglycans in the subendothelial region, accompanied by the separation of endothelial cells from the internal elastic intima, and followed by the migration of SMC's into the subendothelial region. Then, SMC's lose their ability to proliferate and their fibroblast-like appearance and develop contractile fibers. As they mature, SMC's undergo physiological hypertrophy in response to increased load, which is accompanied by a progressive accumulation of cells with a tetraploid nuclear content [9]. At this stage they become quiescent in the adult vessel. When development is substantially complete, there are no more major changes observed in the typical organization of the wall in the large vessels. [9].

The principal function of mature SMC's is contraction. These cells exhibit a high cytoplasmic volume fraction of myofilaments [8], a low rate of proliferation [11], and synthesize only small amount of matrix protein [11]. However, SMC's exhibit a considerable degree of structural and functional plasticity, modulating (usually reversibly) from the mature phenotype towards a less differentiated phenotype (similar to that expressed in developing vessels) with increased capacities for motility, protein synthesis, and proliferation. Such a behavior of SMC's occurs during vascular remodeling related to injury or atherogenesis [12].

1.3 Remodelling of vascular vessels

The vascular vessel wall is dynamically responsive to an array of physiological and biochemical stimuli. First one includes biochemical substances such as inflammatory cytokines, circulating hormones and bacterial products. The biochemical stimuli include fluid shear stresses, cyclic strain and hydrostatic pressure [13]. Under such stimuli, known also as hemodynamic forces, blood vessels can change their structure, a process characterized as a vascular remodelling. Basically, remodelling is a change in the calibre of the vessel, with little or no change in overall tissue mass. The comprehensive analysis of this process has shown the changes in luminal diameter related to movement of tissue to or from the lumen [14].

Remodelling is not only a physiological process but occurs also in pathological events in vessel wall dynamics. Therefore, compensatory enlargement of an artery occurs as an arteriosclerotic plaque within the vessel enlarges. Only when the ability of the vessel to remodel is exceeded does the arteriosclerotic plaque narrow the lumen, a process known as a neointimal formation. Constrictive remodelling of an artery has recently been shown to be the major process involved in restenosis after angioplasty [14].

1.3.1 Hemodynamic forces in vascular remodelling

The passage of blood flow through the vascular system generates hemodynamic forces. The arterial vessel is continuously exposed to such mechanical stresses. Endothelial cells are exposed to shear stress as a result of blood flow, while SMC's within the vascular media are subjected to a significant mechanical strain throughout the cardiac cycle [15]. This includes both a baseline tensile stress, as well as cyclic mechanical strain due to the repetitive pulsative pressure of blood flow induced by a beating heart [15].

1.3.1.1 Shear stress

Shear stress, which endothelial cells are subjected to, is the consequence of blood flow. In detail, fluid shear stress is the frictional force generated at a surface by the flow of a viscous fluid. In the cardiovascular system these are typically defined in dyn/cm^2 [13]. This mechanical force affects mainly endothelial cells and has been implicated as the major hemodynamic factor in initiating some cardiovascular diseases [16]. Shear stress can be calculated taking into consideration flow rate, viscosity and capillary radius of the blood. The viscosity and flow rate of the blood in the blood vessels have been found to affect the magnitude of shear stress, as well as the lumen radius [17]

1.3.1.2 Pulse pressure - induced tensile stress

Tensile stress (tension) is one of the hemodynamic forces resulting from a pulsative nature of blood flow. Precisely, tension in the vessel wall is generated by distending and compressing forces affecting a vessel. During ventricular systole, arterial pressure rises (systolic) pressure and the artery distends. During the ventricular diastole (the semi lunar valve in the aorta is closed and no blood leaves the heart), the arterial pressure falls to the trough of the pressure wave and the artery compresses [18]. Pulse pressure, known also as circumferential pressure, is the difference between systolic and diastolic pressures. Compressing forces tends to decrease the radius of a vessel while distending forces tend to increase the circumference of the vessel [17]. Pulse pressure often increases in hypertension, advanced arteriosclerosis or aortic regurgitation and can decrease in deep shock [18].

1.3.1.3 Cyclic strain

In addition to fluid shear and tensile stress, vascular EC's and SMC's are continuously subjected to cyclic strain, which arises from the periodic change in vessel diameter as a result of pulsative changes in aortic pressure, and thus coronary perfusion pressure. Recent studies indicate that cyclic strain exerts significant effects on VSMC [21], which is widely described in section 1.4.4

1.4 Phenotypic Modulation/Switching Of Smooth Muscle Cells during Vascular Development and Disease.

The VSMC in mature animals are highly specialized cells whose principal function is contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution. SMC within adult blood vessels proliferate at an extremely low rate, exhibit very low synthetic activity, and express a repertoire of contractile proteins, ion channels, and signaling molecules required for the cell's contractile function [131]. Unlike either skeletal or cardiac muscle that are terminally differentiated, SMC within adult animals maintain remarkable plasticity and can undergo profound and reversible changes in phenotype in response to changes in local environment that normally regulate phenotype [131]. The example of SMC plasticity can be seen during vascular development when the SMC play a key role in morphogenesis of the blood vessel and exhibit high rates of proliferation, migration, and production of extracellular matrix components that make up a major portion of the blood vessel wall while at the same time acquiring contractile capabilities. Similarly, in response to vascular injury, the SMC dramatically increases its rate of cell proliferation, migration, and synthetic capacity and plays a critical role in vascular repair [131, 132].

However, an unfortunate consequence of the high degree of plasticity exhibited by the SMC is that it predisposes the cell to abnormal environmental signals

that can lead to unfavorable phenotypic switching and acquisition of characteristics that can contribute to development of vascular disease. Indeed, there is strong evidence that phenotypic switching of the SMC, defined as any change in the normal structure or function of the differentiated SMC, plays a major role in a number of major diseases in humans including arteriosclerosis, cancer, and hypertension [132].

1.4.1 SMC phenotypic switching in Arteriosclerosis

The best example of a disease in which SMC phenotypic switching is believed to play a key role is arteriosclerosis, a disease that is responsible for over 55% of all deaths in Western civilization. Arteriosclerosis is a complex disease involving many cell types including macrophages, lymphocytes, neutrophils, endothelial cells, and vascular SMC [131, 133]. The role of the SMC appears to vary depending on the stage of the disease, with it playing a maladaptive role in lesion development and progression, but likely playing an adaptive role within the fibrous cap in stabilizing plaques before activation of protease cascades that may contribute to end-stage disease events such as plaque rupture [134]. The contributions of the SMC are a function of very complex changes in the differentiated state of the SMC including increased matrix production [135], production of various proteases [134], participation in chronic inflammatory responses including production of inflammatory cytokines and expression of at least some inflammatory cell markers [136], altered contractility and expression

of contractile proteins [137], and a variety of other changes that have collectively been referred to as “phenotypic modulation” [132].

1.4.2 Regulation Of SMC Differentiation

Cellular differentiation is simply the process by which multipotential cells in the developing organism gain cell-specific characteristics that distinguish them from other cell types. Differentiation of the SMC exhibits a wide range of different phenotypes at different stages of development, and even in adult organisms the cell is not terminally differentiated and is capable of major changes in its phenotype in response to changes in its local environment [132]. For example, during early stages of vasculogenesis SMC are highly migratory and undergo very rapid cell proliferation [132].

During vascular injury, “contractile” SMC are capable of undergoing transient modification of their phenotype to a highly “synthetic” phenotype, and they play a critical role in repair of the vascular injury. Upon resolution of the injury, the local environmental signals within the vessel return to normal, and SMC reacquire their contractile phenotype [132].

Taken together, SMC are highly plastic cells capable of profound alterations in their phenotype in response to changes in local environment that is important for their differentiation.

1.4.3 VSMC Differentiation Markers

There is a repertoire of genes expressed by adult VSMC *in vitro* that dictate the phenotype of these cells. A variety of SMC-selective or specific genes and gene products have been identified that serve as useful markers of the relative state of differentiation-maturation of the SMC. These include the smooth muscle isoforms of contractile apparatus proteins: SM α -actin, SM MHC, h1-calponin, SM22, aortic carboxypeptidase-like protein (ACLP), desmin, h-caldesmon, metavinculin, telokin and smoothelin [132,138]. The most widely used SMC marker is SM α -actin. This protein is an excellent SMC differentiation marker and is the first known protein expressed during differentiation of the SMC during development [132,138]. Moreover, it is required for the development of fully differentiated SMC and is by far the single most abundant protein in differentiated SMCs making up to 40% of total cell protein [132, 138]. Similarly, all the remaining SMC differentiation marker genes are expressed in a variety of circumstances in other cell types. SM22, a calponin-like protein of unknown function, exhibits an expression pattern very similar to SM α -actin. It is expressed in skeletal and cardiac muscle during development. H1 calponin, a calcium regulatory protein, is expressed in cardiac myocytes, myofibroblasts, and a variety of tumor cell [139, 140]. ACLP is widely expressed in many tissues. Smoothelin appears to be selectively expressed in differentiated SMCs as two known isoforms: a 55-kDa (type A) and 120-kDa (type B) isoform that

are expressed selectively in visceral SMCs and vascular SMCs, respectively [141].

1.4.4 Effects of mechanical forces on VSMC's

Vascular smooth muscle cells (VSMC's) in the major arteries are constantly exposed to cyclic strain [16]. Recent studies indicated that cyclic strain plays an important role in growth and gene expression in VSMC's under both normal and pathological conditions [16, 19, 20]. In hypertension, cyclic strain increases by as much as 30%, resulting in marked alteration in signal transduction and gene expression that contribute to VSMC hypertrophy and hyperplasia [20, 21, 22]. Moreover, mechanical stress has been shown to induce alterations in vascular smooth muscle cell phenotype, including cell morphology, proliferation, apoptosis [23], production of vasoactive substances [19, 20], gene expression [23, 24, 25] and alignment [23].

Several experimental models have been developed to study effects of mechanical forces on cultured VSMC's. Models such as the Flexercell Strain Unit [26, 27] allow the examination of the effects of mechanical strain on cultured VSMC's. A number of studies have used this model to assess the effect of cyclic strain on proliferation of cultured VSMC's. The results of these studies have been somewhat conflicting, with both increases and decreases in proliferation being reported. Proliferative responses have been reported in both intact aortic cultures [28] and cultured VSMC from humans, rabbits and rats

[29, 30, 31]. Conversely, a decrease in proliferation in response to mechanical stimuli has been reported in porcine VSMC and in A10 cells [32, 33]. The causes of these disparate effects remain to be determined, but are likely to involve the origin of the cells, changes in phenotype occurring in culture, and the conditions under which the cells are maintained [25].

Mechanical strain has been shown to be essential also for the maintenance of VSMC contractile function, vessel wall morphology, and sensitivity to vasoconstriction [34, 35]. It has been found that cyclic strain alters cultured VSMC differentiation characterised by changes of "contractile" phenotype into a "synthetic" phenotype [36]. Such changes in VSMC cells have also been found following injury or during development of arteriosclerosis [21]. It has been proposed that this synthetic phenotype of cultured VSMC's may be similar to that seen in vivo in arteriosclerotic lesions [25]. Synthetic (intimal) SMC's, associated with vascular remodelling following injury, are phenotypically distinct from their contractile (medial) counterparts. Synthetic cells resemble immature, dedifferentiated SMC and have lower level of contractile proteins, fewer myofilaments and express a large number of proteins involved in lesion development [20]. Contractile SMC's express differentiated cell markers associated with contractile function (actin, myosin etc) and the synthesis and maintenance of extracellular components of the vessel wall (elastin, osteopontin etc) [38].

1.5 Involvement of signalling pathways in vascular smooth muscle cell fate.

A number of different intercellular signaling pathways have been identified as key players in the process of vasculogenesis, angiogenesis and vascular remodeling. These pathways include the vascular endothelial growth factor (VEGF) pathway, fibroblast growth factor pathway, the transforming growth factor (TGF) pathway, the Angiopoietin/Tie receptor pathway, platelet-derived growth factor pathway (PDGF) [3], the ephrin/Eph receptor pathway, and many others [2, 4, 5]. However, the molecular mechanism of vascular remodeling is still unclear. Insulin-like growth factor-I (IGF-I) is the most potent for maintaining the differentiated SMC. In the IGF-I-stimulated culture system, phosphoinositide 3-kinase (PI3-K) and its downstream target, protein kinase B (PKB(Akt)), but not mitogen-activated protein kinases (MAPKs), mediate the critical signaling pathways [43]. MAPKs have been implicated in the signaling cascades involved in the proliferation and hypertrophy of SMCs. These include extracellular signal-regulated kinase (ERK) and the stress-activated MAPKs, c-Jun NH₂-terminal protein kinase (JNK) and p38MAPK. ERK is activated in response to growth factors, cytokines, and cellular stresses and is involved in a variety of biological processes. Both the ERK and p38MAPK pathways triggered by PDGF-BB, bFGF, and EGF were found to play an essential role in

inducing SMC de-differentiation, whereas the PI3-K/PKB(Akt) pathway was critical in maintaining a differentiated state.

PDGF is a potent chemo-attractant for SMCs, and TGF- β regulates SMC differentiation. PDGF-B stimulation up-regulates Ang1 expression in SMCs through the PI3K and PKC pathways and produces an acute induction of TGF- β expression in SMCs through the MAPK/ERK pathway [45]. Interestingly, TGF- β negatively regulates Ang1 expression induced by the PDGF-B stimulation in SMCs [40]. Developmental studies indicate that Tie2/ Ang1 signaling mediates recruitment of SMCs during vascular maturation. This suggests a tight regulation of Tie2 activity, PDGF receptor activity, and TGF- β receptor activity, which are required for the appropriate vascular development [45].

Taken together, there are many different signaling pathways involved in vascular development. Recent studies have added the Notch signaling pathway to this list [7].

1.5.1 Notch signalling pathway in vascular development

Evidence that the Notch pathway plays a critical role in vascular development and homeostasis includes the specific expression of Notch pathway ligands and receptors in vascular endothelium or supporting cells [42-51], as well as the phenotypes of several targeted mutants in Notch pathway components. These mutant animals, which include mutations in genes encoding both ligands and receptors, die during embryogenesis from haemorrhaging because of defects in

vascular morphogenesis [52, 53]. Mutations of Notch receptors and ligands in mice lead to abnormalities in many tissues, including the vascular system [54]. Human diseases, such as Alagille syndrome (AGS) and cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which show abnormalities in the cardiovascular system, are caused by mutations of the Notch ligand Jagged-1 and the receptor Notch-3, respectively [55]. Such findings evidently display a crucial role of the Notch pathway in vascular development.

Chapter 2. Notch Signalling Pathway

Notch was identified genetically in a mutant fly with 'notches' in its wings [56], which indicated its requirement in wing outgrowth. Notch has since been found to be crucial for patterning in a great number of other developmental settings throughout the animal kingdom, from worms to humans. Notch signalling is a highly conserved mechanism that regulates cell fate decisions throughout the development of invertebrate and vertebrate species. In vertebrates receptors, ligands, and other components of Notch signaling pathway are expressed in various organs from all three-germ lines, including vessels [56]. Notch is a single spanning transmembrane protein (Fig. 2) with many repeats of a protein module resembling epidermal growth factor (EGF) and three membrane-proximal Lin12/Notch/Glp-1 (LNG) repeats [57]. The

intracellular domain has four distinct regions, the RAM domain, the ankyrin repeats, a transcriptional activator domain (TAD) and the PEST (proline-, glutamate-, serine-, threonine-rich) sequence. Two nuclear localization sequences are present prior to and following the ankyrin repeats [57]. Notch activation involves proteolytic cleavages at three sites S1, S2 and S3 where: S1 cleavage occurs within the secretory pathway so that a processed heterodimeric form is transported to the cell surface; S2 cleavage occurs following ligand binding by Delta or Serrate (Jagged in mammals) through their Delta/Serrate/Lag2 (DSL) domains and releases a membrane tethered form of the Notch intracellular domain; S3 cleavage releases the soluble intracellular domain of Notch (NICD) [58]. NICD is translocated to the nucleus where it binds to a transcription factor Suppressor of Hairless (Su (H)), or CBF1 in vertebrates, via the RAM domain and ankyrin repeats (Fig 3). In the absence of a Notch IC, Su (H)/CBF1 can repress transcription through the recruitment of a histone deacetylase (HDAC) [59]. Binding of NICD displaces HDAC (Fig. 3) and allows recruitment of histone acetylases and the nuclear protein Mastermind, which together activate transcription of downstream target genes, such as Hairy Enhancer of Split (HES) and HRT's [60].

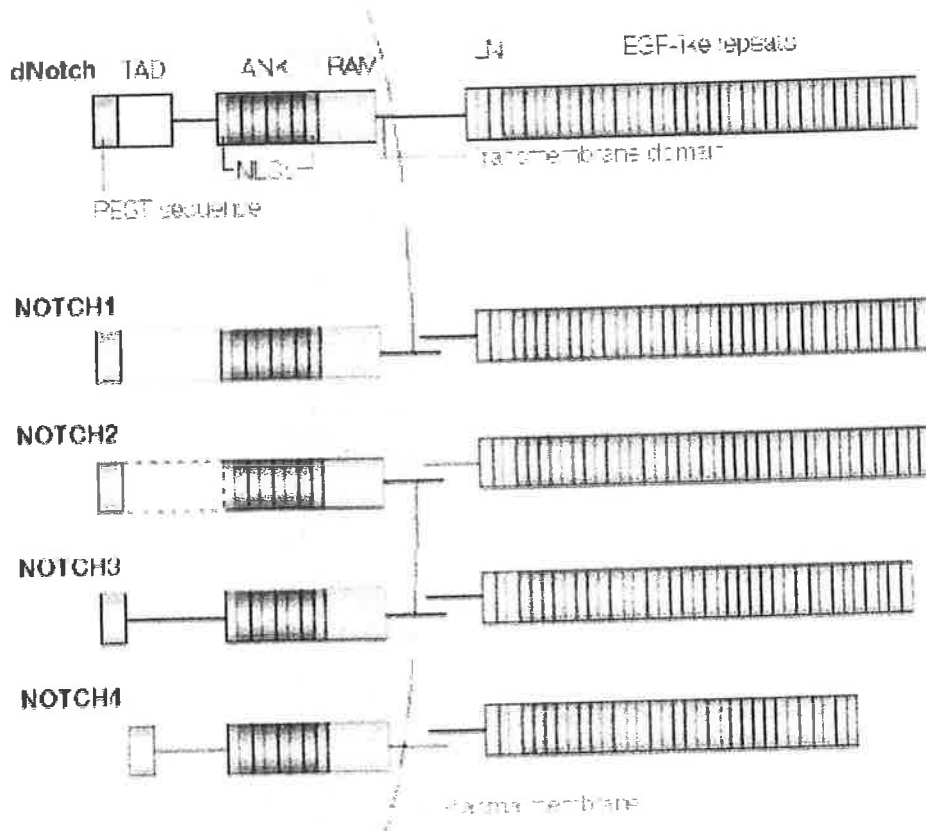


Fig. 2 Domain Organization of Notch Receptors [61]

Proteolytic cleavage by furin at site 1 (S1) produces two subunits, which remain non-covalently associated at the cell surface. EGF-like modules participate in binding to a ligand. Two consecutive proteolytic cleavages induced upon ligand activation release the intracellular domain of Notch (NICD). NICD consists of the RAM domain, NLS (nuclear localization signal), (ANK) ankyrin repeats and TAD (transactivation domain). PEST (proline, glutamate, serine, threonine-rich) sequence regulates protein turnover. EP domain (not shown), which maps C-terminal to the sixth ankyrin repeat, was defined as the region mediating direct interaction with the HAT (histone acetylases) protein p300.

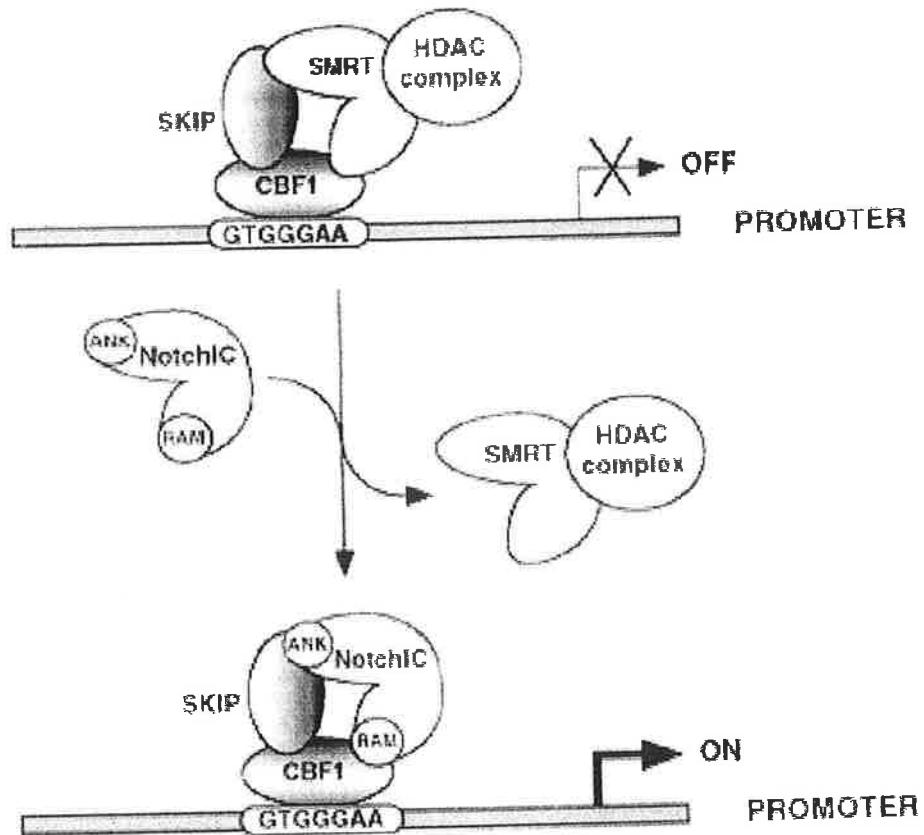


Fig. 3 Activation of Target Genes by Notch Intracellular Domain (NICD)

In the nucleus, NICD converts CBF1 from a transcriptional repressor to a transcriptional activator. This conversion occurs by direct protein-protein interactions between the Notch intracellular domain, SKIP (Ski-related Protein) and CBF1, which leads to SMRT/HDAC dissociation (Silencing Mediator of Retinoid and Thyroid hormone receptors; HDAC, Histone Deacetylase).

2.1 Notch Signal Transduction

The Notch signal is initiated through binding of the ligand, Delta or Serrate (Jagged in mammals). The cytoplasmic tail of Delta allows the ligand/receptor complex to be engaged by the endocytic machinery which may place the receptor under mechanical strain. In some developmental contexts the endocytosis of the ligand together with the Notch extracellular domain may be stimulated by the Ring Finger domain E3 ubiquitin ligase Neuralized (Fig. 4a) [58]. The process of *trans*-endocytosis (Fig. 4b) of the Notch extracellular domain may expose the S2 site for cleavage by an ADAM metalloprotease such as TACE (TNF- α -converting enzyme) or Kuzbanian (Fig 5). The released membrane tethered NIC (Fig. 4c) is recognized by the secretase complex, including the membrane proteins Presenilin and Nicastrin. Notch is cleaved by this complex at the S3 site, which is located within its transmembrane domain. This cleavage releases soluble NICD, which is translocated to the nucleus. A recent report [62] suggests that Presenilin may not be the protein that directly cleaves Notch.

In the nucleus NICD forms a complex with the DNA-binding protein Su (H)/CBF1 (Fig. 4d). The latter can act as a transcriptional repressor as it recruits histone deacetylase (HDAC) (Fig 3), but when complexed with NICD, the histone deacetylase is displaced and histone acetylase (HAT) molecules are recruited which act to promote a chromatin conformation favorable to

transcription. Other transcription activating proteins such as Mastermind are also recruited [62]. The signal is terminated through ubiquitination of NICD by a complex including Sel-10, followed by proteasome-dependent degradation (Fig. 4e). Negative regulation of the Notch signal may also occur prior to ligand-dependent activation through the activity of a Nedd4 family HECT domain E3 ubiquitin ligase, Su(dx)/Itch (Fig. 4f) [62]. Notch ligands also form *cis* complexes with Notch that antagonize the signal (Fig. 4g). The possible alternative models for the activity of Kuzbanian and Neuralized are shown in Fig. 4h-i. Delta undergoes Kuzbanian-dependent cleavage, which may act to remove the *cis*-inhibition by the ligand (Fig. 4h). Neuralized may act to remove excess Delta by triggering its endocytosis (Fig. 4i), relieving the antagonistic *cis*-interaction.

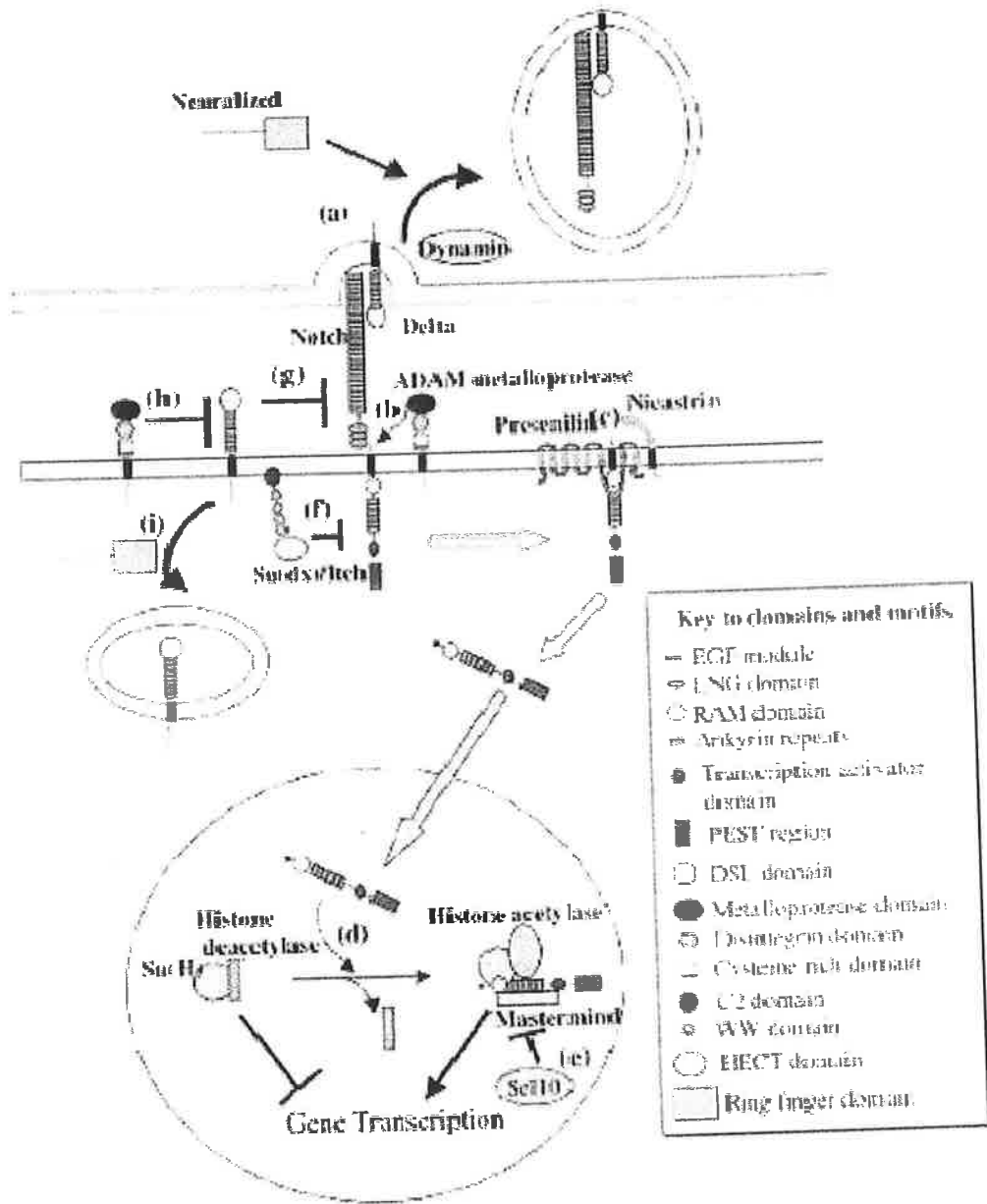


Fig. 4. Multiple enzymatic activities involved in initiating and regulating the Notch signal [63]

2.1.1 S1 site cleavage: Notch processing within the secretory pathway

It has been shown in mammalian cells that Notch-1 and -2 are cleaved within their extracellular domains [64]. The Notch receptor is cleaved in the trans-Golgi network, by a furin-like convertase, and presented on the cell surface as a heterodimer (Fig 5). The two cleavage products remain associated non-covalently but Ca^{2+} dependent at the cell surface.

Cleavage at the site S1 yields an 180-kDa fragment containing the majority of the extracellular domain, and an 120-kDa fragment consisting of a membrane tethered intracellular domain with a short extracellular sequence. The two cleavage products remain associated non-covalently at the cell surface [65]. In mammalian cells this heterodimeric form comprises the majority of the Notch-1 receptor at the cell surface and is responsible for signaling through the Su(H)/CBF1-dependent pathway [66,67]. The situation in *Drosophila* is less clear. Fragments of Notch containing the intracellular domain have been observed in protein extracts, which roughly correspond in size to that expected for the 120 kDa fragment of the heterodimer [64], but the identities of these fragments were not characterized. In fact recent data suggests that it is the uncleaved form of *Drosophila* Notch, which is at the cell surface [68]. Furthermore a region of *Drosophila* Notch, which is in a corresponding position to the Notch-1 Furin-sensitive sequence, was found not to be necessary for signaling [68]. The

situation for the other mammalian Notch receptors, apart from Notch-1, is also not determined and comparison of their sequences suggests there may be differences in their processing.

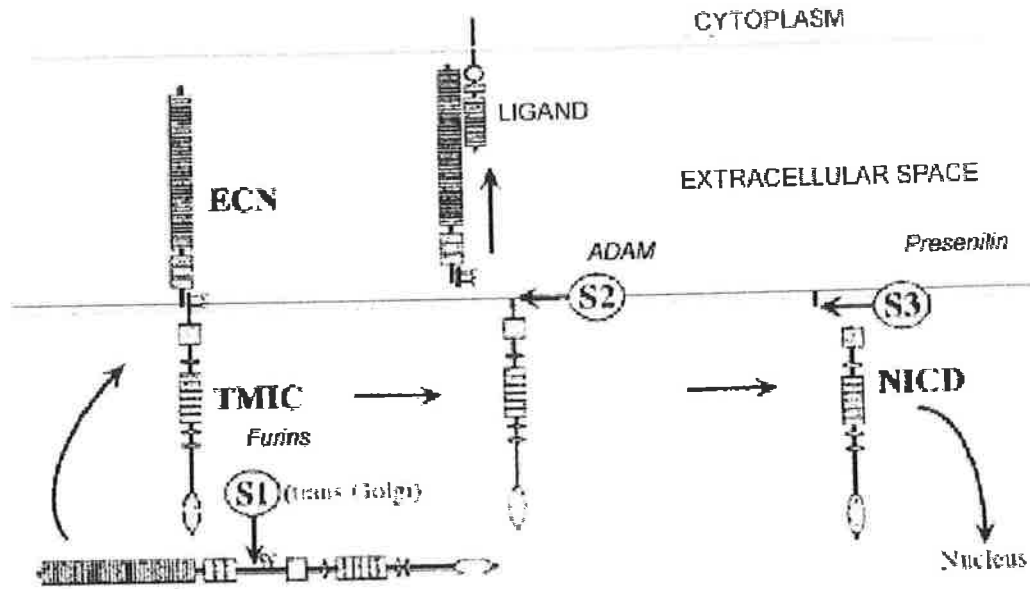


Fig. 5 Notch activation involving proteolytic cleavages at three sites [58]

S1 - Non-activating cleavage mediated by furin-like convertase, occurs during maturation within the secretory pathway and it is thought to be necessary for cell surface expression of heterodimeric full-length receptor. S2 - activating cleavage mediated by metalloproteinases, occurs in response to ligand binding; relieves extracellular inhibition of signaling by releasing the ectodomain. S3 - activating cleavage mediated by γ -secretase-like protease, occurs within the transmembrane domain following S2 cleavage resulting in the release of NICD and activation of downstream target genes.

2.1.2 S2 site cleavage: ligand-dependent cleavage of Notch

The S2 cleavage of Notch occurs following ligand binding by Delta or Serrate (Jagged) to the Notch extracellular domain and is thought to be dependent on a member of the ADAM metalloprotease family (Fig 5) [69]. S2 cleavage releases a membrane-tethered form of the Notch intracellular domain that is a constitutive substrate for the S3 cleavage.

One candidate that may be involved in S1 cleavage is Kuzbanian whose mutation gives phenotypes resembling those of *Notch* [69]. However, the true role for Kuzbanian is clouded by several contradictory reports. The *Kuzbanian* mutations were found to prevent a cleavage of *Drosophila* Notch that was originally thought to be at the S1 site [69], which, as discussed above, may not occur in *Drosophila* Notch. This fragment may therefore reflect *in vivo* S2 site cleavage, which would give a similar sized band, or other cleavage products of Notch. In a later study, expression of a dominant negative form of Kuzbanian had no effect on Notch processing in cell culture experiments, but did inhibit processing of the ligand Delta [70]. A role for Kuzbanian was therefore attributed to the generation of a soluble, secreted form of Delta, potentially capable of activating Notch at a distance from the ligand-expressing cell. However, this does not explain a requirement for Kuzbanian in the cell receiving the Notch signal [68]. In addition, although Notch activation by soluble ligand has been reported [70], other studies have shown soluble Delta

constructs act dominant negatively [71]. Finally, although Delta is capable *in vivo* of acting over several cell diameters, this may be due to cell-to-cell contact by long cytoplasmic extensions of the Delta expressing cell [72]. An alternative role for Kuzbanian-mediated ligand cleavage could be the removal of excess ligand, which, when in a cis-interaction with its receptor on the same cell, can have a dominant negative effect on Notch signaling. This would explain the cell autonomy for Kuzbanian. However a new report supports a role for Kuzbanian acting on *Drosophila* Notch [73]

In mammals, although *Kuzbanian* loss of function has also been linked to *Notch*-like phenotypes, *Kuzbanian* did not co-purify with an enzymatic activity responsible for an *in vitro* S2 site cleavage of Notch-1 [73]. Instead a different ADAM protease, TACE (TNF- α -converting enzyme) has been implicated [73]. It has also been found that *Drosophila* TACE can cause an S2-like cleavage of *Drosophila* Notch although it may be at a site distinct from that which is dependent on *Kuzbanian* [74] (definitive role for TACE in the signal generation step in *Drosophila* awaits identification). Interestingly, one conclusion arising from the data is that the subsequent S3 cleavage step may have a different efficiency depending on whether S2 cleavage was performed by *Kuzbanian* or TACE [30], which if relevant *in vivo* might have implications for the strength and duration of the Notch signal. In mammals the identity of the S2 cleaving enzyme *in vivo* is also not fully established. It is possible that S2 cleavage may involve the activities of more than one enzyme and inspection of the protein

sequences suggests that the S2 cleavage mechanism may not be strictly conserved between different members of the Notch family.

2.1.3 The signal generation step: S3 cleavage

The membrane-tethered NICD product of the S2 cleavage is itself a substrate for a proteolytic activity that cleaves Notch at the S3 site [75]. S3 cleavage happens in the trans-membrane domain of NICD, and is mediated by a multiprotein complex with gamma-secretase activity (Fig. 4, 5). The latter product which is soluble NICD is translocated to the nucleus to activate the Su (H)/CBF1-dependent signal.

In vivo studies in *Caenorhabditis elegans*, *Drosophila* and mice [76] have shown that Presenilin proteins are required for Notch signaling. In mammals, Presenilin-1 and -2 are associated with a large complex of proteins, which make up the secretase activity that cleaves the amyloid precursor protein (APP), also within its transmembrane domain (whose aberrant cleavage may cause Alzheimer's disease)[77]. This suggested that the mechanisms leading to the cleavage of APP and Notch might be related. In support of this, constitutive signaling by membrane tethered NICD, but not soluble NICD, is dependent on Presenilin [78]. It seems that the small size of the extracellular domain is the important factor that triggers the Presenilin-dependent-secretase activity [78]; hence mutations of Notch that remove the mass of the extracellular domain are constitutively active. Other proteins of the large γ -secretase complex must be

involved in this process. For example, the membrane protein, Nicastrin, may be involved in substrate recognition or Presenilin trafficking and turnover [79]. Two further candidate components are Aph-1 and Pen-2, which are multipass membrane proteins that promote γ -secretase cleavage in *C. elegans* [79]. While Presenilin is required for the S3 cleavage, whether it is itself directly responsible is less clear. Aspartyl-protease transition-state analogues were found to bind to Presenilin-1 protein [80] suggesting that Presenilin contains the catalytic site. In addition, two conserved aspartate residues in Presenilin, predicted to form the active site, are required for γ -secretase-dependent cleavage of both amyloid protein and Notch [80]. Some unexplained differences remain between the activity of Presenilin on APP and Notch. Two intra-membrane sites in APP are cleaved by a Presenilin-dependent protease activity, but only the more C-terminal of these resembles the S3 cleavage site of Notch [81]. Even then there are differences between APP and Notch regarding the influence of the amino acid sequence at the cleavage site [82]. In addition, a recent report [83] suggests that S3 cleavage of Notch depends on the presence of Presenilin, but using an *in vitro* assay, Presenilin does not co-purify with the activity responsible for the cleavage.

2.1.4 Switching off the Notch signal

Ubiquitin ligase and proteolytic steps are involved in down-regulation of the Notch signal. Sel-10 was originally identified in *C. elegans* as a negative regulator of Notch signaling and is a substrate-targeting component of an SCF class E3 ubiquitin ligase [84]. Mammalian homologues of Sel-10 protein can stimulate phosphorylation-dependent ubiquitination of nuclear NICD and trigger its proteasome dependent degradation [85]. The latter interaction is dependent on the C-terminal region of Notch, which includes the PEST domain. A proteasome-dependent turnover of *Drosophila* NICD has also been shown *in vivo* [85]. Recent *in vitro* data suggests that Mastermind, the transcriptional activator recruited by NICD into the Su(H)/CBF1 complex, also stimulates phosphorylation and turnover of NICD [85]. Thus activity of Notch is self-limiting. Another E3 ubiquitin ligase implicated in Notch pathway down-regulation in *Drosophila* is the HECT domain Nedd4 family protein Suppressor of deltex (Su (dx)) [86]. A mouse homologue of Su (dx), Itch, associates with Notch and promotes PEST domain-independent ubiquitination in cell culture and *in vitro* assays [87]. Interestingly *itch* mutations result in an inflammatory response although it is not known if this is related to altered Notch signaling.

2.2 Notch ligands

Notch ligands are divided into two subclasses, the Delta family and the Serrate family (Fig 6). All Notch ligands are transmembrane proteins that share some structural features including epidermal growth factor (EGF)-like repeats, a characteristic DSL domain necessary for Notch binding and a transmembrane region. However, an extracellular cysteine-rich domain and insertions that interrupt some EGF-like repeats are common only to the Serrate family. It is these structural differences that categorize a Notch ligand as a Delta or Serrate family member (Fig 6).

Drosophila has only one Delta and one Serrate gene, but mammals contain two members of the Serrate family, Jagged1 and Jagged2, and three members of the Delta family, Dll1, Dll3, Dll4 (Table 1). During *Drosophila* development, Delta and Serrate are distinguished functionally by their sensitivity to Fringe. Fringe is a β -1,3-*N*-acetylglucosaminyltransferase that elongates *O*-fucose on EGF repeats and acts as a key modulator of Notch signaling [88-92]. Expression of Fringe inhibits the activation of Notch by Serrate and, at the same time, potentiates the activation of Notch by Delta [88- 92]. Mammalian Fringe genes have also been shown to be able to influence the activation of Notch by its ligands, and as in *Drosophila* different mammalian ligands exhibit distinct sensitivities to Fringe [88, 91].

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Protein type	<i>Drosophilae</i>	Mammals	<i>Caenorhabditis elegant</i>
Receptors	Notch	Notch1 Notch2 Notch3 Notch4	Lin-12 Glp-1
Ligand	Delta Serrate	Delta 1 Delta 3 Delta 4 Jagged 1 Jagged 2	Lag-2 Apx-1
O-FucT-1	OFUT1	POFUT1	C15C7.1
Kuzibanian	Kuzibanian	Kuzibanian Adam17	Sup-17
Gamma secretase complex	Presenilin Nicastrin	Presenilin1 Presenilin2 Nicastrin	Aph-2 Aph-2 Pen-2

Table 1. Components in core Notch signal pathway

[<http://cell.tulane.edu/Syllabi/Spring%202004/Notch.ppt.pdf>]

Mutations in ligand can result in the disruption of the Notch signalling pathway, leading to developmental abnormalities. Mutations of human Jagged1 have been attributed to the development of Alagille syndrome (AGS) [88-92], an autosomal dominant disorder characterized by developmental abnormalities of the heart, skeleton, muscle, liver, and eyes. Mice homozygous for the pudgy (*pu*) mutation exhibit severe deformations of the ribs and vertebrae, and this mutation has been mapped to the Dll3 locus. Dll1-deficient mice generated by homologous recombination exhibit neonatal lethality, and disruption of murine Jagged2, thus revealing an essential role for this gene in limb, craniofacial, and thymic development [88, 92].

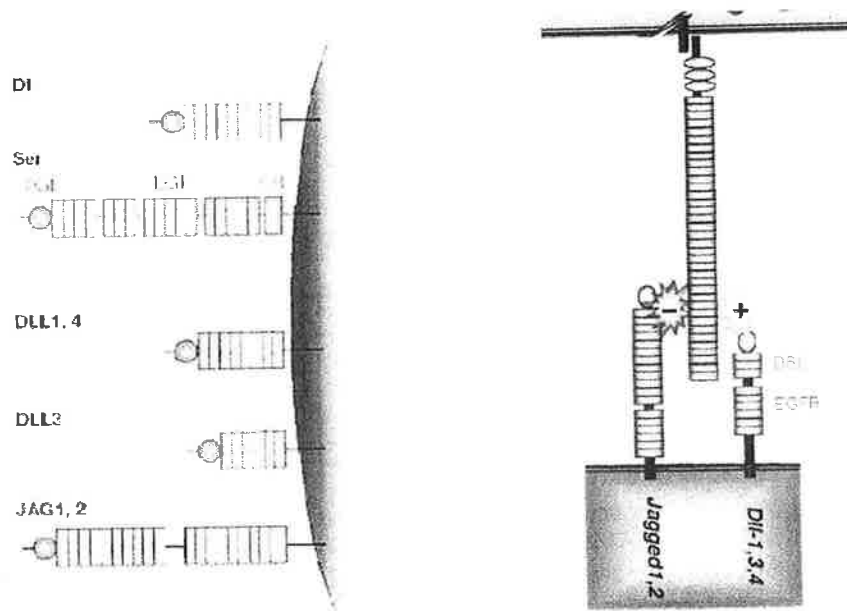


Fig. 6 Notch ligands [61]

Receptor-Ligand interactions are mediated by EGF repeats of the Notch receptor and the conserved extracellular region of the ligand, referred to as DSL domain (Delta/Serrate/LAG-2). Delta3 can function like other ligands as a potent inhibitor of Notch signaling when expressed in responding cells (*cis*-inhibition).

2.3 Notch transcription factors (HES and HERP families)

The NICD-CBF1 complex upregulates the expression of primary target genes of Notch signaling such as HES (Hairy/E (spl)-related) in mammals, and E (spl) (Enhancer of Split) in *Drosophila*. The HES/E (spl) family is a basic helix-loop-helix (bHLH) type of transcriptional repressor and acts as Notch effectors by negatively regulating the expression of downstream target genes such as tissue-specific transcription factors [94]. Recently, a new bHLH family has been

isolated and named as Hey (Hairy/E(spl)-related with YRPW), Hesr (Hairy/E(spl)-related), HRT (Hairy-related transcription factor), CHF (Cardiovascular helix-loop-helix factor), gridlock and HERP (HES-related repressor protein) [Table 2]. The amino acid sequence of HERP and its characteristic domains indicate that HERP is closely related to the HES family. Interestingly, HERP expression is detected in both HES-expressing and non-HES-expressing tissues [94]. Remarkably, HES and HERP may function not only as homodimers but also as HES-HERP heterodimers in those cells co-expressing HES and HERP [94]. Although both HES and HERP act as transcriptional repressors, HERP employs different repression mechanisms than does HES. HERP could thus play a critical role in mediating Notch effects in both HES-expressing and non-HES-expressing tissues either as a hetero- or homo-dimer [94].

The bHLH family of transcriptional regulators plays crucial roles in the development of various organs and cell types including the nervous system, the heart, skeletal muscles, the pancreas, endodermal endocrine organs, and hematocytes [96]. Over 240 HLH proteins have been identified to date in organisms ranging from yeast to human. The bHLH proteins bind to a specific DNA sequence as a dimer. The basic and HLH domains have distinct functions. The basic domain is a major determinant of DNA binding specificities. DNA binding is mediated by a contact between each basic domain of a dimer and a specific half-site of consensus DNA sequences. The HLH domains are

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characterized by hydrophobic residues that allow them to form homo- or hetero-dimers. bHLH proteins can be classified into several groups according to their structural features and biochemical characteristics. Class A proteins are transcriptional activators, class B proteins are bHLH luciferase zipper type proteins, class C proteins are transcriptional repressors such as HES in mammals (hairy and E (spl) in *Drosophila*) and HERP (Hey/Hesr/HRT/CHF/gridlock), and are characterized by an invariant proline residue at a specific site of the basic domain [97].

Abbreviations	Full name	Species	References
HERP 1, 2, 3	HES-related repressor protein	Mouse, rat, human	Iso <i>et al.</i> (2002)
Hesr 2, 1, 3	Hairy/E(spl)-related	Drosophila, mouse, human	Kokubo <i>et al.</i> (1999), Satow <i>et al.</i> (2001)
Hey 2, 1, L	Hairy/E(spl)-related with YRPW	Drosophila, chicken, mouse, human	Leimeister <i>et al.</i> (1999)
HRT 2, 1, 3	Hairy-related transcription factor	Mouse, human	Nakagawa <i>et al.</i> (1999)
CHF 1, 2	Cardiovascular helix-loop-helix factor	Mouse, human	Chin <i>et al.</i> (2000)
Gridlock		Zebrafish, human	Zhong <i>et al.</i> (2000)

Table 2. HERP family nomenclature [98]

2.3.1 Comparison of HES and HERP families

Seven HES members and three HERP members have been isolated in Mammals [99, 100]. The two families share several common features (Fig.7). They contain bHLH domain, and another domain, termed the Orange (or helix3- helix4). The amino acid sequences of these domains are highly conserved within the particular family, but less between the two different families. The most remarkable difference that distinguishes HES from HERP is a proline residue in the basic region. This proline residue is invariant among HES/E(spl) family members across species from *Drosophilae* to human. Because of this proline, HES members are also called proline bHLH proteins. The HERP family has a glycine at the corresponding position and this amino acid also is strictly conserved from *Drosophilae* to human HERPs. Thus, these prolines and glycines are hallmarks for the HES and HERP families, respectively [99, 100]. All HES members share the C-terminal tetrapeptide WRPW motif, whereas the HERP family has YRPW or its variants. In addition to the tetrapeptide motif, the HERP family has an additional conserved region carboxyl-terminal to the tetrapeptide motif, TE (V/I) GAF, which is absent in HES [94]. Both HES and HERP function as transcriptional repressors. A phylogenetic tree shows that they form a distinct subgroup in a large bHLH protein family. These findings indicate that HERPs are closely related to the HES family belonging to class C protein, but it forms a distinct subgroup.

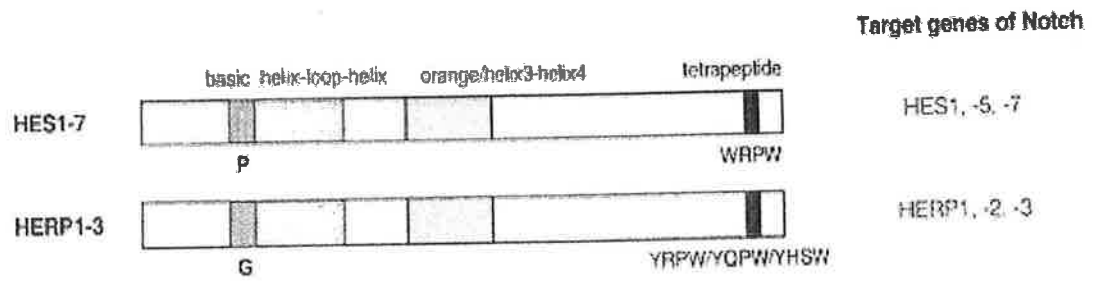


Fig. 7 Schematic diagram of target genes of Notch: HES and HERP [98].

Conserved domains are marked by distinct colors: Blue for the basic domain, green for the helix-loop-helix domain, orange for the Orange domain, and pink for the tetrapeptide motif.

Chapter 3. Modulation of smooth muscle cell fate by

Notch signalling pathway

The Notch signaling pathway has been characterized as a critical determinant of cell fate in a variety of organisms. In mice, for example, Notch-1 and Notch-2 gene deletions are characterized by perturbations in organogenesis that result in embryonic lethality [95]. Human diseases (see section 3.3), which show abnormalities in the cardiovascular system, are caused by mutations of the Notch pathway. Mechanistic studies performed in cell culture models indicate that the Notch pathway influences cell fate by regulating programs governing growth, apoptosis, and differentiation [95]

3.1 Regulation of Cell Development by Notch Signalling

A key to the development of all multicellular life is the ability to form "biological pattern". Pattern formation is possible as a result of a molecular mechanisms of cell-cell signaling, which allow cells to influence each other's fate and behavior. One of the most important mechanisms of cell signaling is mediated by Notch.

Notch is likely to be involved in the development of most tissues in species throughout the animal kingdom, with multiple effects on cell fates including differentiation, migration, proliferation and apoptosis. Often, more cells than needed have the opportunity to become a specialized cell type. An important

role for Notch signaling is to prevent these 'extra cells' from undergoing such specialized fates (Fig. 8A, B) [101].

The classic example of such an inhibitory role of Notch is the *Drosophila* neural-epidermal selection. Special groups of cells known as proneural clusters have neural potential because of their expression of basic helix-loop-helix (bHLH) transcriptional activators, also known as proneural proteins. Notch signaling restricts neural differentiation by repressing the expression of proneural genes. The failure of Notch signaling causes abnormal accumulation of proneural proteins in proneural cells, which, in turns, become neural cells, and manifest the 'neurogenic' phenotype [102]. Constitutive Notch signaling has the opposite effect and suppresses neural differentiation. In vertebrates, Notch also represses neurogenesis and myogenesis via homologous Hairy/E(spl)-related bHLH repressors known as HES.

A second role of Notch signaling is to promote the development of a cell type or body region, often by inducing the expression of positively acting regulatory molecules [Fig. 9 A, B]. Notch signaling appears to create a new cell type as a result of cell-cell interactions at the border between distinct cell populations. This is sometimes referred to as 'inductive' Notch signaling, that is opposite to the 'inhibitory' role of Notch where Notch signaling represses a given cell fate among equipotent cells.

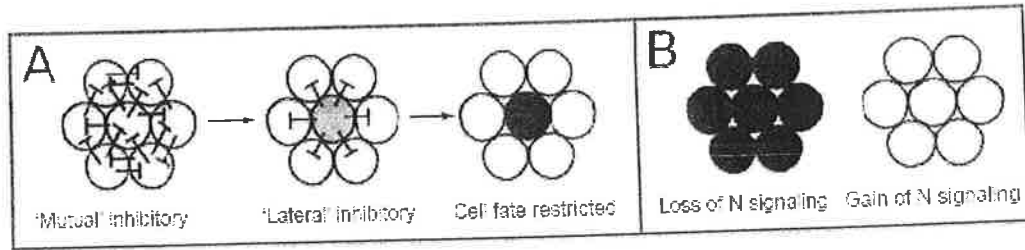


Fig. 8 Inhibitory Notch signaling restricts cell fates [101].

A. Equipotent cells that share a special cell fate potential (gray) send and receive Notch signals, known as 'mutual' inhibitory Notch signaling. Later, one cell undergoes the specialized fate (black) and inhibits surrounding cells (white) from adopting this fate, a situation known as 'lateral' inhibitory Notch signaling. B. The failure of Notch signaling results in extra cells adopting the special cell fate, while excessive Notch signaling prevents the differentiation of these cells.

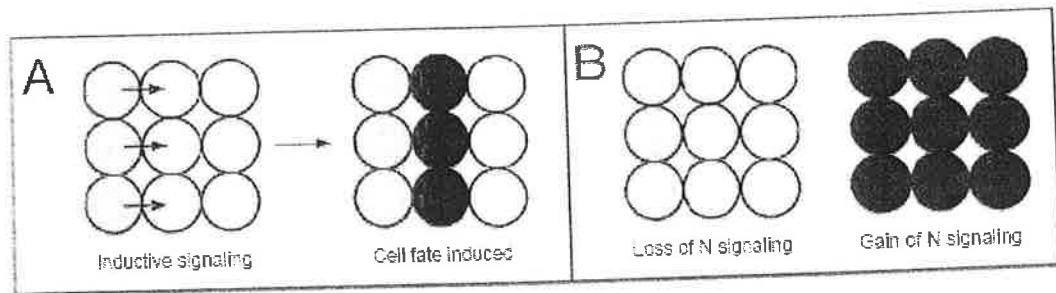


Fig. 9 Inductive Notch signaling specifies cell fate or behavior [101]

A. Schematic of inductive Notch signaling, which occurs between nonequivalent cell populations. In this case, the blue cells give a signal to adjacent white cells to induce a new cell fate or change their behavior (black cells). B. The failure of inductive Notch signaling results in the absence of this cell fate or behavior, whereas excessive Notch signaling has the mutual effect.

3.2 Involvement of Notch in Vascular SMC cell fate

Recent studies performed in cell culture models indicate that the Notch signalling pathway influences VSMC fate by regulating programs governing growth, apoptosis, and differentiation. For example, Notch3 could promote VSMC growth through down-regulation of p27KIP1, a cyclin-dependent kinase (Cdk) cell cycle inhibitor [104]. Mutation in human Notch 3 gene causes CADASIL disease (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) characterized by progressive VSMC death. HRT1, a major downstream target gene of Notch signalling in the vasculature, is also a critical determinant of VSMC fate by modulating growth and apoptosis. HRT1 signalling promotes VSMC growth by inhibiting the expression of a principle cell cycle inhibitor, p21WAF1=CIP1, another Cdk inhibitor closely related to p27KIP1, and attenuates both growth factor deprivation and Fas ligand (FasL)-induced cell death by inducing the expression/activity of Akt, a well-established antiapoptotic mediator [104].

Despite a lot of studies showing that Notch signalling influence SMC differentiation little is known about involvement of Notch components in VSMC diversification. Therefore, this study focused on the role of Notch signalling pathway in controlling VSMC differentiation.

3.2.1 Involvement of Notch in SMC Differentiation

Over the past decade, studies of *Drosophila* notch clearly have identified Notch signalling as an important “switch” controlling cell fate decisions during development in many tissues [103]. Notch has been defined as the “gatekeeper of cell fate”. In many cases, Notch signalling regulates cell fate choices of bipotential precursors through lateral specification resulting from interactions of Notch with other cellular factors or signalling pathways. Generally, Notch modulates the response of a precursor cell to multiple environmental signals. This response is often manifested as an inhibition of progenitor cell differentiation [37, 38].

In mammals Notch is involved in differentiation of a variety of cell types including neurons and blood cells, and also in various diseases such as tumours and hereditary neurological disorders [103]. Notch affects cell differentiation programs in many experimental models. The classical concept is *Drosophila* neurogenesis [103]. In this model, putative stochastic alternations in the expression level of Notch and its ligand Delta in neuroectodermal cells identify cells intended to gain the neuronal phenotype. These neuronal precursors predominantly express Delta. Delta activates Notch in the cells surrounding each neuronal precursor and causes them to upregulate Notch expression. The activation of Notch in these cells prevents them from differentiating toward the neuronal lineage. Subsequently, these cells switch to an epidermal

differentiation program. Thus, Notch mutations or deletions result in profusion of cells that assume a neuronal cell fate [103]. Several studies have reported the involvement of Hes 1 and Hes 5 downstream target genes of Notch in neuronal differentiation. Hes 1 and Hes 5 mutation have been found to lead to premature neuronal differentiation and together been essential for Notch activity in regulation of mammalian neuronal diversity [105].

In lung chick retina explants, expression of constitutively active Notch 1 inhibits differentiation of retinal progenitors to ganglion cells, while *notch -1* antisense oligonucleotides increase differentiation toward a neuronal phenotype [106, 107]. Similarly, ligand – induced activation of Notch 1 inhibits oligodendrocyte maturation *in vitro* [108]

The effect of Notch signalling on differentiation of many other cells has also been studied. Expression of constitutively active forms of Notch receptors, lacking the extracellular subunit, inhibited terminal differentiation *in vitro* in murine models of myogenesis and granulocytopoiesis [109]. A dual effect of Notch signalling on myeloblast cells has also been reported. Notch signalling has been found to promote progression from myeloblast to promyelocytes, but also inhibit postmitotic differentiation from metamyelocytes to mature granulocytes [109]. It has been observed that activated Notch caused more cells to progress from myeloblast to promyelocytes, resulting in a decreased percentage of undifferentiated cells. Cells transfected with a different length of deletion constructs of Notch exhibited various behavioural effects. These cells

INTRODUCTION

transfected with Notch construct containing RAM, ANK (necessary for CBF-1 binding) and C-terminal domain have revealed the strongest phenotype of enhanced survival and differentiation, whereas cells containing just a partial RAM domain have shown altered differentiation. There is a suggestion that Notch functions through a CBF-1 dependent pathway in these cells [110].

A variety of evidence suggests that Notch has an important role during blood vessel development and that Notch is important for arterial-venous differentiation [111]. For example, it has been found that Zebrafish *Notch 3* was expressed in the dorsal aorta (DA) but not the posterior cardinal vein (PCV) during vascular development [111]. Embryos lacking Notch activity exhibited ectopic expression of venous markers within DA. Activation of Notch caused loss of venous – specific markers within DA [111]

In lung development, activated Notch 3 has been found to inhibit terminal differentiation of epithelial cells [112]. In pulmonary epithelium of transgenic mouse, activated Notch 3 IC inhibited differentiation of equivalent cells, resulting in the failure of type II cells to differentiate into type I pneumocytes. Several studies have been shown that Notch 1 may be involved in the regulation of epidermopoiesis. In murine keratinocytes, enforced expression of active Notch 1 induces expression of early differentiation markers and prevents expression of late differentiation markers [112].

Overall, these studies suggest that in many instances, Notch activation inhibits or delays differentiation along with developmental pathway until the cell is able to respond to signals that determine its subsequent fate.

3.3 Mutations of Notch Signalling Pathway

Notch pathway is linked to several human diseases such CADASIL, AGS (Alagille syndrome), and spondylocostal dysostosis, which are associated with alteration in *Notch3*, *Jagged1* and *Delta3* genes, respectively. The distinct nature of these diseases, which include a neoplasia, a developmental and a late onset neurological disorder, points to the broad spectrum of Notch activity in humans.

3.3.1 CADASIL (Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy)

CADASIL is an autosomal-dominant adult onset vascular disorder characterized by a variety of symptoms, including migraine with aura, mood disorders, recurrent subcortical ischemic strokes, progressive cognitive decline, dementia and premature death. The pathological hallmark of the disease is a systemic nonamyloid and nonatherosclerotic vasculopathy, which affects predominantly the small cerebral arteries. Vessel changes are characterized by progressive degeneration of smooth muscle cells, as well as by the

accumulation, in the vessel wall, of an unknown material that appears by electron microscopy as granular osmiophilic material (GOM) [92, 101].

CADASIL is caused by mutations in the Notch3 gene [92]. All mutations associated with CADASIL are highly stereotyped leading to a gain or loss of a cysteine residue in one of the 34 EGF-like repeats in the extracellular domain of the Notch3 protein. Most mutations are missense mutations that are clustered near the amino terminus of the protein [92, 97, 103]. Some splice site mutations have also been described. All EGF repeats contain six conserved cysteine residues that form three intradomain disulfide bonds. Splicing mutations invariably cause in-frame deletions that result in the loss of these cysteine residues [92, 97, 103].

EGFR 2-5, which have no predicted functional role, have been identified as forming a mutational hotspot [92]. Pathogenic mutations in EGF 10 and EGF 11 that are predicted to be required for ligand binding, by homology to the *Drosophila* Notch receptor are the primary targets of the pathogenic process. In normal human adults, expression of Notch 3 is highly restricted to these cells. In patients with CADASIL, there is an abnormal accumulation of the Notch 3 extracellular domain that takes place at the plasma membrane of vascular smooth muscle cells. However, the molecular pathways linking Notch 3 mutations to degeneration of vascular smooth muscle cells are poorly understood.

3.3.2 Alagille syndrome

Alagille syndrome (AGS) is one of the most common genetic causes of chronic liver disease in childhood, with a frequency as 1 in 70,000 live births. It is an autosomal dominant condition characterized by cholestasis caused by intrahepatic bile duct paucity, congenital heart defects involving primarily the pulmonary arteries, butterfly vertebrae, anterior chamber defects of the eye, typically posterior embryotoxon, and facial dysmorphism. Several other consistent clinical findings have been reported including renal abnormalities, retinal pigmentary changes, and pancreatic insufficiency [92, 113]. Vascular anomalies have been noted in AGS from some of the earliest descriptions of this syndrome. Pulmonary artery involvement is a hallmark feature of the condition. The literature documents case reports of intracranial vessel abnormalities and other vascular anomalies in AGS [92, 103, 113]. The latter include involvement of the aorta, renal, celiac, superior mesenteric, and subclavian arteries

The AGS locus has been assigned to chromosome 20p12. The Notch ligand, *Jagged1* has been identified as the defective gene [92, 103, 113]. The majority of mutations identified so far as frame shift, non-sense and splice site mutations results in truncated proteins. The resulting protein, if translated, carries C-terminal deletions of various lengths including at least the transmembrane and

the intracellular domain. A few missense mutations have been also identified [92, 103, 113].

3.3.3 Spondylocostal dysostosis

Spondylocostal dysostosis (SD) is a family of related diseases where vertebral defects are associated with rib abnormalities and short trunk dwarfism. One particular form of autosomal recessive SD is caused by mutation in Dll-3 and is characterized by abnormal segmentation of the spine, likely due to loss of Dll-3 functions in somitogenesis and the segmentation clock (an inner molecular oscillator based on periodic activation of genes involved in the Notch-signaling pathway, such as HES, which controls the formation of paraxial mesoderm called somites) [92].

Currently 17 different mutations in Dll-3 have been found, which include protein truncations, addition or deletion of cysteine residues within EGF repeats, or the replacement of an essential amino acid. The discovery of Dll-3 mutations in SD patients accentuates the importance of the Notch signaling pathway in skeletal development [92].

Although there has been much recent progress in defining the developmental functions of Notch, many aspects of this pathway remain poorly understood. Notch signaling also plays important roles in myogenesis, cardiogenesis and likely others [92]. Thus, it seems likely that Notch defects will be discovered in other human diseases in the future.

AIM OF STUDY

Based on literature it appears that the Notch signaling pathway may control VSMC fate decisions [3, 34, 54, 55, 95, 101, 116]. Our central objective was to explore the role of this novel developmental signaling pathway in controlling vascular cell fate decisions following a physiologically relevant stimulus. Thus, we examined the regulatory role of Notch 1 and 3-receptor expression and signaling in controlling human VSMC differentiation in static cells and cells exposed to cyclic strain.

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Chapter 4. Materials

4.1 Suppliers

<p><i>Amersham Pharmacia Biotech</i></p> <ul style="list-style-type: none"> • ECL Plus Western Blotting detection system • ECL Hybond • ECL Hyperfilm <p><i>QIAGEN</i></p> <ul style="list-style-type: none"> • QIAGEN purification plasmid kit <p><i>Biolabs</i></p> <ul style="list-style-type: none"> • Rainbow Protein Marker, Broad range (250 – 10 kDa) <p><i>Oxiod</i></p> <ul style="list-style-type: none"> • Skimmed milk powder <p><i>Pierce</i></p> <ul style="list-style-type: none"> • SuperSignal West Pico Luminol/Enhancer solution • SuperSignal West Pico Stable Peroxide Solution • BCA reagents • Albumin standard <p><i>Santa Cruz</i></p> <ul style="list-style-type: none"> • Polyclonal anti-smoothelin antibody <p><i>Molecular Probes</i></p> <ul style="list-style-type: none"> • Alexa Fluor 488 F (ab)2 fragment of rabbit anti-α-actin • Alexa Fluor 488 F (ab)2 fragment of goat anti-mouse IgG 	<p><i>Sigma Aldrich (Poole, Dorset. UK)</i></p> <ul style="list-style-type: none"> • Agarose • Ammonium Persulfate • N, N-Methylene-Acrylamide-bis-Acrylamide solution • Foetal Calf Serum • Hanks Balanced Salt Solution • Penicillin-Streptomycin Solution Stabilized (10,000 units penicillin and 100mg streptomycin/ml) • Ponceau S • RPMI-1640 Medium • DME Medium • TEMED • SDS • Trypsin-EDTA Solution (10x) • Anti - mouse IgG - peroxide conjugate • Anti - goat IgG - peroxide conjugate • Monoclonal anti - myosin (smooth) antibody • Monoclonal anti - alpha smooth muscle actin clone 1A4 peroxide conjugate • Monoclonal anti-calponin clone hCP • BCIP/NBT colour substrate
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4.2 Solutions

4.2.1 LB Agar

In order to prepare LB Agar following ingredients were used and dissolved in 100 ml of distilled water:

- 1g bacto – tryptone
- 0.5g bacto – yeast
- 0.5g NaCl

Then, LB Agar was autoclaved in 121°C for 20 min. After sterilization, LB Agar was cooled down to 55°C in water bath and then, 1.5g of Agar and 1.25 µg / ml of Amp (50mg/1 ml) were added. LB Agar was stored at room temperature.

4.2.2 LB Broth

In order to prepare LB Broth following ingredients were used and dissolved in 950 ml of distilled water:

- 10g Bacto – tryptone
- 5g Bacto – yeast extract
- 10g NaCl

Solution was then mixed until the solutes have dissolved and the pH was adjusted to 7.0 with NaOH. The volume of LB Broth was adjusted to 1 L with distilled H₂O and LB Broth was sterilised by autoclaving for 50 min at 15 lb / sq. in. on liquid cycle. After sterilization, LB Broth was cooled down to 55°C in water bath and 1.25 µg / ml of Ampicilin (50mg/1 ml) was added. LB Broth was stored at room temperature.

4.2.3 Plasmid preparation solutions

Buffers were supplied with QIAGEN Kit. Contents of each buffer was as follow:

Buffer	Composition	Storage
Buffer P1 (resuspension buffer)	50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A	2–8°C, after addition of RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)	15–25°C
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5	15–25°C or 2–8°C
Buffer QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)	15–25°C
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)	15–25°C
Buffer QF (elution buffer)	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)	15–25°C

4.2.4 Cell culture solutions

4.2.4.1 RPMI media (500 ml)

RPMI media were purchased from Sigma Aldrich in 500 ml bottles. Foetal calf serum (FCS) and Penicillin/Streptomycin (Pen/Strep) were added aseptically to each bottle to obtain final concentration 10% for FCS and 1% for Pen/Strep.

4.2.4.2 Protein Lysis Buffer

Following ingredients were used in order to prepare Protein Lysis Buffer:

- 25 mM Hepes
- 300 mM NaCl
- 1.5 mM MgCl₂
- 200 μM EDTA
- 1% Triton-X
- 200 mM β-glycerophosphatase
- 0.1% sodium dodecyl sulphate
- 0.5% sodium deoxycholate
- 0.5 mM DTT
- 100mM Na₃VO₄
- 100 μg/ml PMSF
- μg/ml aprotinin

The pH was adjusted to 7.5 and Protein Lysis Buffer was stored at 4°C.

4.2.4.3 Phosphate Buffered Saline (PBS) 10x

Following ingredients were used in order to prepare 10x PBS:

- 80g NaCl
- 2g KCl
- 12g Na₂HPO₄

- 2g KH₂PO₄

The pH was then adjusted to 7.3 and ultra pure water was added to adjust volume of 10x PBS to 1L. 10x PBS was stored in 4°C.

4.2.4.4 Transfection media (500 ml)

Dulbecco Modified Eagle's Media were purchased from Sigma Aldrich in 500 ml bottles. No FCS and Pen/Strep were added to the medium.

4.2.4.5 Puromycin media

Dulbecco Modified Eagle's Media were purchased from Sigma Aldrich in 500 ml bottles and supplemented with 0.8 µg/ml puromycin, 10% FCS and 1% Pen/Strep.

4.2.5 Western blot solutions

4.2.5.1 Resolving Gel Buffer

In order to prepare Resolving Gel Buffer, 90.78g of Tris base was added to 30 ml of ultra pure water and the pH was adjusted to 8.8 with HCl. Finally, ultra pure water was added to adjust volume of buffer to 500 ml. Resolving Gel Buffer was stored at 4°C.

4.2.5.2 Stacking Gel Buffer

In order to prepare Stacking Gel Buffer, 15.125g Tris base was added to 30 ml of ultra pure water and the pH was adjusted to 6.8 with HCl. Finally, ultra pure water was added to adjust volume of buffer to 250 ml. Stacking Gel Buffer was stored at 4°C.

4.2.5.3 10% SDS

In order to prepare 10% SDS, 10g sodium dodecyl sulphate was added to 100 ml of ultra pure water and solution was stored at room temperature.

4.2.5.4 Ammonium Persulphate AP (10%)

In order to prepare 10% Ammonium Persulphate, 0.1g AP was added to 1 ml of ultra pure water and solution was stored in room temperature. AP solution was made up fresh daily.

4.2.5.5 7.5% Resolving gel preparation (500 ml)

Following ingredients were used in order to prepare 500 ml of 7.5% Resolving gel:

- 5 ml 10% SDS
- 125 ml resolving gel buffer
- 125 ml Acrylamide-bis acrylamide solution
- 245 ml ultra pure water
- 50- μ l 10% AP / 20 ml solution
- 15 μ l TEMED / 20 ml solution

7.5% Resolving gel was stored at 4°C.

4.2.5.6 12% Resolving gel preparation (500 ml)

Following ingredients were used in order to prepare 500 ml of 12% Resolving gel:

- 5 ml 10% SDS
- 125 ml resolving gel buffer
- 200 ml Acrylamide-bis acrylamide solution
- 170 ml ultra pure water
- 50 μ l 10% AP / 20 ml solution

- 15 µl TEMED / 20 ml solution

12% Resolving gel was stored at 4°C.

4.2.5.7 4% Stacking gel preparation (500 ml)

Following ingredients were used in order to prepare 500 ml of 4% Stacking gel:

- 5 ml 10% SDS
- 125 ml resolving gel buffer
- 125 ml Acrylamide-bis acrylamide solution
- 285 ml ultra pure water
- 50 µl 10% AP / 20 ml solution
- 15 µl TEMED / 20 ml solution

4% Stacking gel was stored in 4°C.

4.2.5.8 Tris-Glycine stock (1L of 10x)

30.27g Trizma base and 144g Glycine were added to 800 ml of ultra pure water, and the pH was adjusted to 8.2 with HCl. Then, ultra pure water was added to adjust volume of solution to 1 litre. Tris-Glycine stock was store at 4°C

4.2.5.9 Semi-dry transfer buffer (1x: 25mM Tris/192 mM Glycine/20% MeOH)

200 ml MeOH and 100 ml 10x Tris-Glycine stock were added to 700 ml of ultra pure water, mixed and stored at 4°C

4.2.5.10 PBS-Tween (1xPBS, 0.1% Tween)

200 ml 10xPBS (see section 4.2.4.3) and 2 ml Tween 20 were added to 1798 ml of ultra pure water, mixed and stored at 4°C

4.2.5.11 Blocking solution

5g skimmed milk and 2 ml Tween 20 were added to 100 ml 1 X PBS/Tween (see section 4.2.5.10, mixed and stored at 4°C for 2 to 3 days.

4.2.6 Immunocytochemistry solution

4.2.6.1 PBS/BSA solution (1x PBS, 3%BSA)

3g Bovine Serum Albumin was added to 100 ml of 1x PBS (see section 4.2.4.3), mixed and stored at 4°C for 2 to 3 days.

4.2.7 Quantitative Real Time PCR solutions

4.2.7.1 Reverse Transcriptase

Oligo dT and dNTP's were purchased from Bio-Sciences Ltd and stored at 4°C. MLV 5x reaction buffer, MLV-RT, Rnase H were purchased from Medical Supply Co. Ltd and stored at 4°C.

4.2.7.2 Real Time Polymerise Chain Reaction

The SYBR®Green Quantitative RT-PCR kit was purchased from Sigma. It combined:

- eAMV Reverse Transcriptase,
- JumpStart® Taq DNA polymerase
- SYBR Green I fluorescent dye
- SYBR Green Taq ReadyMix

Chapter 5. Methods

5.1 Cell culture

5.1.1 Maintenance and sub-culturing

Human Aortic Smooth Muscle Cells were purchased from Cell Applications Inc. (CA, USA). Cells were seeded into T75 cm² flasks and cultured in 15 ml of RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS) (or with 0.1% FCS in order to stimulate cell differentiation) and 1% penicillin/streptomycin. Cells were incubated in a humidified atmosphere of 5% CO₂, 95% air. Cells were routinely subcultured (passage 3 to 18) on reaching confluency, by taking off the media and washing cells with Hanks Balanced Salt Solution (HBSS) to remove FCS and unadhered cells. Cells were treated with 2X Trypsin-EDTA (diluted in HBSS) at 37°C for 2-5 min. RPMI containing 10%FCS, 1% Pen/Strep was added to inhibit the effect of trypsin. The suspension of cells was transferred to 15 ml Falcon tubes, centrifuged and a pellet resuspended in 3 ml of media and used to seed three T75 flasks. The cells used in the experiments ranged from passage 3 to passage 18. The wide range of passage numbers used in experiments was not associated with any changes in expression differentiation markers in cultured cells.

5.1.2 Seeding of 6-well Bioflex plates or regular 6-well plates

HASMC were seeded into regular 6-well plates or a flexible, pronectin-coated Bioflex at a concentration of 5×10^5 cells/well. The cells were left to reach 90-100% confluence and then those seeded on Bioflex plates were exposed for 24 hours to 10% cyclic strain using the Flexercell Tension Plus System (FX-4000, Flexcell, McKeesport, PA). It is a computer driven apparatus, which was developed to offer a mechanical load to cells cultured *in vitro*. The cells were harvested using Trypsin as described in section 5.1.1

5.2 Purification of plasmids

A single colony of *E. coli* was picked from a freshly streaked plate and inoculated into 3 ml of LB broth (containing 125 μ g/ml of ampicillin) as a "starter culture". Starter culture was then incubated for 8 hours at 37°C with vigorous shaking, then, diluted into 30 ml of LB broth (containing 125 μ g/ml ampicillin) and grown at 37°C for 16 hours with vigorous shaking. Culture was decanted into 50 ml tube and centrifuged at 5,000 rpm for 10 minutes at 4°C. All traces of supernatant were removed. Bacterial pellet was resuspended in 4 ml of buffer P1 (+RNase A). The pellet was vortexed and aspirated to ensure complete resuspension. 4 ml of buffer P2 was added to the cell suspension. The cell suspension was then mixed gently but thoroughly by inverting 4-6 times and incubated at room temperature for no more than 5 minutes. 4 ml of chilled buffer P3 was added. Suspension was mixed immediately but gently by

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inverting the tube 4-6 times and incubated on ice for 15 minutes. Cell suspension was centrifuged at 10,000 rpm for 60 minutes at 4°C. Supernatant containing plasmid DNA was then removed, transferred to a new 50 ml tube and re-centrifuged at 10,000 rpm for 30 minutes at 4°C. A QIAGEN-tip 100 was equilibrated by applying 4 ml of buffer QBT and allowing to the column to drain. Supernatant was applied to a QIAGEN-tip and allowed to enter by gravity flow. QIAGEN-tip was washed twice with 10 ml of buffer QC. DNA was eluted with 5 ml of buffer QF and precipitated by adding 3.5 ml of room-temperature isopropanol. Eluate was then mixed and centrifuged at 10,000 rpm for 30 minutes at 4°C. Supernatant was carefully decanted. DNA pellet was washed with 2 ml of room temperature 70% ethanol and centrifuged at 10,000 rpm for 20 minutes. Supernatant was carefully decanted without disturbing the pellet. Pellet was then air-dried for 5-10 minutes, redissolved in suitable volume of TE buffer (10mM Tris-HCl, pH 8.0; 1 mM EDTA) and transferred to the eppendorf. Eppendorf was left at 4°C overnight. DNA was quantified by the $A_{260/280}$ nm method, e.g.:

- 750 μ l dH₂O was used to zero OD at 260 nm. $A_{260/280}$ nm ratio was also zeroed.
- 10 μ l eluate was diluted in 750 μ l dH₂O and OD at 260 nm and $A_{260/280}$ nm ratio was measured for diluted ratio

Plasmid DNA was quantified using the equation given below:

- DNA conc. ($\mu\text{g}/\mu\text{l}$) = $\frac{A_{260\text{nm}} \times 50\text{mg/ml} \times \text{dilution factor}}{1000}$

The ratio A260/AD280 was used to assess the purity of the sample. If ratio was 1.7-1.9, the absorption was due to nucleic acid. A ratio less than 1.7 indicated that there might be proteins or other absorbers in the sample, in which it was necessary to reprecipitate the DNA with ethanol.

5.3 Transfection of plasmid DNA into cells

HVSMC cells were used over the course of this study. The following protocol is specified for transfection of cells growing in T75 flask and 6-well plates. Total concentration of 20 μg or 3 μg of plasmid DNA mixed with 45 μl or 15 μl of lipofectamine (LFA) was added to T75 flask or one well in 6-well plate, respectively. If co-transfection of three different plasmid DNAs was performed, e.g. Notch 1, RPMS-1, puromycin plasmid, 6.6 μg (for T75) or 1 μg (for one well) of each DNA was prepared in DMEM (excluding FCS, Pen/Strep) and combined. If co-transfection of two different plasmid DNAs was performed, e.g. Notch 1, puromycin plasmid, 10 μg (for T75) or 1.5 μg (for one well) of each DNA was prepared in DMEM (excluding FCS, Pen/Strep) and combined. LFA reagent was prepared by diluting 45 μl (for T75) or 15 μl (for one well) of LFA in 300 μl DMEM (excluding FCS, Pen/Strep). 300 μl of LFA mixture was added to combined DNAs. The resulting mixture was incubated at room temperature for 15 minutes. Media was removed from flask or well, which then were washed

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twice with HBSS to dispose FCS remnants. Mixture of LFA/DNA was added to flask or well. 3 ml or 200 μ l of DMEM (excluding FCS) was added to flask or well, respectively. The flask or well was then mixed by shaking and incubated at 37°C in humidified chamber for 3-5 hours. 12 ml or 500 μ l of RPMI (+10% FCS) was added to flask or well, respectively, to let the cell recover after transfection, overnight at 37°C in humidified chamber. Media was then removed from the flask or well. Flask or well was washed with HBSS. RPMI with 0.1% FCS and 0.8mg/ml puromycin was added to the flask or well to stimulate cell differentiation. The puromycin was added to select only cells that has been transfected. The flask or well was then incubated at 37°C in humidified chamber for 24 hours. Media was then removed from the flask or well. Flask or well was washed twice with HBSS. Lysis Buffer was added to flask or well to prepare a protein lysate for Western Blot (section 5.4.1.1). Alternatively, cells from 6-well plate were fixed with -20°C MeOH for immunocytochemistry (section 5.6).

5.4 Western blot analysis

5.4.1 Sample preparation

5.4.1.1 Preparation of whole cell lysate

Media was removed from flask. Cells were washed with HBSS and trypsinized as described in section 5.1.1. The suspension of cells was transferred to 15 ml Falcon tube, centrifuged and a pellet resuspended in 500 μ l of lysis buffer (see section 4.2.4.2). The sample was then sonicated for 20 seconds to burst cells and the protein lysate was aliquoted and stored at -20°C .

5.4.1.2 Bicinchoninic acid (BCA) protein microassay

The BCA assay was used to assess protein concentration in whole cell lysate. This method combined the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the selective colorimetric detection of the cuprous cation using a reagent containing bicinchoninic acid. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a broad working range of 20 – 2.000 $\mu\text{g}/\text{ml}$. The two separate reagents used, A and B, were supplied in the commercially available assay kit (Pierce Chemicals). A working solution was prepared by mixing 1 part of reagent B with 50 parts of reagent A. On a microlitre plate 200 μ l of the working solution was added to 2 μ l of the whole cell lysate or bovine serum albumine (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 spectrophotometer. All samples and standards were tested in triplicate. Quantitation was carried out by interpolation from a BSA standard curve (0-10 $\mu\text{g}/\mu\text{l}$).

5.4.1.3 Sample preparation

An aliquot of the sample, giving a concentration of 10-20 μg (conc. determined by BCA assay in section above), to be analysed was mixed 1:4 v/v with 4x Sample Solubilisation Buffer (SSB) (4g Sodium dodecyl sulphate, 20ml glycerol, 2ml β -mercaptoethanol, 0.04g Bromophenol blue, 24ml 0.25M Tris-HCL, made up to 50ml, pH 6.8) and made up to 20 μl with lysis buffer in a sealed screw-cap microcentrifuge tube. The sample was then heated to 94 $^{\circ}\text{C}$ for 5 min. The sample was then placed on ice until ready to load onto a gel.

5.4.2 SDS polyacrylamide gel preparation and electrophoresis

An Atto AE-6450 Dual Mini Slab Kit was used for all protein electrophoresis carried out during the study. All gel cast parts were cleaned with ethanol prior to use. The gel cast was prepared as described in the manufacturers operating instructions. Stock solutions of 4% (stacking), 7% (resolving) and 12% (resolving) Bis-Acrylamide were made up and stored at 2-8 $^{\circ}\text{C}$ as described in section 4.2.5.5 – 4.2.5.7

An aliquot of the resolving gel stock solution (10ml per gel) was mixed with 50 μl of freshly prepared 10% Ammonium persulphate and 15 μl of TEMED; this

was then poured into the gel cast. A few drops of 95% ethanol were then added to remove any bubbles, the gel was then allowed to polymerise for 10-30 min. When set, the unpolymerised liquid was poured off and gel was washed with stacking gel buffer. The stacking gel was prepared in the same manner. This was then poured onto the resolving gel, the 12-well comb was then put in place and the gel was allowed to polymerise for 10-30 min. The chamber was then half filled with reservoir buffer (25mM Tris, 192mM Glycine, 0.1% Sodium dodecyl sulphate). When the stacking gel polymerised, the comb, clips and casket were removed and the gel cast was clipped into the electrophoresis chamber. The chamber was then filled with reservoir buffer and the lanes flushed out to remove unpolymerised Bis-Acrylamide. The lanes were loaded with 20 μ l of samples. The gel was then run at 170V, 90mA and 150W until the dye front had migrated to the end of the gel (approximately 90 min).

5.4.3 Semi-dry electro blotting

An Atto AE-6675 semi-dry blotting apparatus was used for all electro blotting during this study. Following electrophoresis, the gel was removed from the cast and the stacking gel was removed from the resolving gel. The resolving gel was then soaked in semi-dry transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 5 min. Two stacks of 8 sheets of Whatman chromatography paper were cut to exactly the same size of the resolving gel (app. 9x6cm²). A sheet of nitrocellulose membrane was cut to the same size as the resolving gel (9x6cm²)

and soaked in semi-dry transfer buffer. The transfer stack was set up as follows: 8 sheets of Whatman chromatography paper, the nitrocellulose membrane, the resolving gel and finally 8 sheets of Whatman chromatography paper. A pen was rolled over the stack to remove any air bubbles. The apparatus was then closed and run at 100V, 500mA, 150 W for 1 hour.

5.4.4 Ponceau S staining, blocking and antibody incubations

When the transfer was finished the nitrocellulose membrane was removed and stained in Ponceau S for 5 min. The membrane was then rinsed briefly in ultra pure water to remove any background staining. The membrane was placed between 2 acetates and an image was taken with an Epson perfection 1200S scanner. The image was saved and used to assess equality of protein loading and transfer across the gel. The membrane was then destained in 1x Phosphate buffered saline-Tween20 (1x PBS-T20) (10mM Tris, 100mM NaCl, 0.1% Tween 20) until all Ponceau S stain was removed.

The membrane was then blocked in 5% skimmed milk for 30-60 min at room temperature on an orbital shaker. The membrane was then washed three times for 15 min in PBS-T20. The primary antibody was diluted in a 2.5% skimmed milk solution (for α -actin 1:2000, calponin, myosin 1:500). The dilution factor for each antibody was determined empirically. The membrane was incubated in primary antibody overnight at 4°C on an orbital shaker.

The membrane was then washed 3 times for 10 min in 1x PBS-Tween 20. The secondary antibody was diluted in a 2.5% skimmed milk solution. The membrane was incubated in an appropriate secondary antibody (conjugated to horseradish peroxidase or alkaline phosphatase, 1:1000 for calponin, myosin or 1:2000 for α -actin) for 3-4 hours at room temperature on an orbital shaker. The membrane was then washed 3 times for 10 min in 1X PBS-T20. Excess wash buffer was drained from the membrane by holding gently in a forceps and touching the edge against a tissue.

5.4.5 Detection and development of blot

Supersignal west pico chemiluminescent substrate (Pierce) or BCIP/NBT colour substrate (Sigma) were the detection reagents used to develop Western blots carried out in this study. The detection reagents were removed from storage at 2-8°C and allowed to equilibrate to room temperature before opening. An equal volume of reagent A was mixed with reagent, usually a total of 1ml for one membrane. The membrane was placed, protein side up, on the acetate and the detection reagent placed on top ensuring the whole membrane was covered. A second acetate was then placed on top. The membrane was incubated for 5 min at room temperature. The excess detection reagent was then drained off by holding gently in a forceps and touching the edge against a tissue. The blot was placed onto a fresh piece of cling film and wrapped up; any air bubbles were gently removed.

In the dark room, the wrapped blot was placed, protein side up in an x-ray film cassette. A sheet of Amersham Hyperfilm ECL autoradiography film was placed on top of the membrane, the cassette was closed and the film was exposed for between 10 seconds and 5 min depending on the antibody. The film was then developed in an Amersham hyperprocessor automatic developer and results scanned or photographed. The density of each band was determined using NIH image software.

5.4.6 Dot blot analysis

A dot blot analysis was used to determine the concentration of antibody required to pick up differentiation markers using monoclonal and polyclonal antibodies prior to Western blot analysis. Filter paper and nitrocellulose membrane were hydrated and blocked as for Western blot analysis. Using the dot blot apparatus, 10 µg of protein from cell lysate was added to each well and dilutions of 1:100, 1:300, 1:500, 1:700, 1:1000 of primary antibodies were used. The membrane was then probed with secondary antibodies and immunodetection was performed as described in section 5.6.

5.5 Real Time PCR analysis

5.5.1 Preparation of total RNA from HVSMC's

Total RNA was isolated from cells using Trizol® reagent according to the manufacturer's specification. Growth media was removed from cells and cells were washed twice with HBSS. Cells were then lysed by adding Trizol reagent directly to the flask. The lysate was transferred to a Falcon tube and incubated for 10 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Subsequently, chloroform was added and the tube was shaken vigorously for 15 seconds and then centrifuged at $12.000 \times g$ for 15 min at 4°C . The resulting aqueous phase was then transferred to a fresh tube and isopropanol was added in order to precipitate RNA. The sample was incubated for 10 min at room temperature and centrifuged at $12.000 \times g$ for 10 min at 4°C . The supernatant was then removed and a gel – like pellet of RNA was washed with 75% ethanol. The sample was centrifuged again at no more than $7.500 \times g$ for 5 min at 4°C . The supernatant was removed and the pellet was air-dried. Dry RNA was then resuspended in approximately $50\mu\text{l}$ of RNase free water. All total RNA preparations were stored at -80°C .

5.5.2 Quantitation of total RNA in sample.

Quantitation of total RNA in a sample was carried out by absorption spectroscopy. The absorption of the sample was measured at several different wavelengths to assess its purity and concentration. By measuring absorbance at 280 nm and 260 nm, purity was estimated using the A_{260}/A_{280} ratio. A ratio of 1.9 – 2.0 was indicative of a highly purified preparation of RNA. A ratio lower than this indicated possible protein contamination. Absorbance carried out at 230 nm reflected contamination by phenol, while absorbance at 325 nm indicated contamination by a dirty cuvette. The concentration of RNA in the sample was determined using the A_{260} reading as follows:

$$\text{Concentration of single stranded RNA } \mu\text{g}/\mu\text{l} = \frac{A_{260} \times \text{Dilution factor} \times 40}{1000}$$

All sample were tested in triplicate.

5.5.3 Design of PCR primers

A web based program called “Multialign” was used to design the primer sets for α -actin and GAPDH. This program allows alignment of sequences from a number of different species so primers can be designed from highly conserved areas. Primers were designed with ~ 50% GC content and the annealing temperature for α -actin was set at 60 °C.

- **α -actin primer set, from human sequence**

Forward primer 5' – atc tgg cac cac tct ttc ta - 3'

Reverse primer 5' – gta cgt cca gag gca tag ag - 3'

This primer set generated fragment size of 200 base pare (bp)

- **calponin primer set, from human sequence**

Forward primer 5' – cca acc ata cac agg tgc ag - 3'

Reverse primer 5' – atc tca ctc cca cgt tca cc - 3'

This primer set generated fragment size of 213 base pare (bp)

- **myosin primer set, from human sequence**

Forward primer 5' – tgc aac ttg aga agg tca cg - 3'

Reverse primer 5' – tct tta gcc gca ctt cca gt - 3'

This primer set generated fragment size of 228 base pare (bp)

- **smoothelin primer set, from human sequence**

Forward primer 5' – cga gtg aac aaa gca cca ga - 3'

Reverse primer 5' – tgc tct gat cca gca tct tg - 3'

This primer set generated fragment size of 474 base pare (bp)

- **GAPDH primer set, from rat sequence**

Forward primer 5' – tcc tgc acc acc aac tgc tt - 3'

Reverse primer 5' – tgc ttc acc acc ttc ttg at - 3'

This primer set generated fragment size of 350 base pare (bp)

5.5.4 Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

5.5.4.1 Reverse transcriptase

Reverse transcription was carried out using Promega Murine Leukemia Virus Reverse Transcriptase (MLV RT) as follow. The initial amount of RNA used for each primer set was determined empirically to ensure a semi quantitative analysis. 1 µg of RNA was transferred to an Rnase free microcentrifuge tube. Subsequently, 1 µg of Promega Oligo dT primer was added. The volume was then made up to 15 µl with Rnase free water. The tube was heated to 70°C for 5 min to melt a secondary structure within the template. The tube was cooled immediately on ice to prevent a secondary structure from reforming. The following components were added to the annealed primer/template in this order and made up to a total of 25 µl with Rnase free water:

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	200 units

The components were gently mixed in the tube and spun down for a few seconds. The mix was then incubated for 60 min at 42°C. 1 µl of RNase H (2 units/µl). Subsequently, the mix was incubated for 20 min at 37°C. A negative control was also carried out. The negative control lacked reverse transcriptase and no amplification by RT PCR was possible. All RT samples (cDNA) were stored at 80°C.

5.5.4.2 Real Time Polymerase Chain Reaction

Quantitative Real Time Polymerase Chain Reaction was carried out using the Rotor Gene (RG-3000, Corvett Research, Australia) and the SYBRGreen PCR kit (Qiagen) as described by the manufacturer. PCR was performed using primers listed in section 5.5.3

5.6 Immunocytochemistry

Cells were seeded on 6-well plates as described in section 5.1.2 until they reached 90-100% confluence. Then cells were permeabilized and fixed with methanol (-20°C) for 10 min. The cells were rehydrated with PBS containing 3% BSA for 10 min. The cells were incubated with specific antisera diluted in PBS containing 3% BSA (anti- α actin 1:500, Alexa Fluor conjugate, Molecular Probes; calponin, myosin and smoothelin 1:50, Sigma) overnight at 4°C. The cells were washed five times in PBS, before they were incubated with Alexa Fluor anti-mouse IgG (Molecular Probes) for 2 hours at 37°C in a humidified chamber.

Cells were washed 3 times in PBS before wells were mounted and visualized using an Olympus microscope fitted with a 20X and 40X objective lens. Images were recorded using "Studio Life" software.

5.7 Cell proliferation measurement

Cell proliferation was examined by cell counting and determining proliferating cell nuclear antigen expression using Western blot.

Transient transfection of HVSMC with Notch 1 or 3 IC and mock controls was performed on 6-well plates as described in section 5.3. Cells were then aseptically removed from one well on day one after transfection and resuspended in 1 ml of RPMI media. 6-well plate was then put back to the incubator. Cells from one well were then counted under the microscope by using hemocytometer. The counting procedure was repeated until day five after transfection.

Cell proliferation was also examined by determination of proliferating cell nuclear antigen (pCNA) expression in cells transfected with constitutively active Notch 1 and 3 using Western blot.

RESULTS

Chapter 6. Role of Notch Signalling Pathway in

Differentiation of VSMC

The effect of the Notch signaling pathway on differentiation of human VSMC's is reported in this section. The pretext for this study has been outlined in section 3.2. Briefly, a number of studies have shown that the Notch signaling pathway influences smooth muscle cell fate, through increasing or decreasing their differentiation, proliferation, migration and apoptosis [3, 34, 54, 55, 95, 101, 116]. The experiments were designed to examine the effects of the Notch signaling pathway on HVSMC differentiation. Both changes in protein and mRNA levels of differentiation markers were monitored in cells following transfection with Notch signaling components and Notch inhibitors (RPMS-1, ED4) as well as treatment with pharmacological inhibitors of Notch (Brefeldin A). It has been proposed by us [34] and others [95, 101] that Notch-induced changes in VSMC fate may be via a CBF-1 dependent pathway. In support of this notion, several studies have demonstrated the utility of over-expressing a dominant negative CBF-1 (associates with Notch IC but lacks DNA binding) in the context of Notch IC expression to determine whether Notch-cellular events occur via a

CBF-1 dependent or independent pathway [9, 10, 11]. The experiments demonstrating that both Notch 1 and 3 IC signal via a CBF-1 dependent pathway in VSMC have been published by our lab previously [34].

6.1 Validation of over-expression of Notch 1 and Notch 3 IC in HVSMC's

This study was initiated by showing the presence of Notch 1 and Notch 3 receptors in human VSMC in conjunction with studies to validate the over-expression of these receptors in HVSMC's by using Western blot analysis and Immunocytochemistry. Therefore, cells were co-transfected with Notch 1 IC or Notch3 IC or mock vector and pGK-puro vector encoding puromycin resistance as described in section 5.3. Cells were then selected following treatment of cells with 0.8 mg/ml puromycin for 24 h. At this concentration of puromycin, around 70-80% of cells that survived were transfected.

Western blot analysis revealed the presence of both receptors in HVSMC's as indicated in Fig. 10 and 11. The predominant form of the receptor was the IC fraction (~90 kDa) as antibodies specific for the IC fraction of Notch were employed. The accuracy of the antibodies was confirmed by competing with peptides selective for Notch 1 and Notch 3 receptors, respectively (data not shown). The expression of both receptors was enhanced at protein level following transient transfection with Notch 1 IC and 3 IC when compared to mock-transfected cells (60% and 40% increase in expression of these receptors).

To confirm the presence of Notch 1 and Notch 3 IC in HVSMC's, immunocytochemical staining was carried using anti-Notch 1 and 3 IC antibodies, which stained both native and transfected Notch 1 and 3 IC receptors within the cell (Fig. 12 and 13).

In addition, anti-HA antibodies specific for both Notch 1 and 3 IC proteins were used in immunocytochemical staining in order to determine the exact location of transiently transfected Notch 1 and 3 IC in the cell. Anti-HA antibody binds only to transiently transfected Notch 1 and 3 IC tagged plasmids in these cells. This study demonstrated predominant cytoplasmatic localization of Notch IC domain in the cell (Fig. 14).

The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6.

The results are as follows:

6.1.1 Western blot analysis.

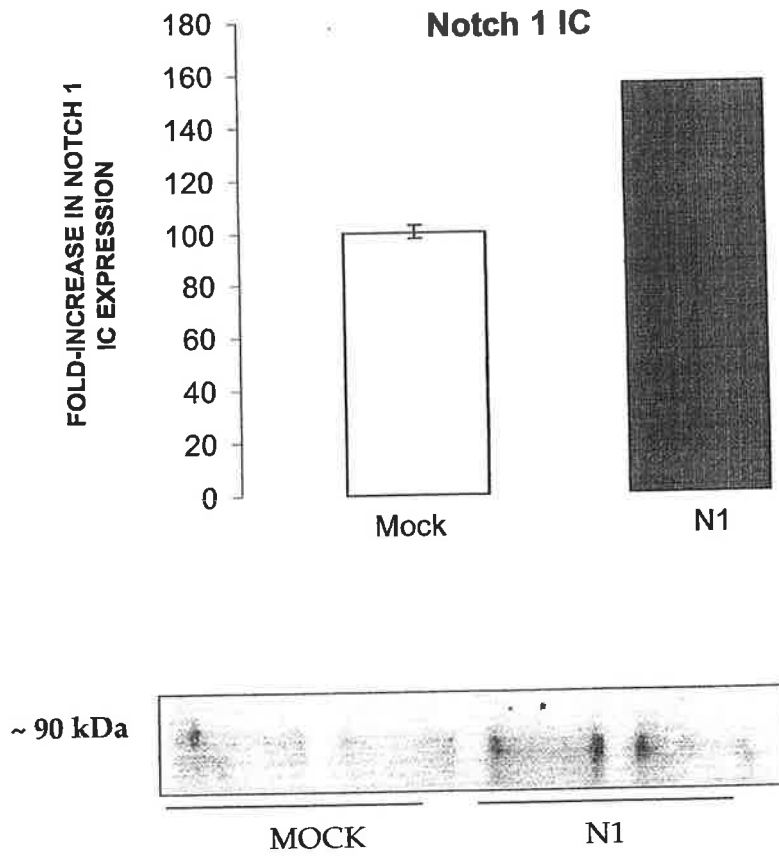


Fig. 10 Inset shows representative graph displaying enhanced expression of Notch 1 IC receptor expression level in Notch1 IC transfected cells (N1) as compared to mock-transfected cells (control cells). Equal protein was loaded and confirmed by staining each blot with Ponceau S stain. Data are means \pm SEM of 2 individual experiments.

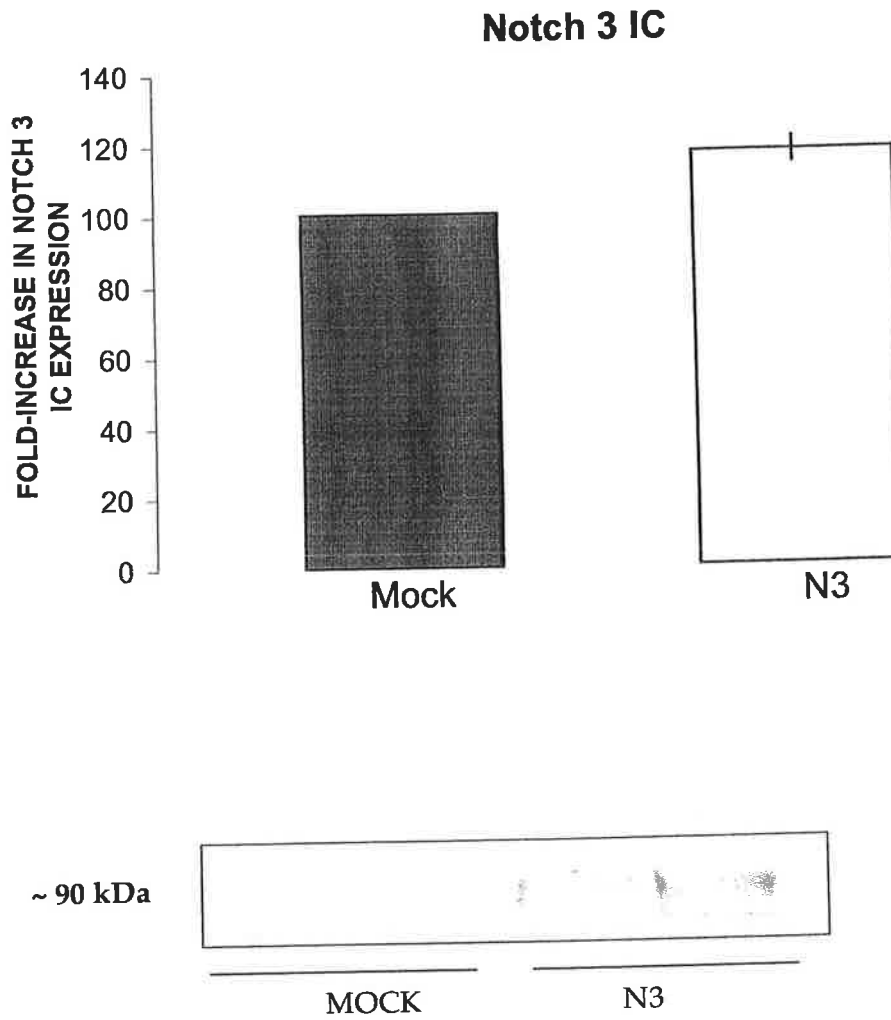


Fig. 11 Inset shows representative graph displaying enhanced expression of Notch 3 IC receptor expression level in Notch3 IC transfected cells (N3) as compared to mock-transfected cells (control cells). Equal protein was loaded and confirmed by staining each blot with Ponceau S stain. Data are means \pm SEM of 2 individual experiments.

6.1.2 Immunocytochemical staining with anti-N1 and N3 antibodies.

Notch 1 IC

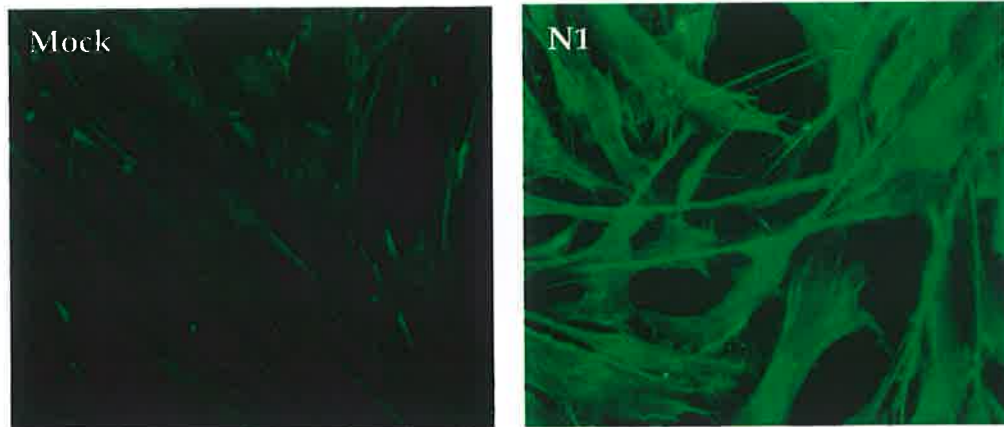


Fig. 12 Immunofluorescent staining of HVSMC's: Notch 1 IC in mock and Notch 1 IC (N1) transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

Notch 3 IC

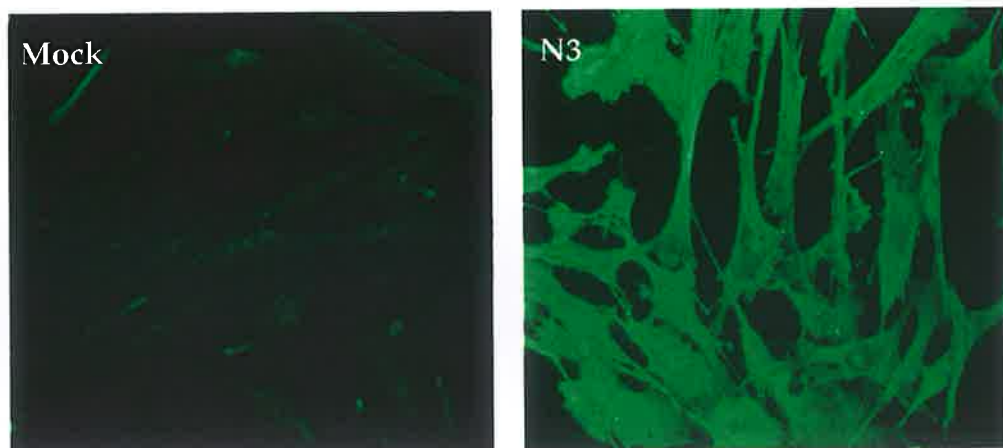


Fig. 13 Immunofluorescent staining of HVSMC's: Notch 3 IC in mock and Notch 3 IC (N3) transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

6.1.3 Immunocytochemical staining using anti-HA antibody.

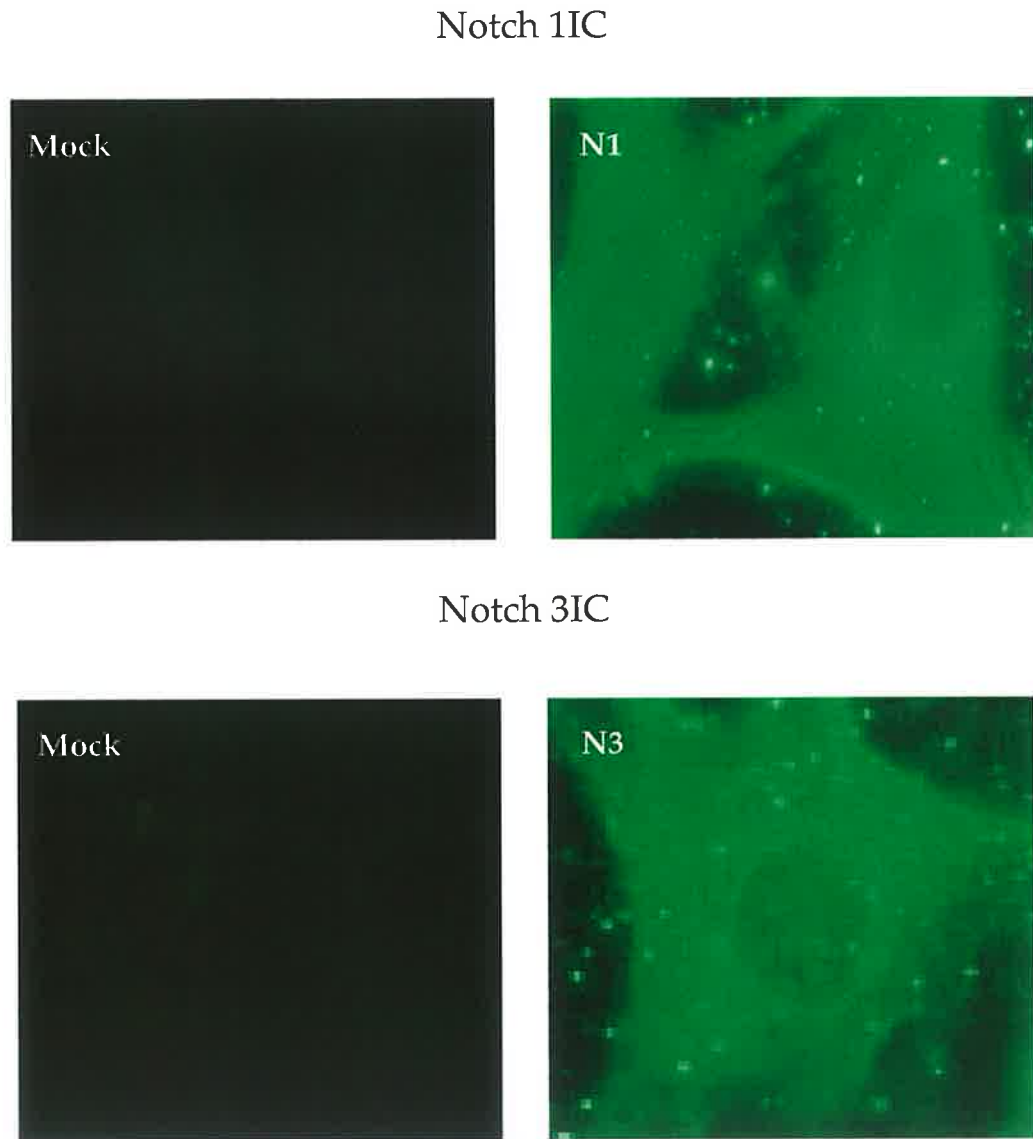


Fig. 14 Immunofluorescent staining of HVSMC's: Notch 1 and 3 IC in mock and Notch 1 IC (N1) and Notch 3 IC (N3) transfected cells using anti-HA antibody. Antibody binds only transfected Notch IC in cells, therefore no staining is observed in mock-transfected cells. This experiment demonstrating predominant cytoplasmatic localization of transfected Notch 1 and 3 IC in the cell. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 2 individual experiments.

6.2 Validation of Expression of Differentiation Markers in VSMC.

During development the cells acquire a repertoire of proteins, which may reflect their functional state. Several proteins were described as specific markers of SMC's like for instance: Smooth Muscle α - Actin (*SM α A*), Smooth Muscle Myosin Heavy Chain (*SMMHC*), basic Calponin and Smoothelin.

A basal protein expression of α -actin, calponin and myosin in HVSMC was assessed in this section by Western blot analysis and Immunocytochemical staining. α -Actin and calponin were abundantly expressed in HVSMC, while the expression of myosin was much less intensive in these cells as monitored by Western blot analysis (Fig. 15). Smoothelin was undetectable by Western blot analysis. The presence of differentiation marker protein expression was further confirmed by immunocytochemical staining (Fig. 16) and QRTPCR analysis (Fig 17-20)

The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6. mRNA and QRTPCR was carried out as described in section 5.5

The results are as follows:

6.2.1 Western blot analysis

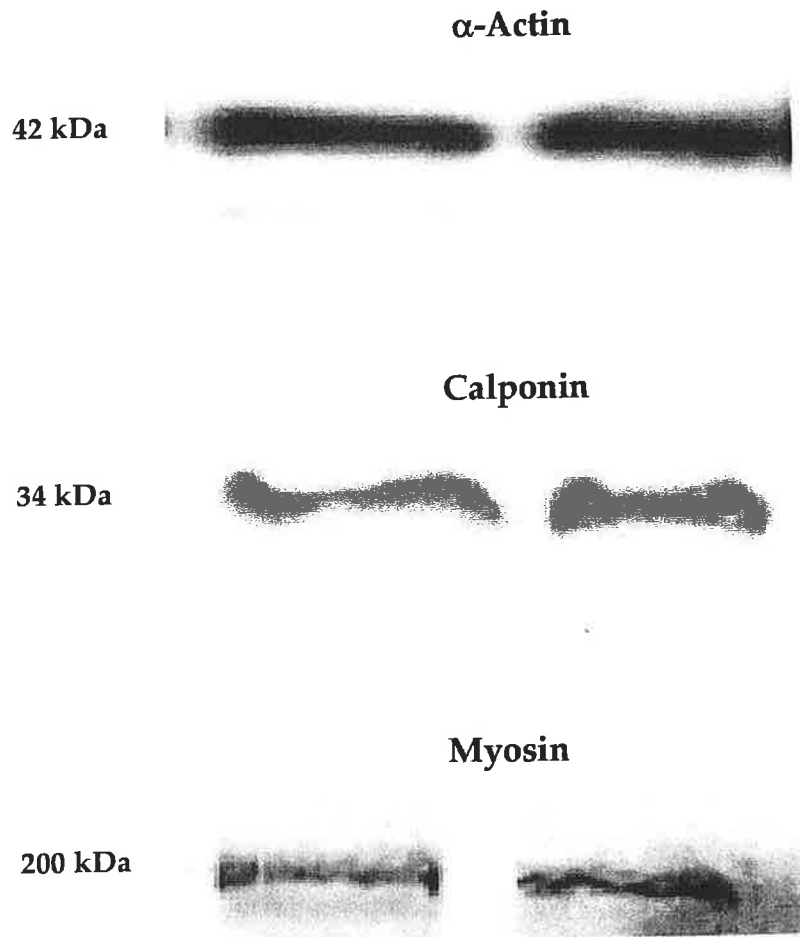


Fig. 15 Inset shows representative graphs displaying Smooth muscle α -actin, basic calponin and Smooth muscle myosin heavy chain in HVSMC's. Equal protein was loaded and confirmed by staining each blot with Ponceau S stain (data not shown). Data presented is representative of at least 3 individual experiments.

6.2.2 Immunocytochemical staining.

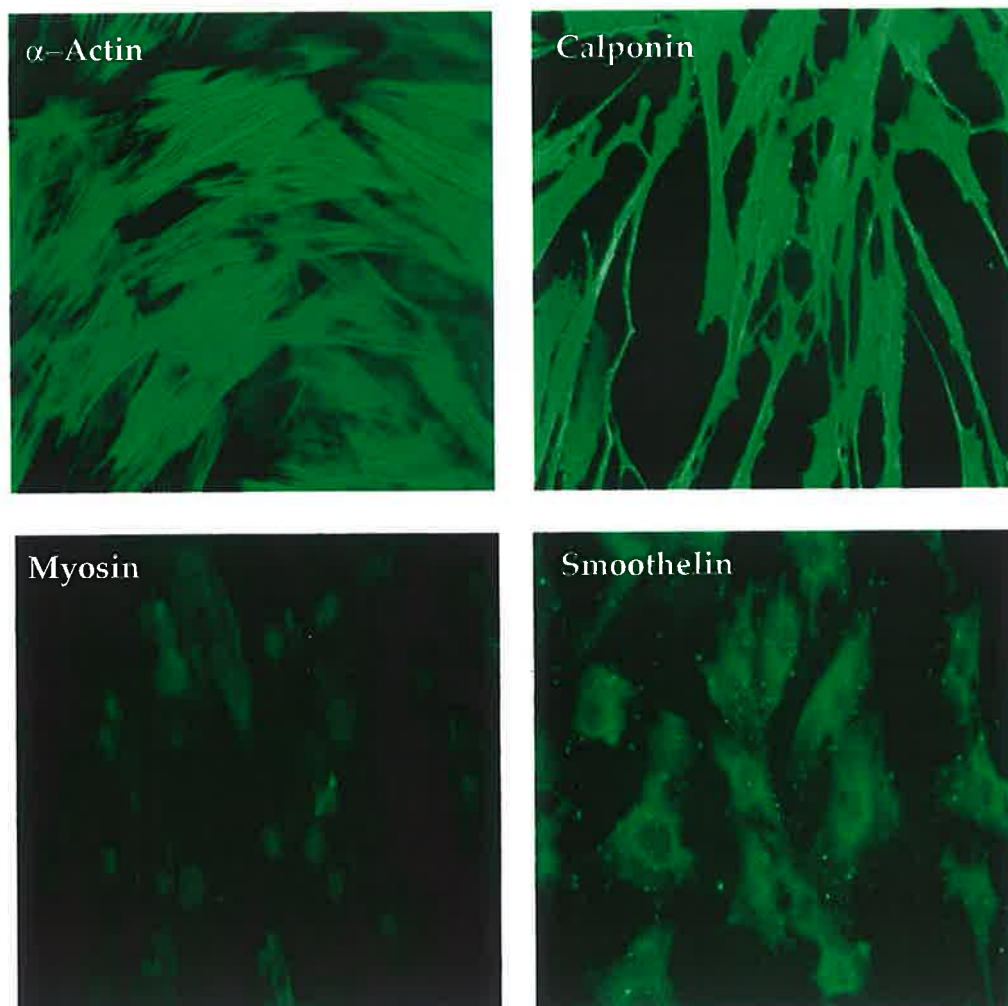


Fig. 16 Immunocytochemical staining of static HVSMC's: Smooth Muscle α -Actin, basic Calponin, Smooth Muscle Heavy Chain Myosin and Smoothelin. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

6.2.3 QRT-PCR analysis.

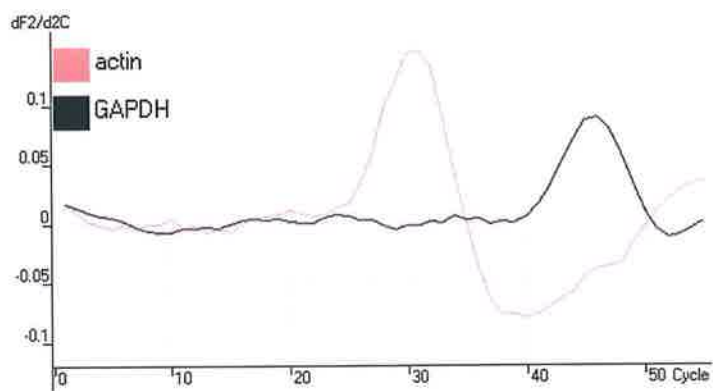


Fig. 17 Representative graph shows mRNA level of α -actin in HVSMC. Results were normalized by amplification of GAPDH. Data presented is representative of 2 individual experiments.

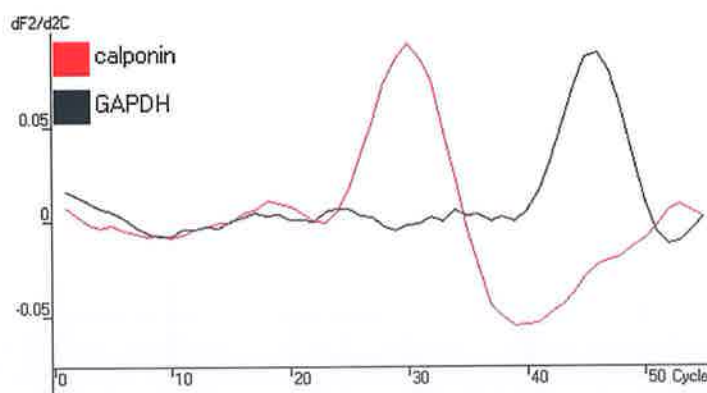


Fig. 18 Representative graph shows mRNA level of calponin in HVSMC. Results were normalized by amplification of GAPDH. Data presented is representative of 2 individual experiments.

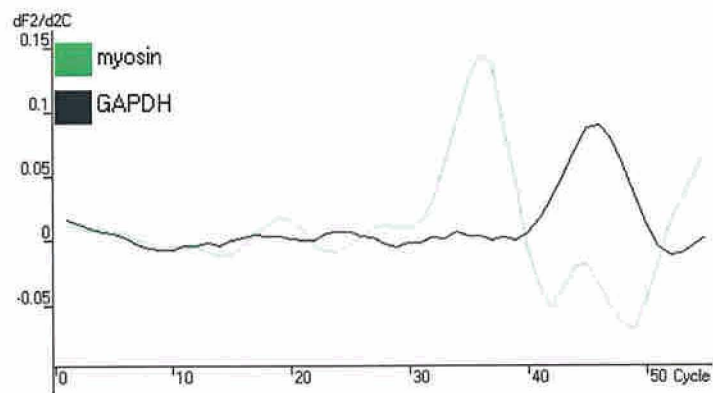


Fig. 19 Representative graph shows mRNA level of myosin in HVSMC. Results were normalized by amplification of GAPDH. Data presented is representative of 2 individual experiments.

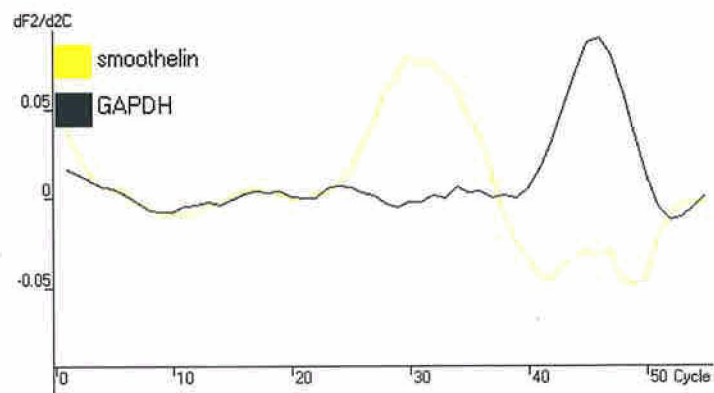


Fig. 20 Representative graph shows mRNA level of smoothelin in HVSMC. Results were normalized by amplification of GAPDH. Data presented is representative of 2 individual experiments.

6.3 Endogenous Notch induces changes in differentiation marker expression.

To determine the influence of the Notch Signalling Pathway (NSP) on human VSMC differentiation, the levels of VSMC differentiation markers were analysed and compared following both inhibition of the NSP with RPMS-1 and over expression of constitutively active Notch IC, respectively. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6. mRNA and QRTPCR was carried out as described in section 5.5

The results are as follow:

Expression of the Epstein_Barr virus encoded gene product (RPMS-1), which has been shown to negatively regulate the activity of Notch IC by specifically binding to CBF-1, was used to inhibit CBF-1 dependent Notch signalling. Western blots revealed a significant up-regulation of SMC differentiation markers in cells that express RPMS-1 to inhibit Notch 1 and 3 IC CBF-1 dependent gene expression, when compared to mock transfected cells (Fig 21, 22, 23). These results were confirmed by immunocytochemical staining of these cells. Namely, there was a marked increase in α -actin, calponin and myosin

staining in cells that expressed RPMS-1 when compared to mock controls (Fig 24, 25 and 26). Smoothelin was undetectable by Western blot analysis. However, immunocytochemical staining revealed increased smoothelin staining in RPMS-1 transfected cells as compared to mock-transfected cells (Fig 27). The up-regulation of VSMC differentiation markers following CBF-1/RBP-Jk inhibition with RPMS-1 was further confirmed by measuring mRNA levels for α -actin using QRT-PCR. There was an increase in α -actin mRNA levels in cells expressing RPMS-1 when compared to mock controls (Fig 28). Taken together, these data suggest that inhibition of endogenous Notch CBF-1/RBP-Jk signalling with RPMS-1 results in a marked increase in VSMC differentiation markers, α -actin, myosin, calponin and smoothelin.

6.3.1 Western blot analysis

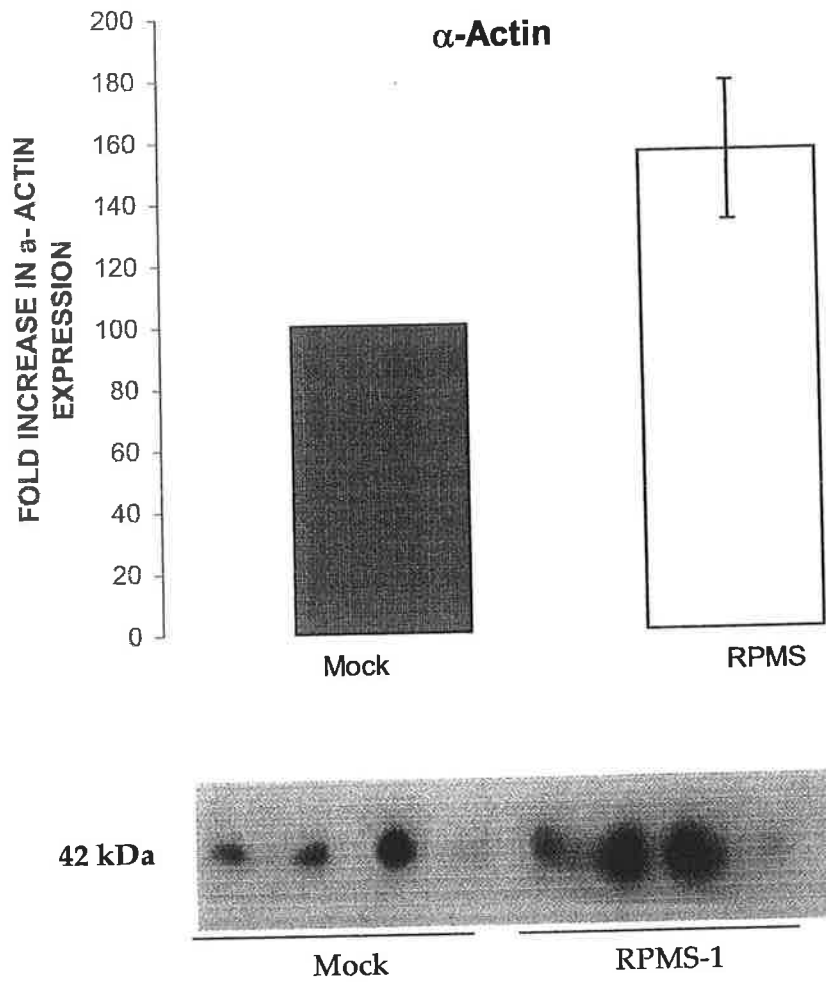


Fig. 21 Inset shows a representative graph displaying 50% increase of α -actin protein expression in RPMS-1 transfected HVSMC's as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

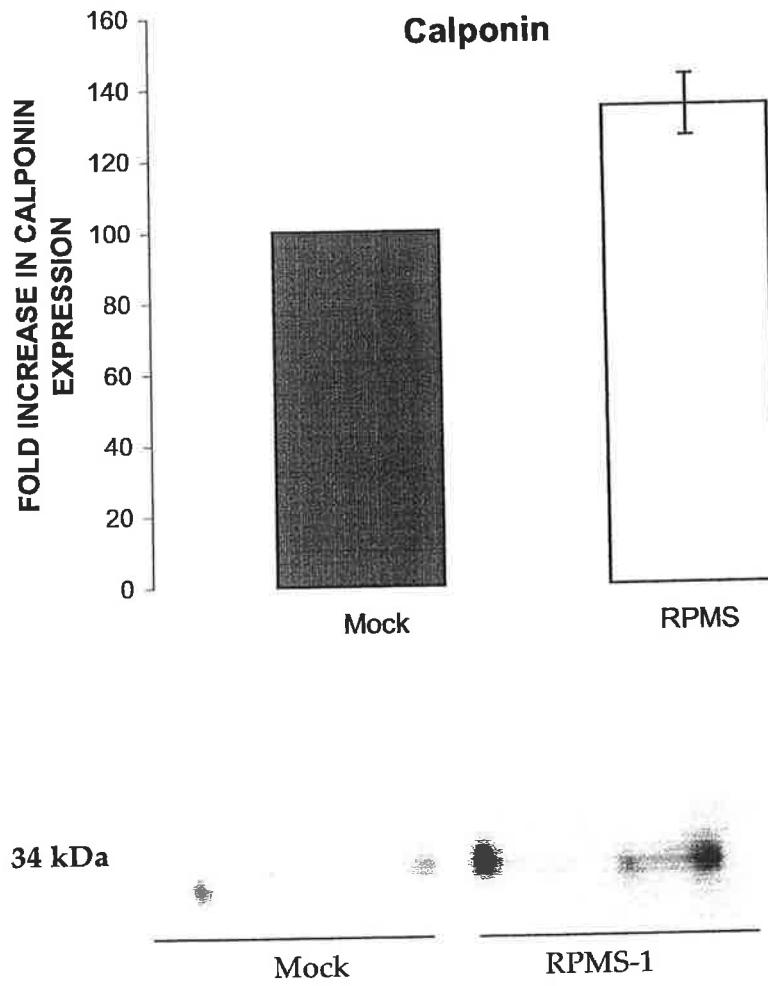


Fig. 22 Inset shows a representative graph displaying 34% increase of calponin protein expression in RPMS-1 transfected HVSMC's as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

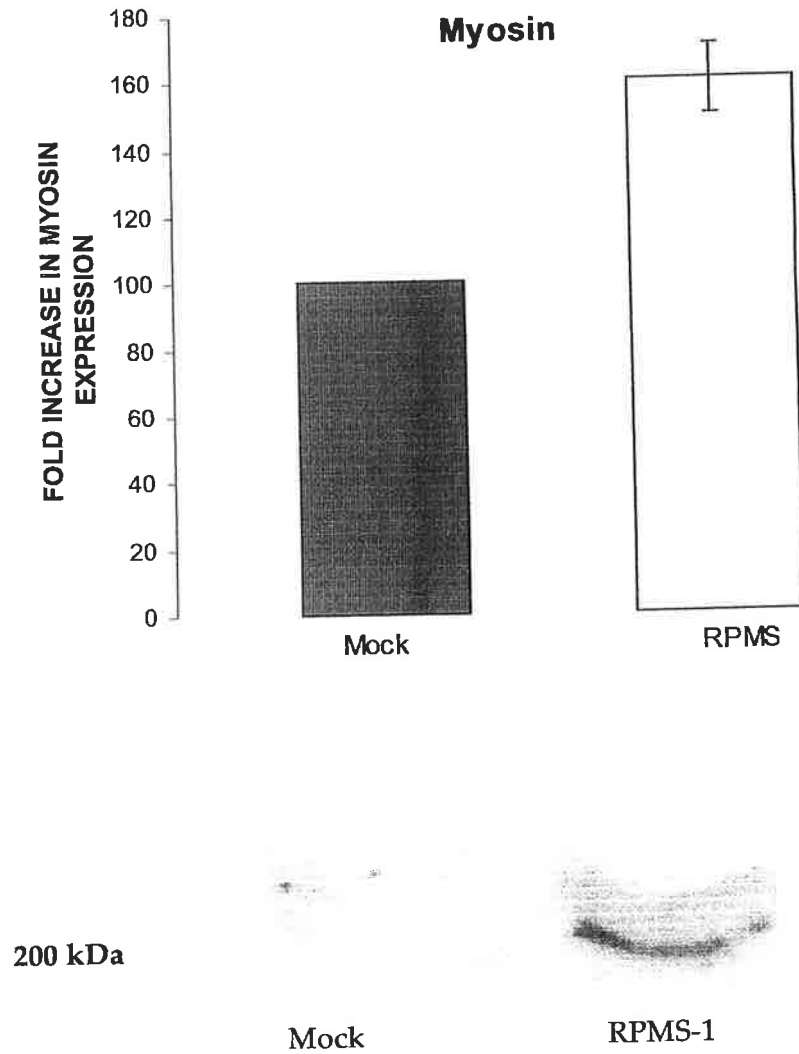


Fig. 23 Inset shows a representative graph displaying 60% increase of myosin protein expression in RPMS-1 transfected HVSMC's as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

6.3.2 Immunocytochemical staining

α -Actin

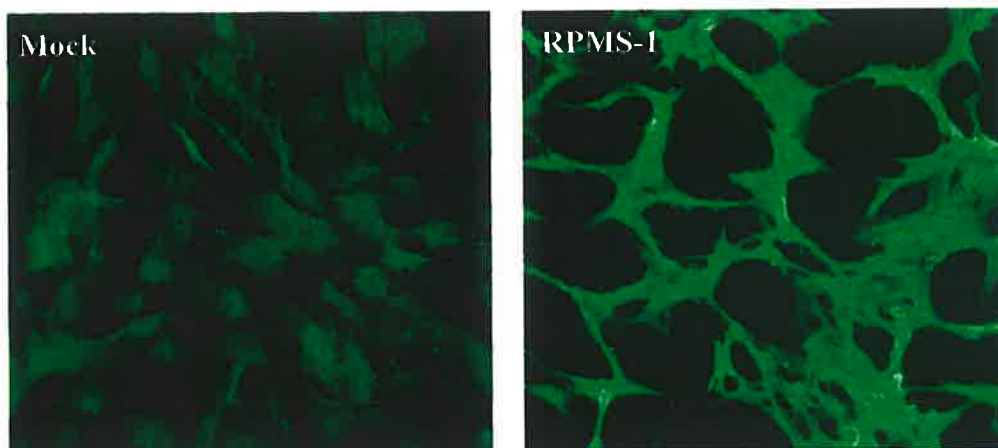


Fig. 24. Immunocytochemical staining of HVSMC's: α -actin in mock and RPMS-1 transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

Calponin

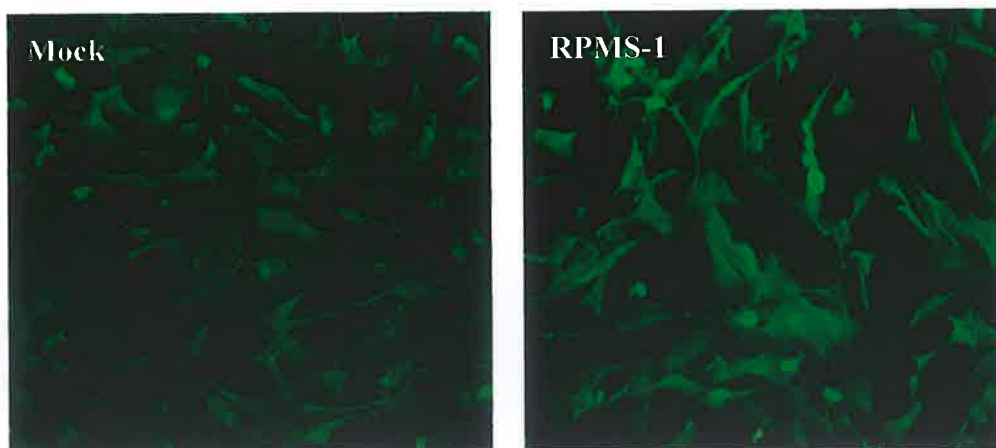


Fig. 25. Immunocytochemical staining of HVSMC's: calponin in mock and RPMS-1 transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

Myosin

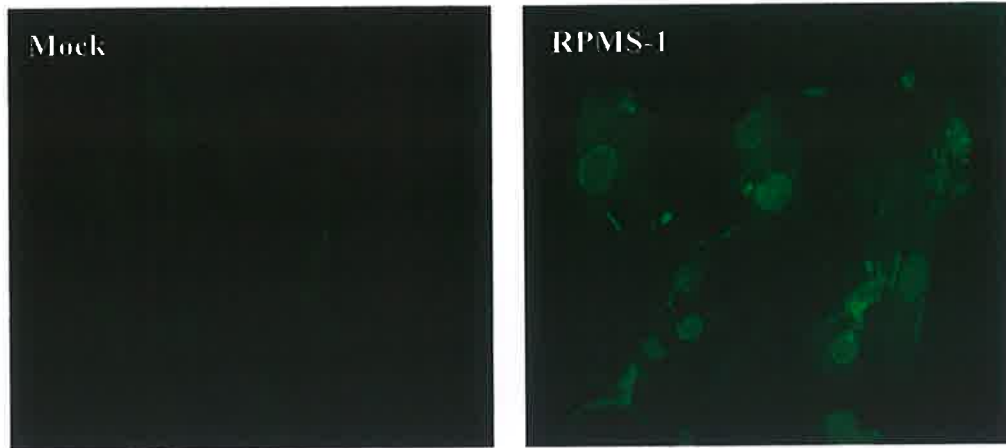


Fig. 26 Immunocytochemical staining of HVSMC's (passage 12): myosin in mock and RPMS-1 transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

Smoothelin

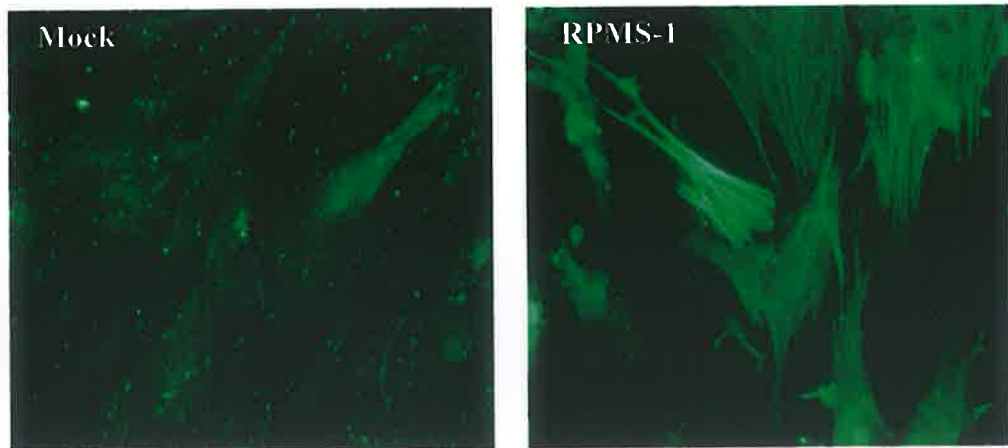


Fig. 27 Immunocytochemical staining of HVSMC's (passage 12): smoothelin in mock and RPMS-1 transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

6.3.3 QRT-PCR analysis

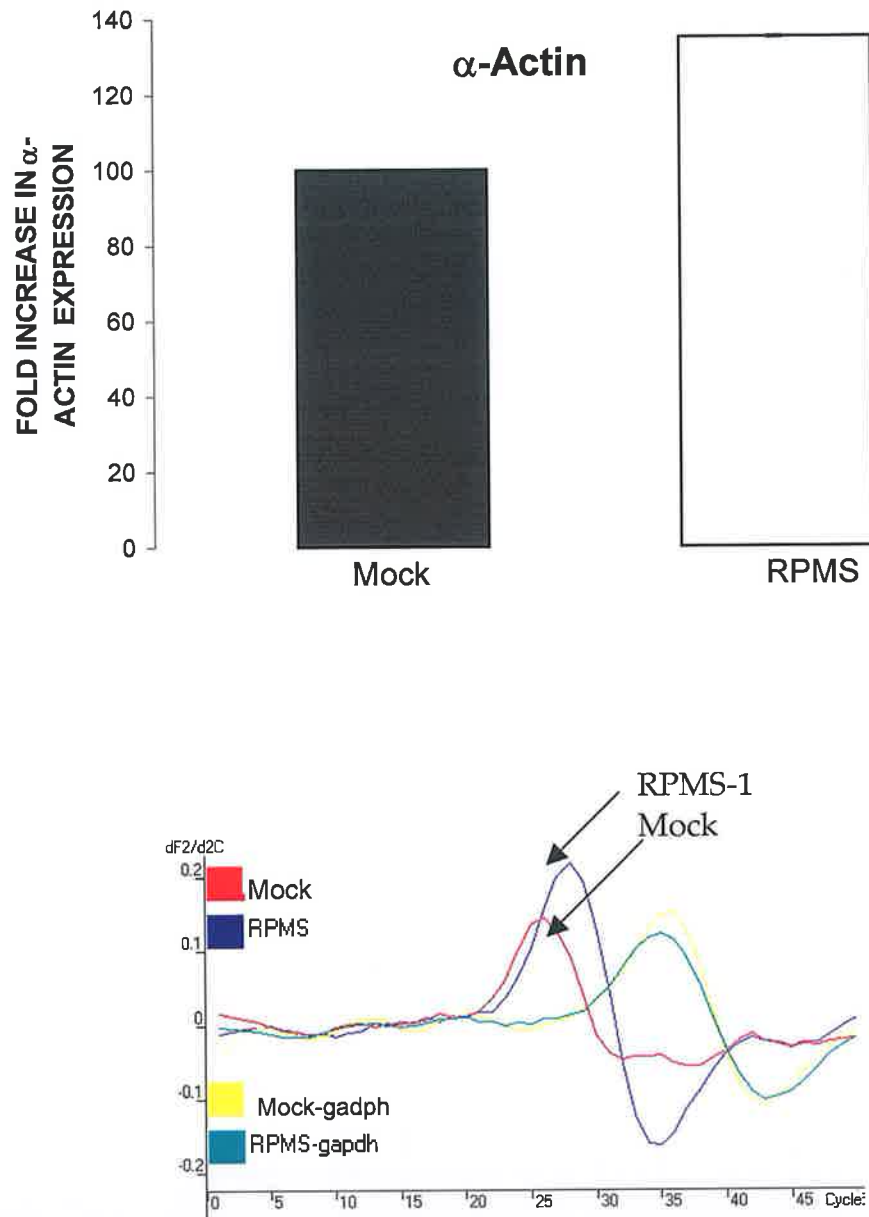


Fig. 28 Representative graph shows 35% increase in α -actin mRNA in RPMS-1 transfected cells as compared to mock-transfected cells. Results were normalized by amplification of GAPDH.

6.4 Overexpression of Notch IC inhibits differentiation in HVSMC's

To further confirm that the Notch Signalling Pathway inhibits differentiation of human VSMC, transient transfection of these cells with constitutively active Notch 1 or 3 IC was carried out to determine the influence of over-expression of Notch 1 IC and Notch 3 IC on human VSMC differentiation marker protein expression. Cells were also co-transfected with with pGK-puro vector and puromycin selected for 24 hrs. Over expression of Notch 1 and 3 IC was confirmed by Western blot analysis and immunocytochemistry as described in section 6.1.1. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6. mRNA and QRTPCR was carried out as described in section 5.5

The results are as follows:

Western blot analysis revealed a significant down-regulation of differentiation markers in Notch 1 and 3 IC transfected cells when compared to mock controls. There was a 43% and 42% decrease of α -actin protein expression in Notch 1 IC and Notch 3 IC transfected cells, respectively (Fig. 29).

RESULTS

In a similar manner, expression of constitutively active Notch 1 and 3 IC resulted in a marked decrease in calponin (Fig 30) and myosin expression (Fig 31) when compared to mock controls. The results were further confirmed by immunocytochemical staining of these cells (Fig. 32, 33 and 34). There was a marked decrease in α -actin, calponin, myosin and smoothelin staining in cells expressing constitutively active Notch 1 and 3 IC when compared to mock controls (Fig 32, 33 and 34). Smoothelin was undetectable by Western blot analysis. However, immunocytochemical staining revealed decreased smoothelin expression in Notch 1 and 3 IC transfected cells as compared to mock-transfected cells (Fig 35). Finally, the down-regulation of VSMC differentiation marker expression by active Notch 1 and 3 IC was further confirmed by measuring mRNA levels for α -actin using QRT-PCR. There was a 64% and 79% reduction in α -actin mRNA levels in cells expressing constitutively active Notch 1 and 3 IC when compared to mock controls (Fig 36).

6.4.1 Western blot analysis

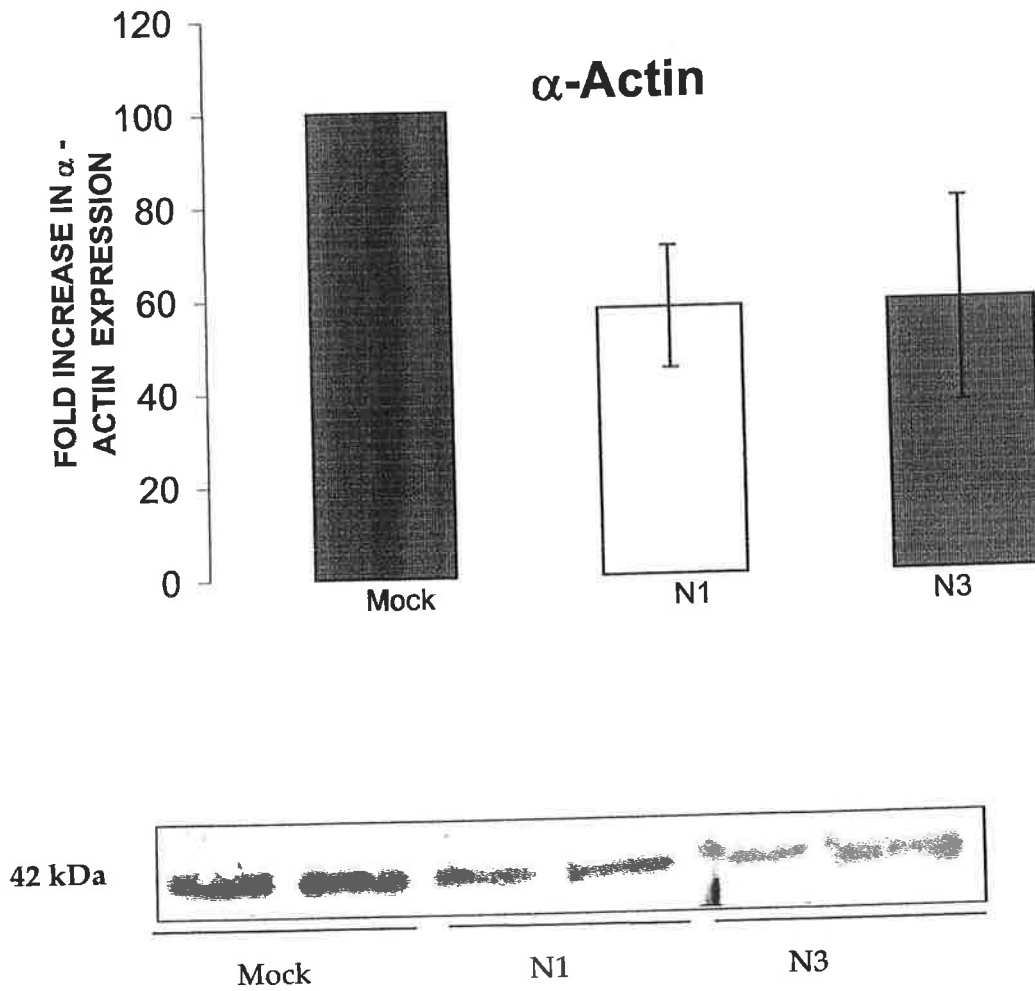


Fig. 29 Inset shows a representative graph displaying 43% and 42% decrease of α -actin protein expression in Notch 1 and 3 IC transfected cells as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

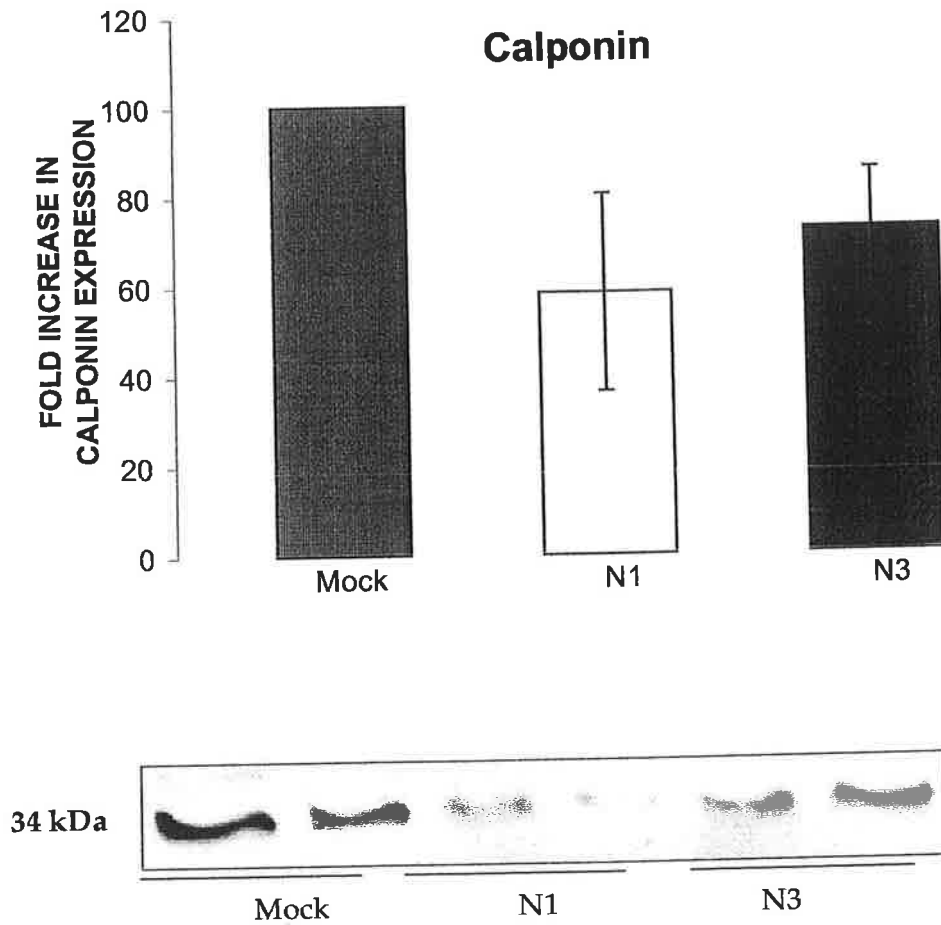


Fig. 30 Inset shows a representative graph displaying 42% and 28% decrease of calponin protein expression in Notch 1 and 3 IC transfected cells as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

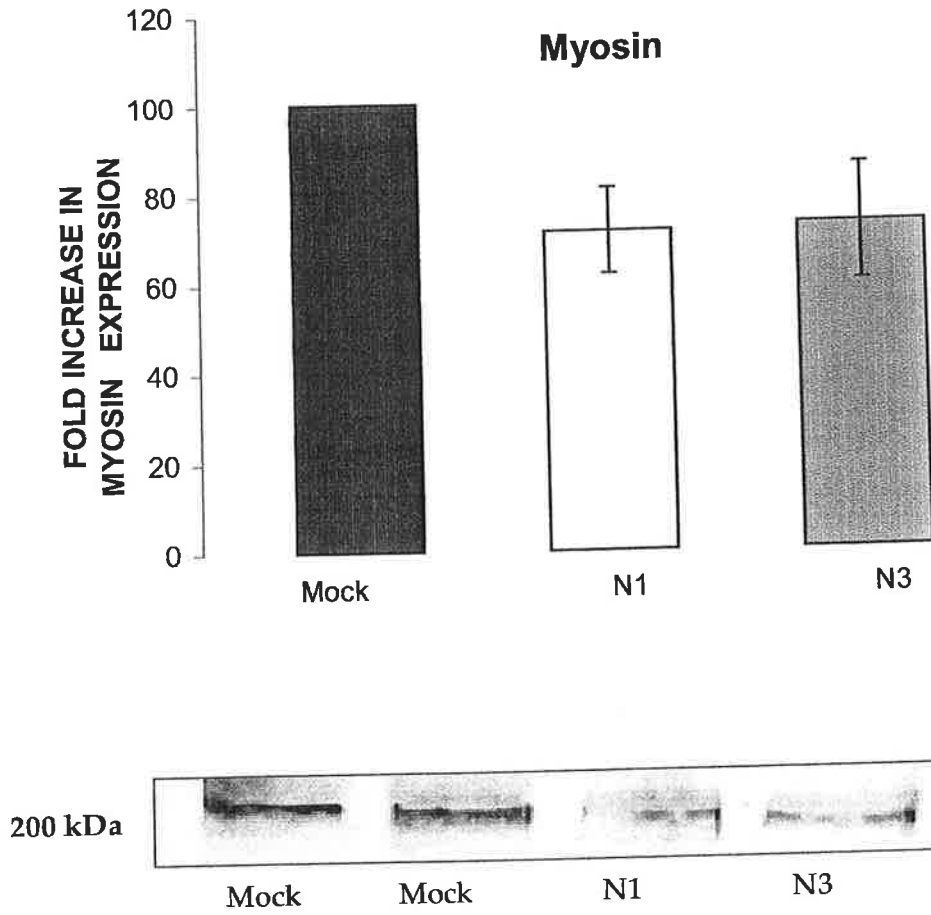


Fig. 31 Inset shows a representative graph displaying 29% and 27% decrease of myosin protein expression in Notch 1 and 3 IC transfected cells as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

6.4.2 Immunocytochemical staining

α -Actin



Fig. 32 Immunocytochemical staining of HVSMC's: α -actin in mock, Notch 1 IC (N1) and Notch 3 IC (N3) transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments.

Calponin

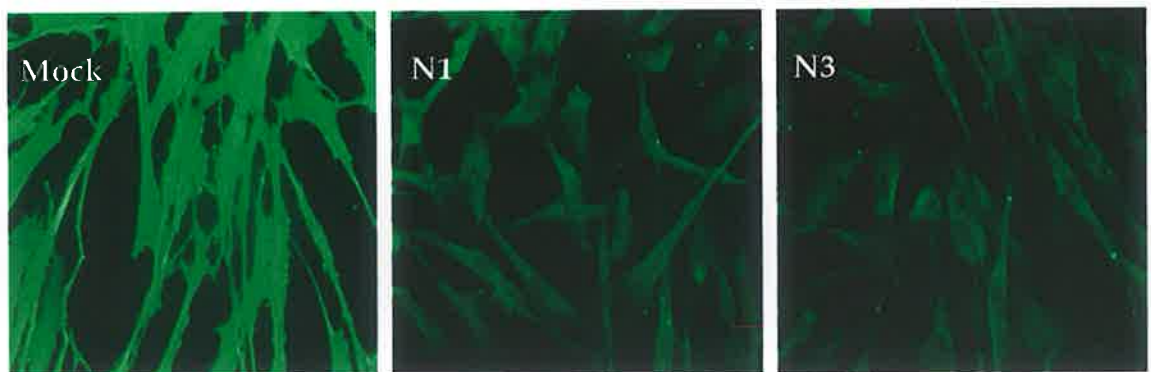


Fig. 33 Immunocytochemical staining of HVSMC's: calponin in mock, Notch 1 IC (N1) and Notch 3 IC (N3) transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments

Myosin

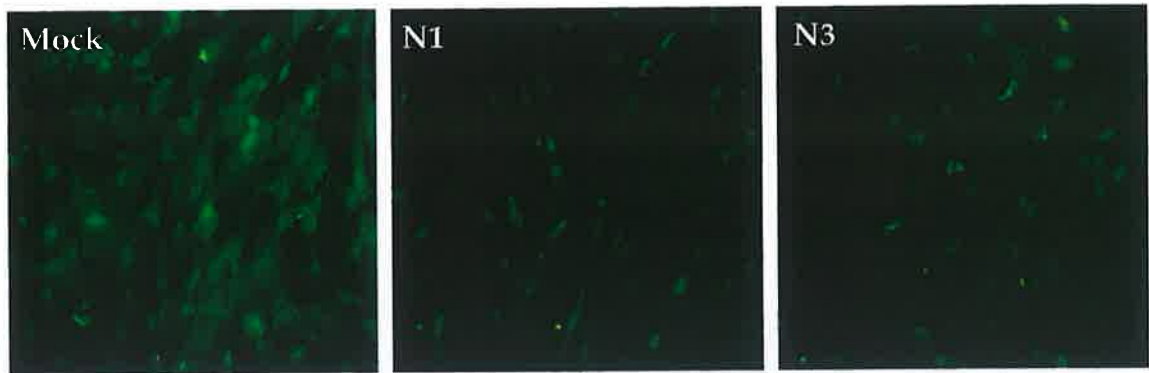


Fig. 34 Immunocytochemical staining of HVSMC's: myosin in mock , Notch 1 IC (N1) and Notch 3 IC (N3) transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments

Smoothelin

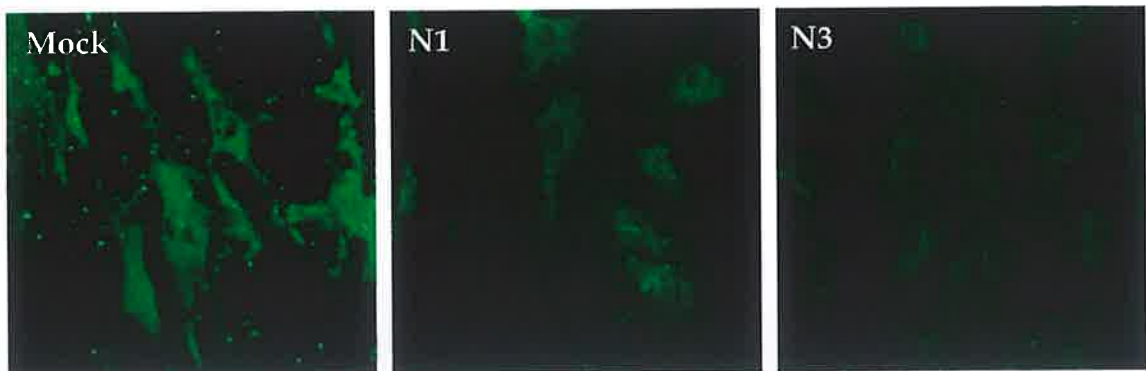


Fig. 35 Immunocytochemical staining of HVSMC's: smoothelin in mock , Notch 1 IC (N1) and Notch 3 IC (N3) transfected cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of at least 3 individual experiments

6.4.3 QRT-PCR analysis

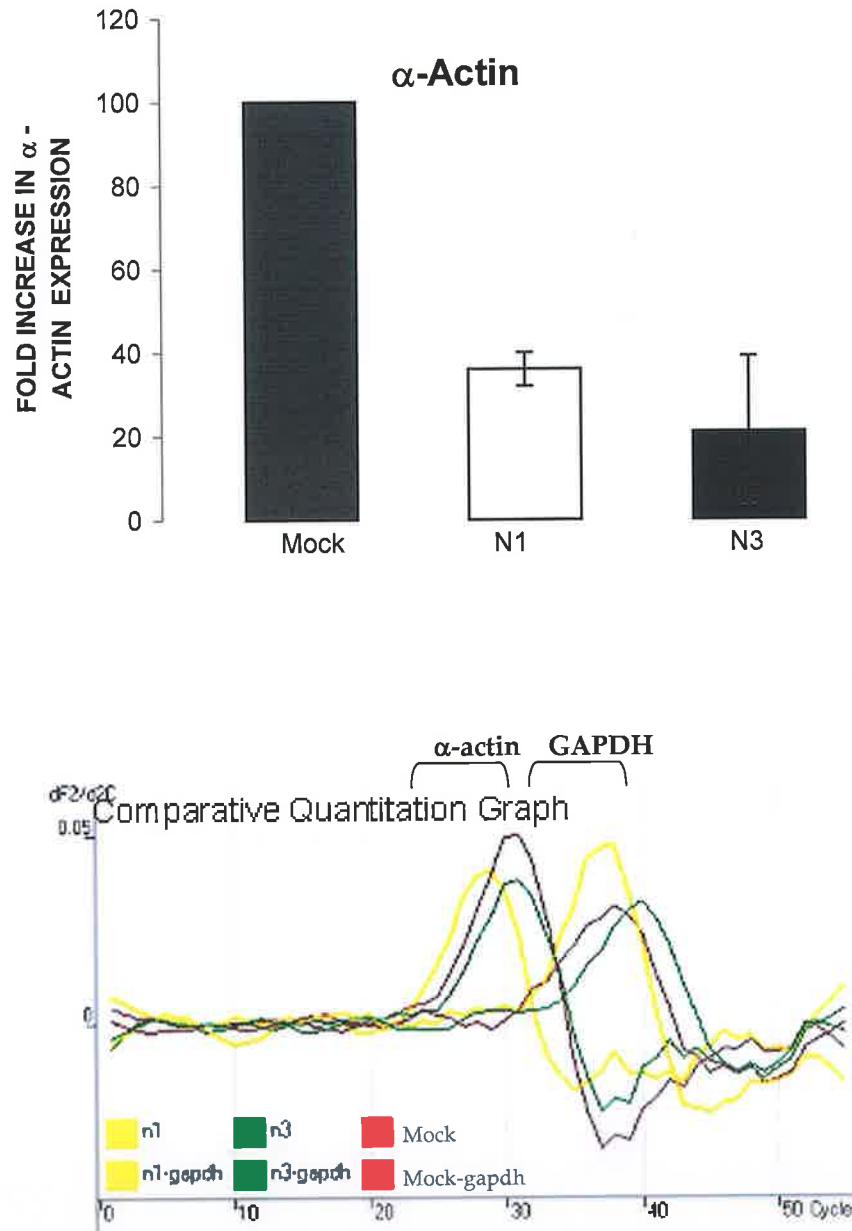


Fig. 36 Representative graph displaying 64% and 79% decrease in α -actin mRNA in Notch 1 and 3 IC transfected cells as compared to mock-transfected cells. Results were normalized by amplification of GAPDH. Data presented is representative of at least 2 individual experiments

6.5 Notch IC promotes HVSMC proliferation

The results from the previous section demonstrate that Notch signalling may promote the de-differentiation of HVSMC. The de-differentiation of human VSMC by Notch 1 and 3 IC suggests that Notch IC may promote de-differentiation of human VSMC to enhance proliferation of these cells. To examine this possibility, transient transfection of HVSMC with Notch 1 or 3 IC or P7-CMV mock controls was performed and the proliferative capacity of these cells was examined. Transient transfection of human VSMC and puromycin selection of Notch IC transfected cells was performed as described in section 5.3. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Cell proliferation was examined by cell counting and determining proliferating cell nuclear antigen expression using Western blot.

The results are as follows:

There was an increase in cell number after 5 days following over expression of constitutively active Notch 1 and 3 (Fig. 37), concomitant with a marked increase in proliferating cell nuclear antigen (pCNA) expression, a marker of DNA synthesis, after 24 h (Fig 38).

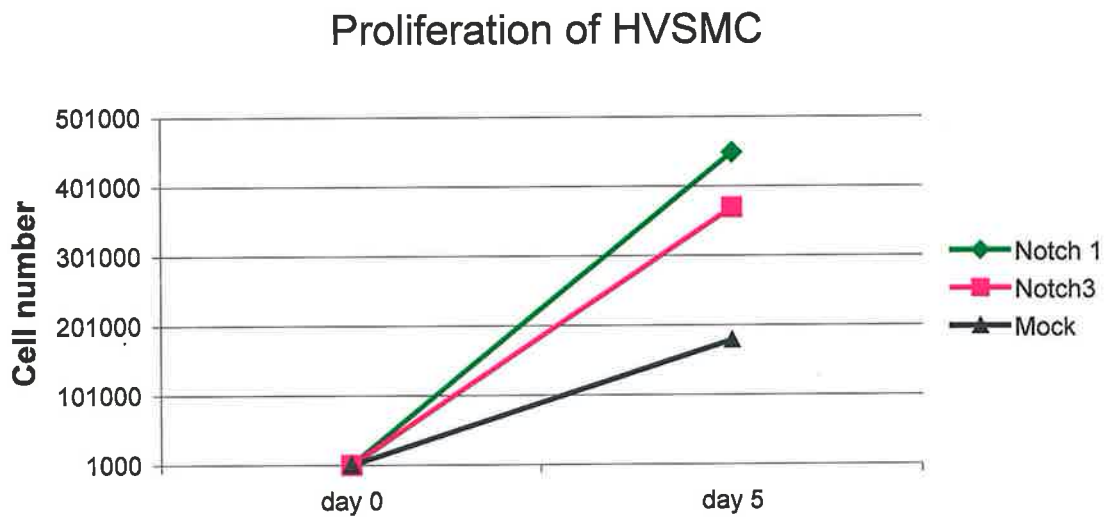
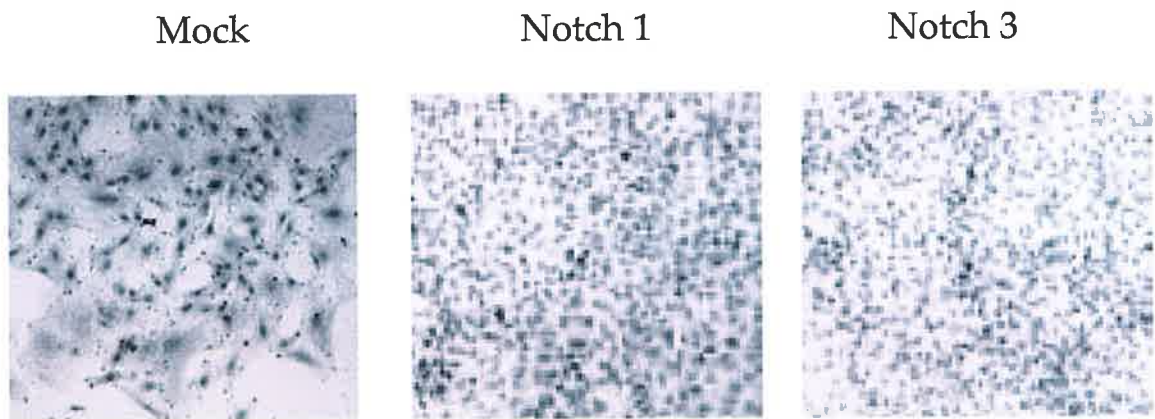


Fig. 37 Inset shows an increase in proliferation of HVSMC transfected with Notch 1 and Notch3 transfected as compare to mock transfected cells. Cell counts were carried out five days after transfection. Data presented is representative of at least 3 individual experiments

RESULTS

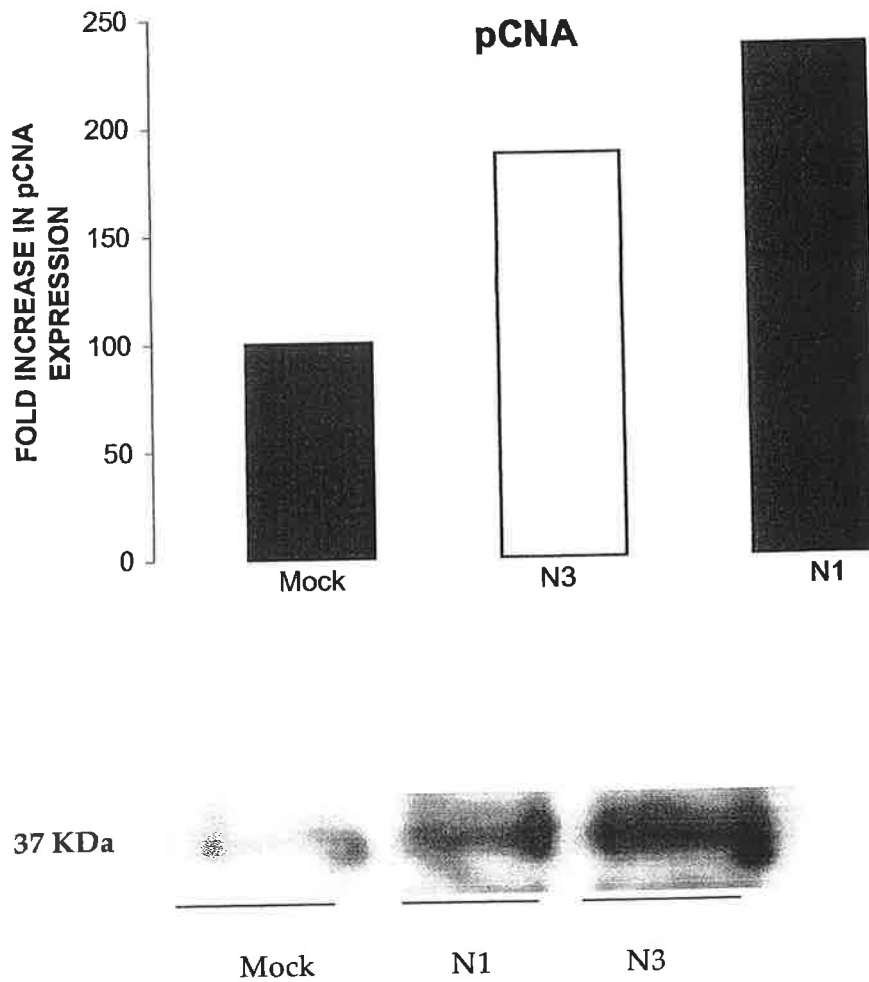


Fig. 38 Inset shows a representative graphs displaying 87% and 136% increase of pCNA protein 24 h following over expression of constitutively active Notch 1 and 3 IC as compared to mock-transfected cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

6.6 Notch IC inhibits HVSMC differentiation in a CBF-1/RBP-Jk dependent manner.

In order to determine whether changes observed in human VSMC differentiation following expression of constitutively active Notch 1 and 3 IC are indeed driven by enhanced expression of these receptors acting through CBF-1/RBP-Jk dependent mechanisms, human VSMC were co-transfected with inhibitors of Notch IC activation of CBF-1/RBP-Jk dependent signalling using RPMS-1 or a mutant Notch 1 IC, (ED4, a delta RAM deleted Notch 1 IC that acts as a selective inhibitor of Notch IC/CBF-1/SKIP interactions).

Human VSMC differentiation marker protein expression was determined by Western blot analysis, immunocytochemistry and QRTPCR analysis. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6. mRNA and QRTPCR was carried out as described in section 5.5

The results were as follows:

There was a marked decrease in α -actin protein expression in cells over expressing constitutively active Notch 1 and 3 IC, an effect that was fully reversed in cells co-expressing RPMS-1 (Fig 39). In a similar manner, the

decrease in calponin levels following expression of constitutively active Notch 1 and 3 IC was reversed in full in cells by co-expression with RPMS-1 (Fig 40) and partially reversed following expression of a mutant Notch 1 IC (ED4, Fig 41). Myosin expression was decreased following expression of constitutively active Notch 1 and 3 IC, an effect that was reversed following co-expression with RPMS-1 (Fig 42). Immunocytochemical analysis of α -actin, calponin and myosin further confirmed that the inhibitory effect of constitutively active Notch 1 and 3 IC on VSMC differentiation was reversed following co-expression with RPMS-1 (Fig 43, 44 and 45). Smoothelin was undetectable by Western blot but immunocytochemical staining revealed a relative decrease in levels following constitutive Notch IC expression, an effect that was reversed with RPMS-1 (Fig. 46). In addition, a pharmacological inhibitor of the Notch signalling pathway, brefeldin A that inhibits translocation of Notch to the nucleus by disassembling the Golgi apparatus, was also employed in this study to inhibit Notch signalling. Immunocytochemical analysis of α -actin and calponin expression in HVSMC transfected with Notch 1 or 3 IC and treated with 0.25 mg/ml of brefeldin A revealed there was not a reversal of Notch IC-induced decrease in α -actin and calponin expression in brefeldin A-treated cells as compared to untreated cells (Fig 47 and 48). Finally, the down-regulation of VSMC differentiation marker expression by constitutively active Notch 1 and 3 IC was further confirmed by measuring mRNA levels for α -actin using QRT-PCR. There was a marked reduction in α -actin mRNA levels in cells expressing

RESULTS

constitutively active Notch 1 and 3 IC when compared to mock controls, an effect that was reversed following co-expression with RPMS-1 (Fig 49).

Taken together, the results demonstrate that inhibition of CBF-1/RBP-Jk dependent signalling with RPMS-1 resulted in a significant reversal of Notch IC induced down-regulation of human VSMC differentiation marker expression.

6.6.1 Western blot analysis

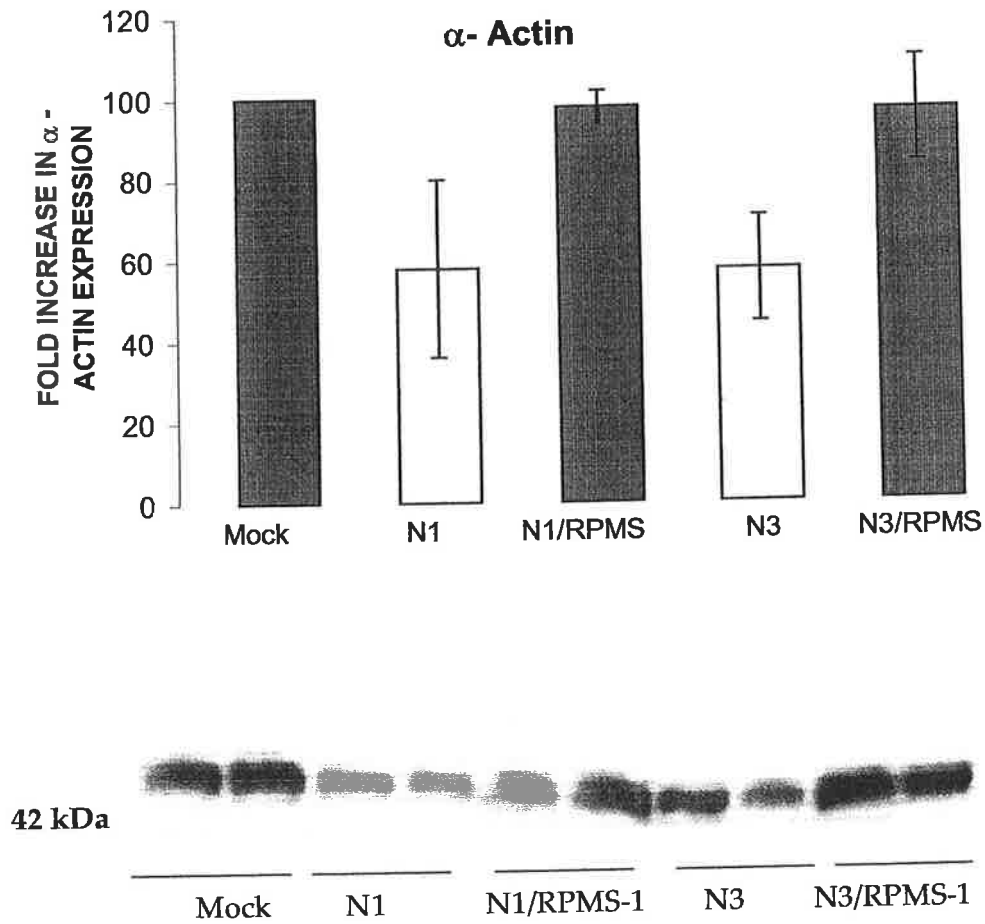


Fig. 39 Inset shows representative graph displaying the effect of inhibition of over-expression of Notch 1 and 3 on α -actin protein expression in HVSMC. 41% and 43% decrease of α -actin protein expression in Notch 1 and 3 IC transfected cells is fully reversed in cells co-transfected with Notch and RPMS-1. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

RESULTS

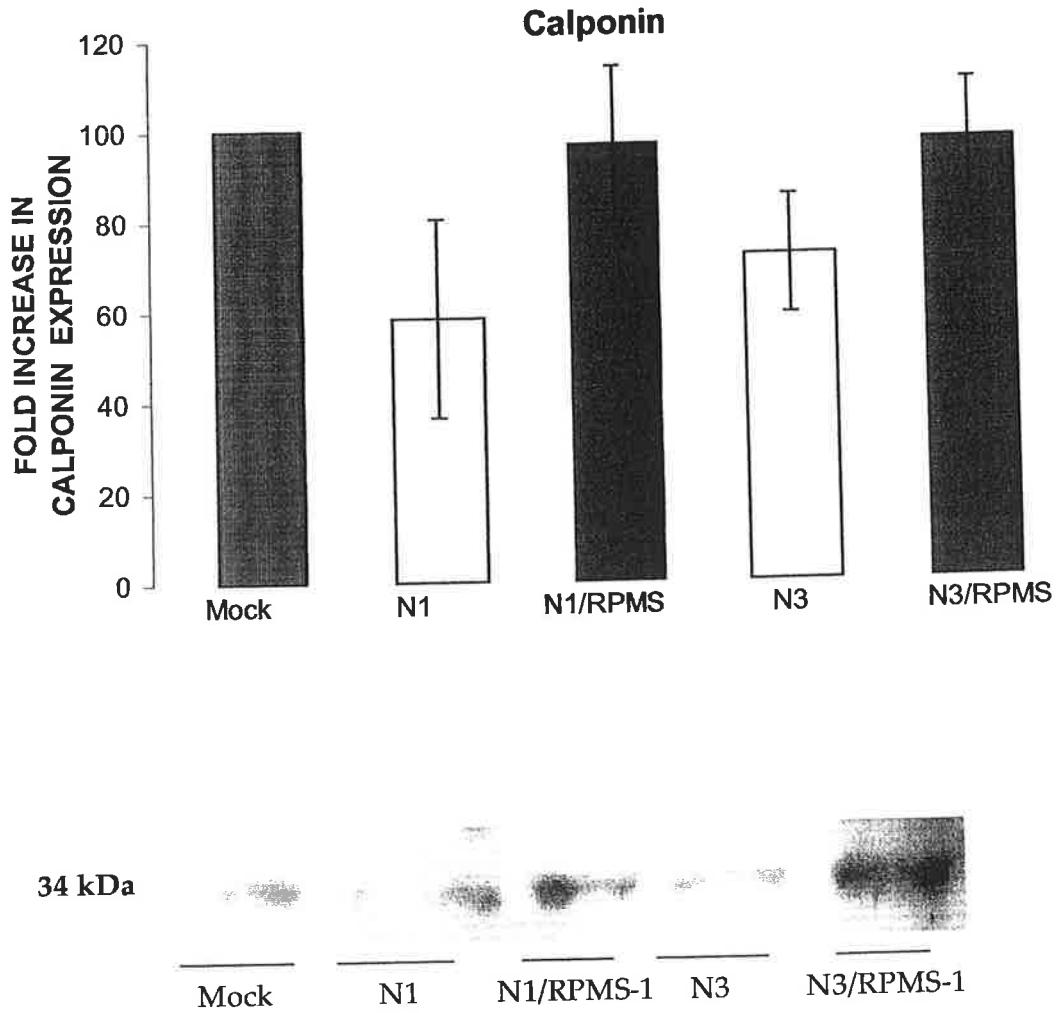


Fig. 40 Inset shows representative graph displaying the effect of inhibition of over-expression of Notch 1 and 3 on calponin protein expression in HVSMC. 42% and 28% decrease of α -actin protein expression in Notch 1 and 3 IC transfected cells is fully reversed in cells co-transfected with Notch and RPMS-1. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

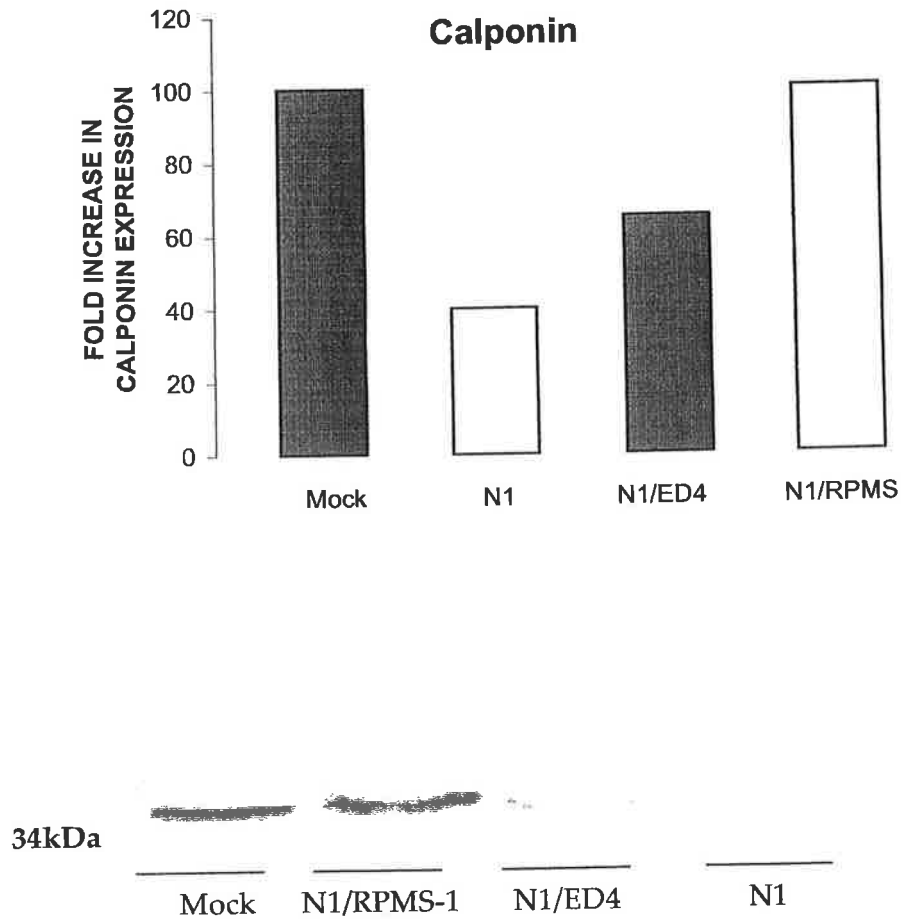


Fig. 41 Inset shows representative graph displaying the effect of inhibition of over-expression of Notch 1 on calponin protein expression in HVSMC. 60% down-regulation of calponin protein expression in Notch 1 transfected cells is reversed in cells co-transfected with Notch and RPMS-1 and partially reversed in cells co-transfected with Notch and ED4. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 1 experiment.

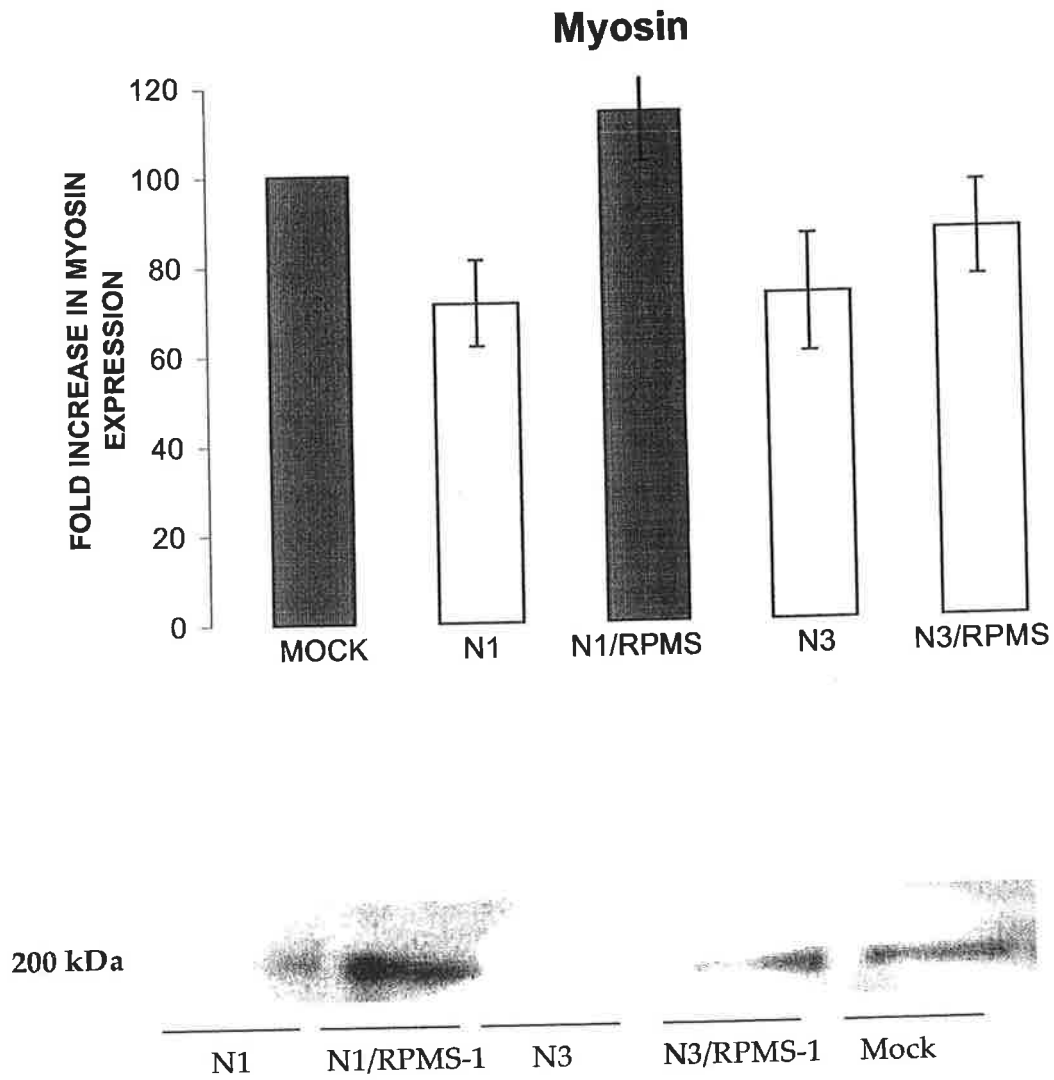


Fig. 42 Inset shows representative graph displaying the effect of inhibition of over-expression of Notch 1 and 3 on myosin protein expression in HVSMC. 29% and 27% down-regulation of myosin protein expression in Notch 1 and 3 IC transfected cells is reversed in cells co-transfected with Notch and RPMS-1. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

6.6.2 Immunocytochemical staining

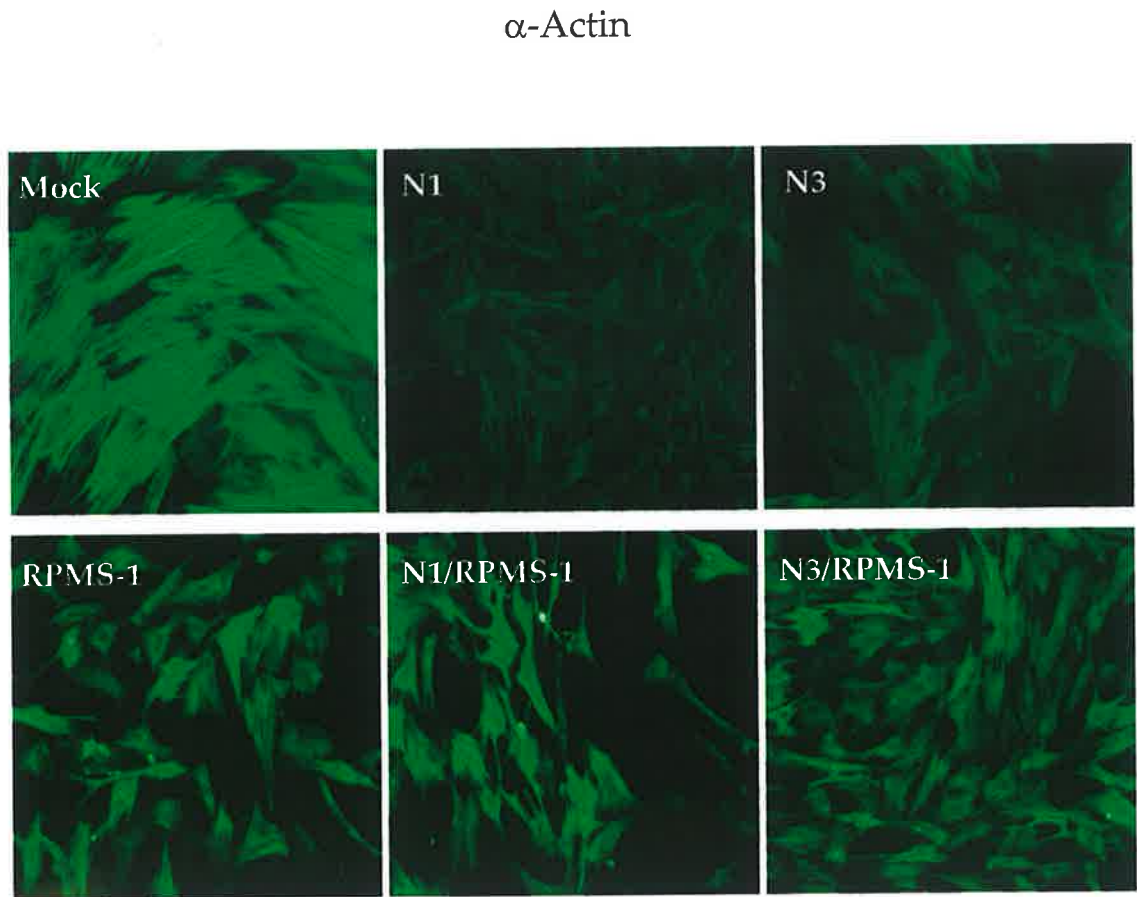


Fig. 43 Immunocytochemical staining of HVSMC's: decrease of α -actin protein expression in Notch 1 IC (N1), Notch 3 IC (N3) transfected cells is reversed in cell co-transfected with Notch 1 IC and RPMS-1 (N1/RPMS-1) or Notch 3 IC and RPMS-1 (N3/RPMS-1); mock transfected cells and cells transfected with RPMS-1 alone (RPMS-1). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

Calponin

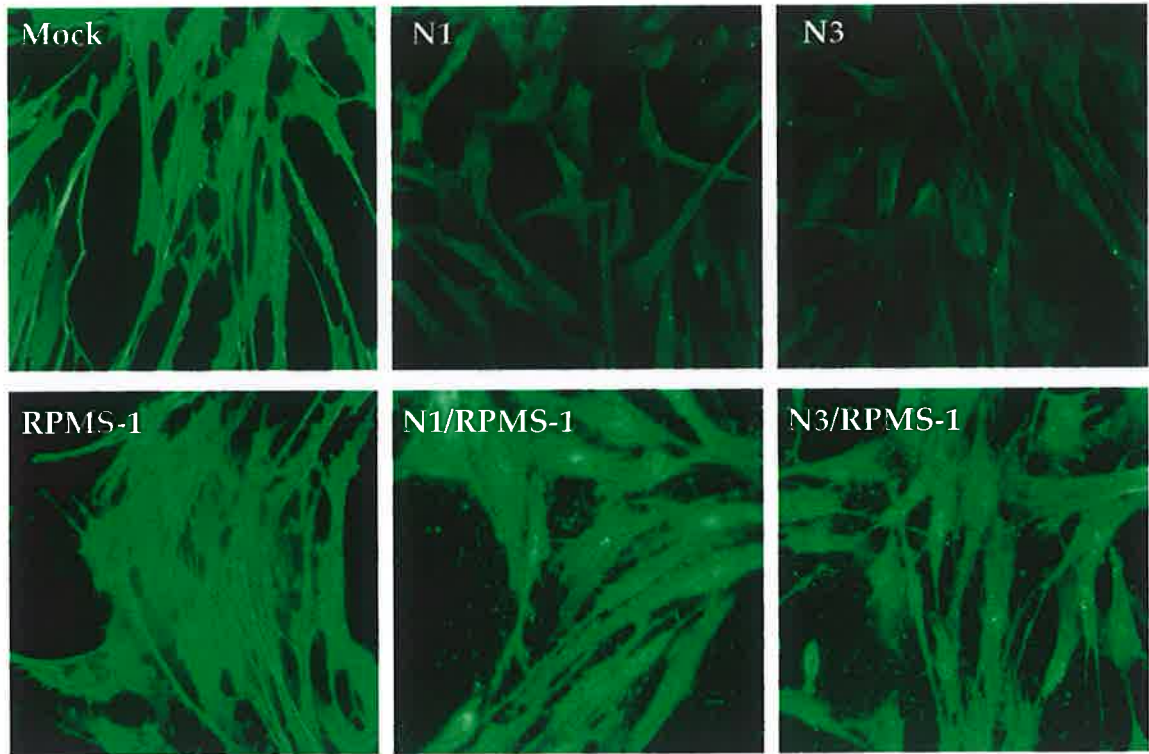


Fig. 44 Immunocytochemical staining of HVSMC's: decrease of calponin protein expression in Notch 1 IC (N1), Notch 3 IC (N3) transfected cells is reversed in cell co-transfected with Notch 1 IC and RPMS-1 (N1/RPMS-1) or Notch 3 IC and RPMS-1 (N3/RPMS-1); mock transfected cells and cells transfected with RPMS-1 alone (RPMS-1). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

Myosin

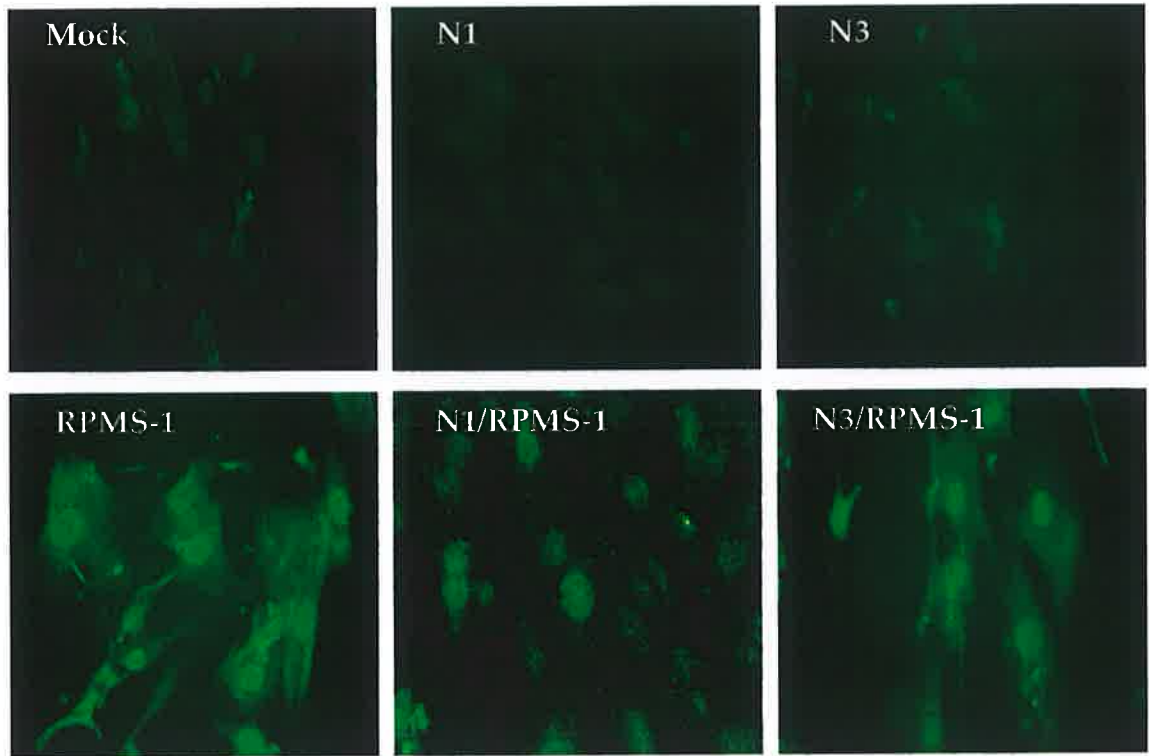


Fig. 45 Immunocytochemical staining of HVSMC's: decrease of myosin protein expression in Notch 1 IC (N1), Notch 3 IC (N3) transfected cells is reversed in cell co-transfected with Notch 1 IC and RPMS-1 (N1/RPMS-1) or Notch 3 IC and RPMS-1 (N3/RPMS-1); mock transfected cells and cells transfected with RPMS-1 alone (RPMS-1). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

Smoothelin

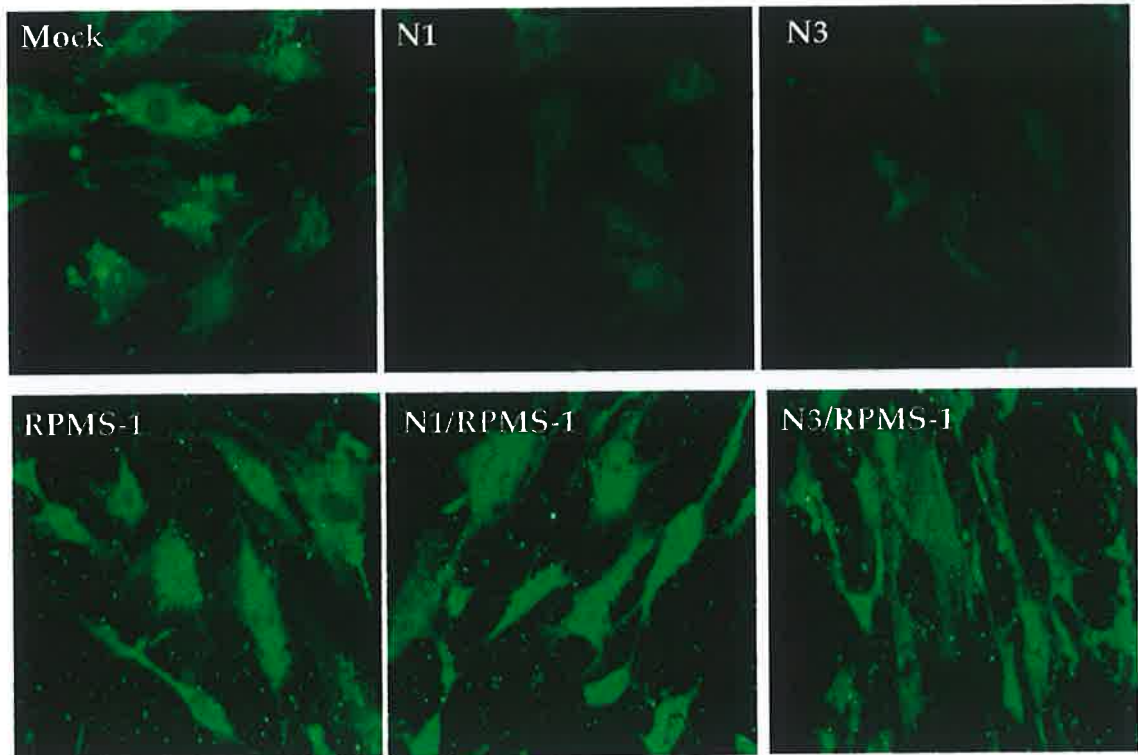


Fig. 46 Immunocytochemical staining of HVSMC's: decrease of smoothelin protein expression in Notch 1 IC (N1), Notch 3 IC (N3) transfected cells is reversed in cell co-transfected with Notch 1 IC and RPMS-1 (N1/RPMS-1) or Notch 3 IC and RPMS-1 (N3/RPMS-1); mock transfected cells and cells transfected with RPMS-1 alone (RPMS-1). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

6.6.3 Immunocytochemical staining of cells treated with brefeldin

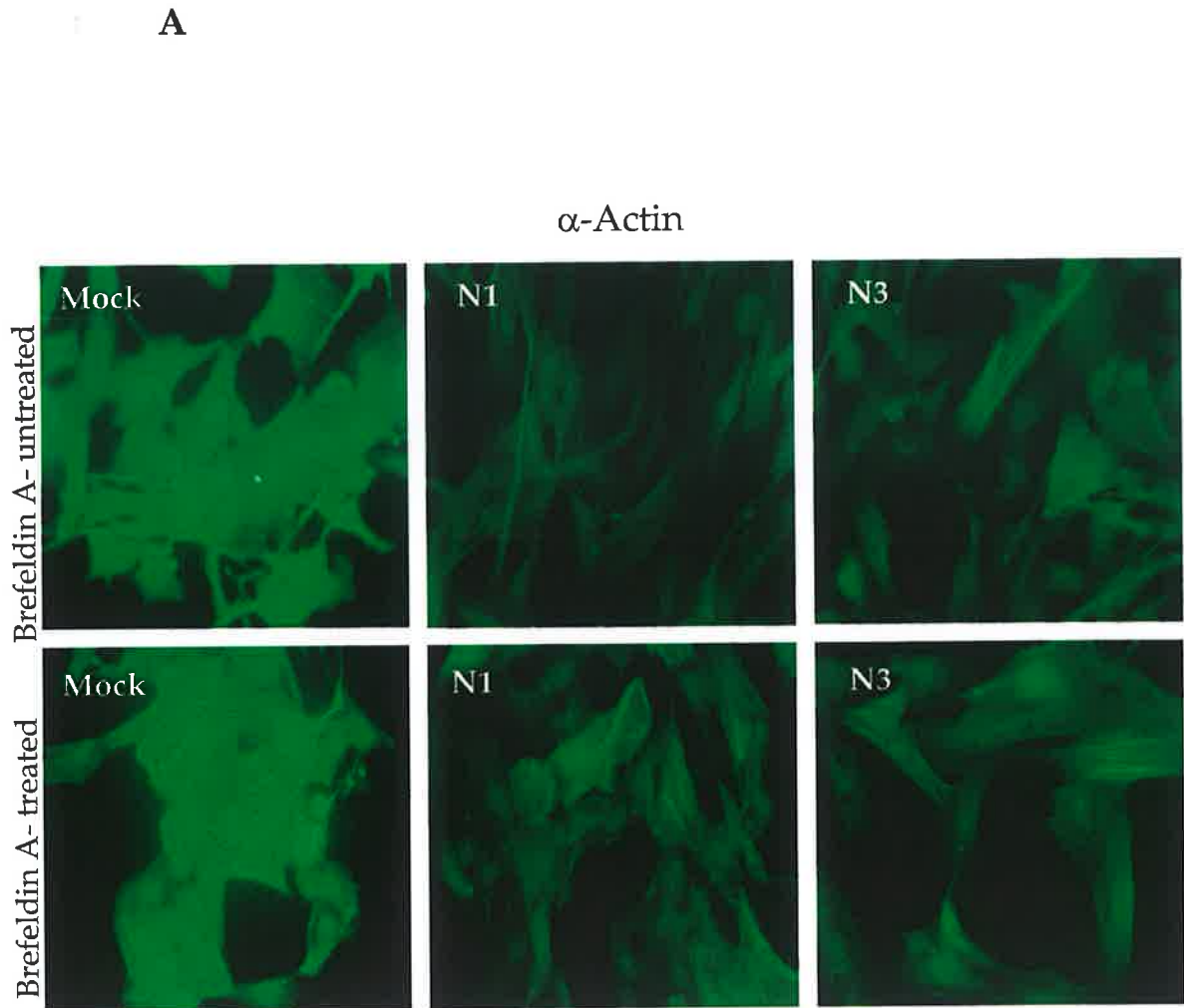


Fig. 47 Immunocytochemical staining of HVSMC's: down-regulation of α -actin protein expression in Notch 1 IC (N1), Notch 3 IC (N3) as compared to mock transfected cells is not reversed in these cells following treatment with brefeldin A. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 1 experiment.

Calponin

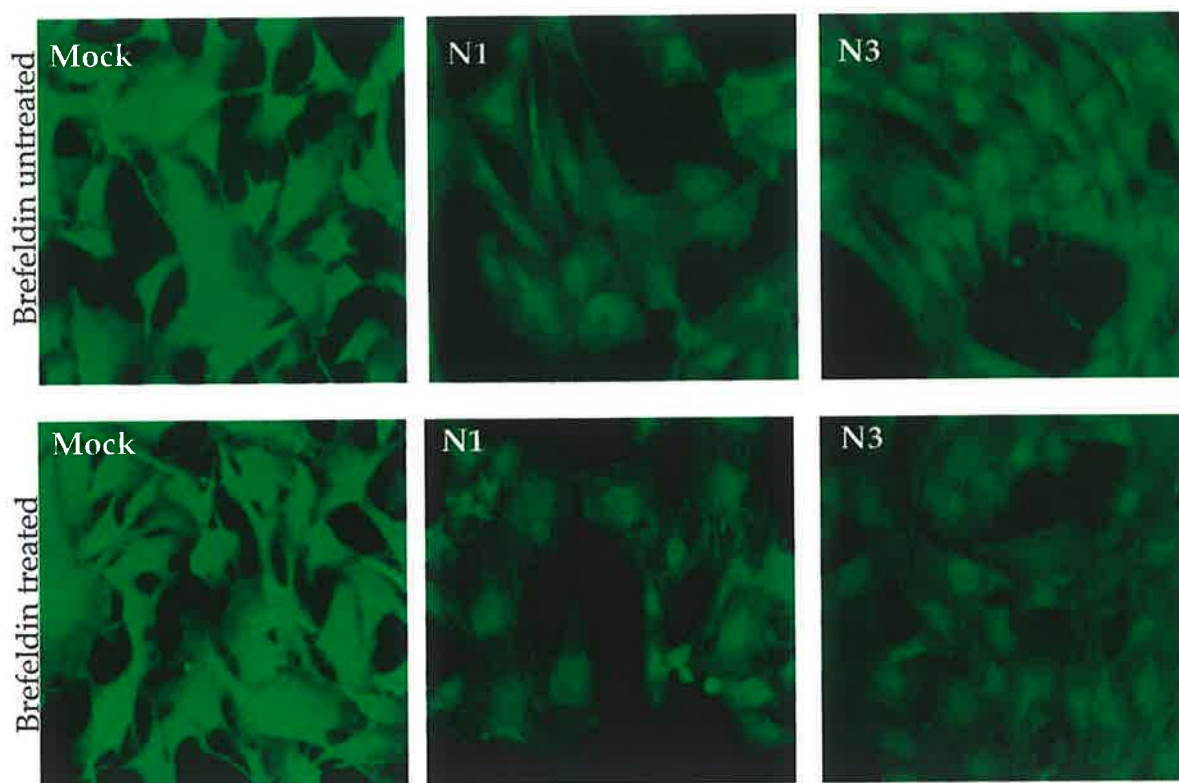


Fig. 48 Immunocytochemical staining of HVSMC's: down-regulation of calponin protein expression in Notch 1 IC (N1), Notch 3 IC (N3) as compared to mock transfected cells is not reversed in these cells following treatment with Notch inhibitor, brefeldin A. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 1 experiment.

6.6.4 QRTPCR analysis

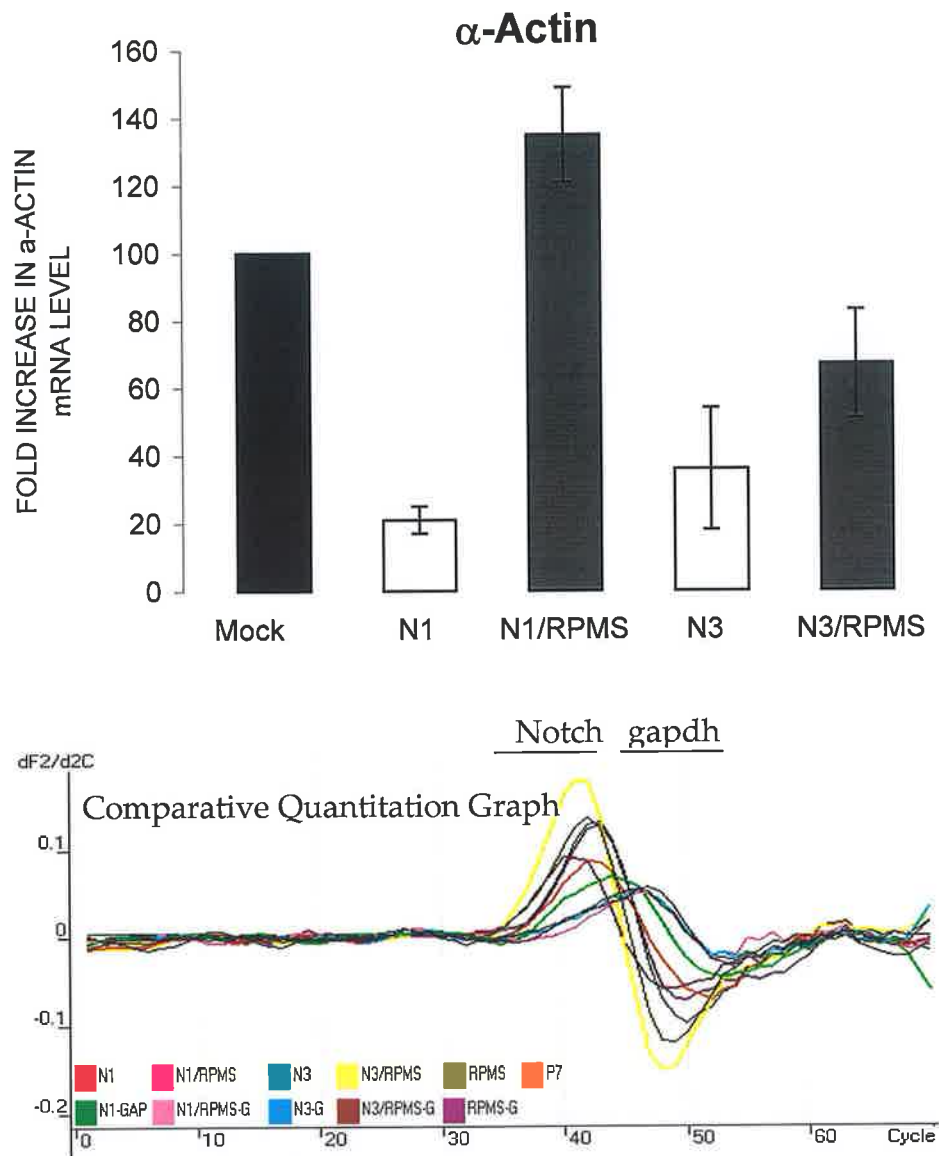


Fig. 49 Inset shows representative graph displaying the effect of inhibition of over-expressed Notch 1 and 3 IC in HVSMC on α -actin mRNA level. 80% and 65% down-regulation in α -actin mRNA level in Notch 1 and 3 IC transfected cells is reversed in cells co-transfected with Notch 1 and RPMS-1 and partially reversed in cells co-transfected with Notch 3 and RPMS-1. Results were normalized by amplification of GAPDH. Data presented is representative of 2 individual experiments.

6.7 DISCUSSION:

This study was initiated by showing the presence of Notch 1 and Notch 3 receptors in human VSMC in conjunction with studies to validate the over-expression of these receptors in HVSMC's by using Western blot analysis and Immunocytochemistry. The data revealed the presence of both receptors in HVSMC's, which provides evidence of the presence of Notch in VSMC as well as the enhanced expression of both receptor proteins following transfection with Notch 1 IC and 3 IC when compared to mock-transfected cells. The localization of Notch in the cell has also been monitored. Immunocytochemical staining of HVSMC revealed the cytoplasmatic and nuclear presence of Notch protein.

In order to examine the differentiation profile of HVSMC, basal protein expression of α -actin, calponin, myosin and smoothelin in HVSMC was assessed in these cells. The Western blot results revealed abundant expression of α -actin and calponin in HVSMC and much less intensive expression of myosin in these cells. Smoothelin was undetectable by Western blot analysis. The presence of differentiation marker proteins expression was further confirmed by immunocytochemical staining and QRT-PCR. Smoothelin has been successfully detected in HVSMC by Immunocytochemistry and QRT-PCR but its expression in cells remained low.

RESULTS

To determine the influence of the Notch Signalling Pathway on differentiation of HVSMC, the levels of VSMC differentiation markers have been analysed and compared following both inhibition of the NSP with RPMS-1 and over expression of constitutively active Notch IC, respectively. This study revealed that inhibition of endogenous Notch CBF-1/RBP-Jk signalling with RPMS-1 results in a marked increase in VSMC differentiation markers, α -actin, myosin, calponin and smoothelin.

The up-regulation of α -actin, calponin and myosin in cells that express RPMS-1 to inhibit Notch 1 and 3 IC dependent CBF-1 dependent gene expression was measured by quantitative method as Western blot. The up-regulation of VSMC differentiation markers was confirmed by qualitative method, Immunocytochemistry as well as by measuring mRNA levels for α -actin using QRTPCR. Actin was considered as an index for other differentiation markers.

In parallel studies, RPMS-1 has been shown as a selective inhibitor that inhibited both endogenous Notch and over-expressed constitutively active Notch IC, respectively [34, 149].

Transient transfection of HVSMC with constitutively active Notch 1 or 3 IC was carried out to further confirm that the Notch Signalling pathway inhibits differentiation of these cells. Western blot analysis revealed a significant down-regulation of α -actin, calponin and myosin in Notch 1 and 3 IC transfected cells when compared to mock controls. These data were confirmed by Immunocytochemistry analysis. Smoothelin was undetectable by Western blot

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analysis but immunocytochemical staining revealed decreased smoothelin expression in Notch 1 and 3 IC transfected cells as compared to mock-transfected. Inhibition of differentiation of transfected HVSMC was also confirmed by measuring mRNA levels for α -actin using QRT-PCR.

The above results demonstrate that Notch signalling may promote the de-differentiation of HVSMC by enhancing the proliferation of these cells. An increase in cell number following over expression of constitutively active Notch 1 IC and 3 IC, in parallel with a marked increase in proliferating cell nuclear antigen (pCNA) expression found in this study confirm the above suggestion.

This study also reveals that changes observed in human VSMC differentiation following expression of constitutively active Notch 1 and 3 IC are indeed driven by enhanced expression of these receptors acting through CBF-1/RBP-Jk dependent mechanisms. In this case three different inhibitors of NSP were employed, RPMS-1, ED4 and a pharmacological inhibitor brefeldin A. Co-transfection of HVSMC with inhibitors of Notch IC activation of CBF-1/RBP-Jk dependent signalling using RPMS-1 or ED4 demonstrates that inhibition of CBF-1/RBP-Jk dependent signalling with RPMS-1 resulted in a significant reversal of Notch IC induced down-regulation of HVSMC differentiation marker expression. In a similar manner, the decrease in calponin levels following expression of constitutively active Notch 1 and 3 IC was reversed partially following expression of a mutant Notch 1 IC, ED4.

RESULTS

The down-regulation of VSMC differentiation marker expression by constitutively active Notch 1 and 3 IC was further confirmed by measuring mRNA levels for α -actin using QRT-PCR.

Treatment with brefeldin A that inhibits translocation of Notch to the nucleus by disassembling the Golgi apparatus was very problematic and didn't bring expected results. Brefeldin A did not reverse Notch IC-induced decrease in α -actin and calponin expression in HVSMC as expected. However, in parallel study we successfully demonstrated an inhibition of Notch IC signaling events in brefeldin A-treated RASMC [34].

Problems experienced with brefeldin A were probably associated with concentration of brefeldin A used in this study that might not have been sufficient to obtain expected results on HSMC. Titration experiment should have been done to determine the required concentration of brefeldin A necessary to influence Notch signalling events.

Chapter 7. Role of cyclic strain on Notch signalling pathway-induced changes in differentiation of HVSMC

Hemodynamic forces generated by the passage of blood flow through the vascular system have been implicated in SMC cell fate. Due to combined effects of changes in arterial lumen diameter, the pulsative nature of blood flow, elevation in shear stress and arterial branching are produced in areas of the vascular tree where the velocity vector of blood flow becomes non-linear. Arteriosclerosis is predominantly likely to occur in the aorta and disturbing arteries as well as at junctions and bends. Thus the transduction of mechanical forces to SMC in the blood vessel may be a significant pathogenic factor in arteriosclerosis or hypertension [114, 115].

SMC do not directly experience the shear stress or turbulent forces because of their medial location within arterial wall. They, in turn, experience the longitudinal and radial deformation and stresses imposed on the acquiescent arterial wall by blood pressure [114].

Several studies have shown that cyclic strain influences SMC phenotypic modulation (section 1.4.4). The Notch Signaling Pathway has also been found to influence SMC fate (section 3.2). In this section we decided to prove our hypothesis that Notch signalling dictates, in part, VSMC fate in response to mechanical stimuli, which is, among others, cyclic strain. This chapter mostly focuses on the role of Notch signalling in mechanical force-induced changes in

vascular structure. The Flexercell-Tension Plus System was used to mimic stress that SMC undergo in normal physiological conditions. This model was developed to provide mechanical load to cells cultured *in vitro* and was used in this study to expose HVSMC to an equiaxial cyclic strain mimicking that induced by a normal cardiac cell cycle.

7.1 Cyclic strain up-regulates differentiation marker proteins expression in HVSMC

HVSMC were subjected to 10% cyclic strain for 24 hours and protein expression of differentiation markers was analyzed using Western blot analysis and Immunocytochemical staining. Western blot revealed that cyclic strain significantly increased protein expression of differentiation markers. There was a 72% up-regulation of α -actin protein expression in cells subjected to cyclic strain as compare to static cells (Fig. 50). The results were further confirmed by Immunocytochemical staining of these cells (Fig. 53).

A 33% up-regulation of calponin protein expression was also observed in strained cells, as monitored by Western blot (Fig. 51). Immunocytochemistry confirmed these results as seen in Fig. 54.

Myosin expression was also enhanced in cells subjected to cyclic strain. A 60% up-regulation of myosin protein expression was observed as compared to static cells (Fig. 52). Increase of expression myosin in strained cells was also confirmed by Immunocytochemical staining of these cells (Fig. 55). Smoothelin

RESULTS

was undetectable by Western blot analysis but Immunocytochemical analysis revealed significant up-regulation of smoothelin expression in strained cells as compared to static cells (Fig. 56).

Exposure of HVSMC to cyclic strain was described in section 5.1.2. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.5

The results are as follows:

7.1.1 Western blot analysis

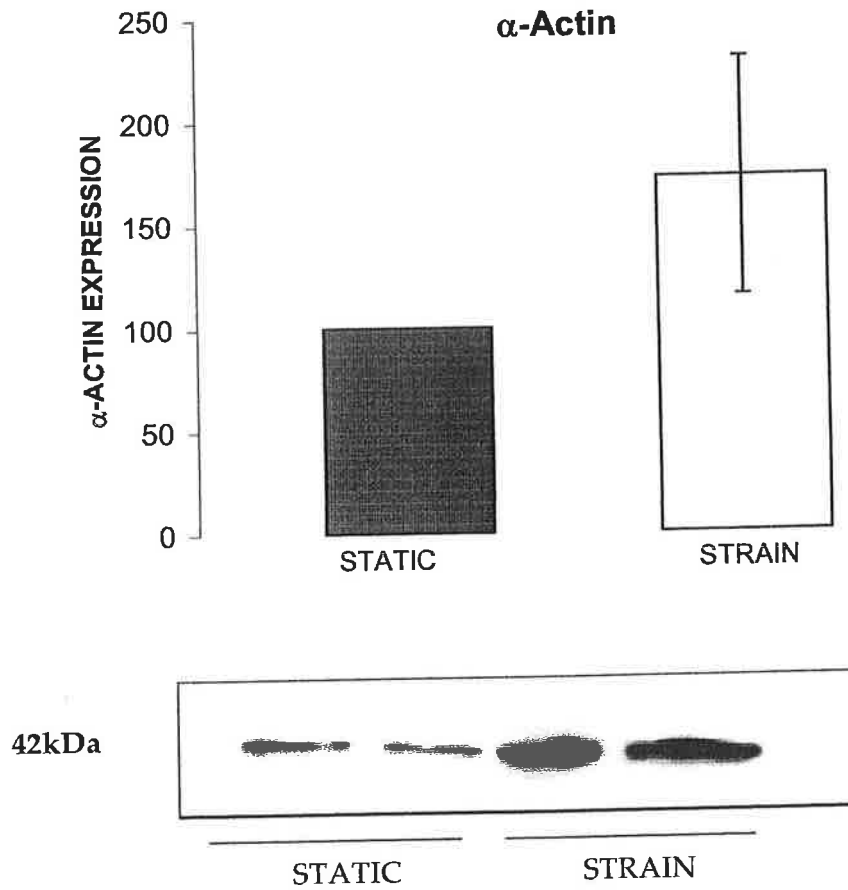


Fig. 50 Inset shows a representative graph displaying 72% up-regulation of α -actin protein expression in HVSMC in response to cyclic strain (10%, 24 hours) as compared to static cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

RESULTS

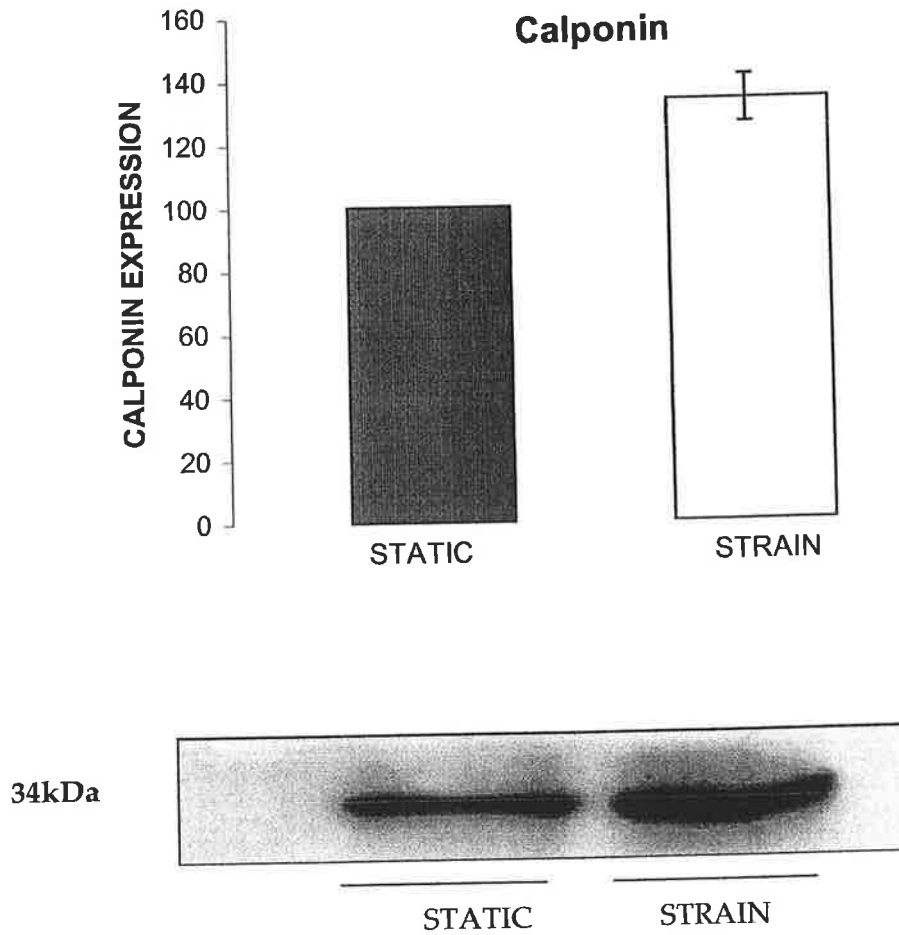


Fig. 51 Inset shows a representative graph displaying 33% up-regulation of calponin protein expression in HVSMC in response to cyclic strain (10%, 24 hours) as compared to static cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

RESULTS

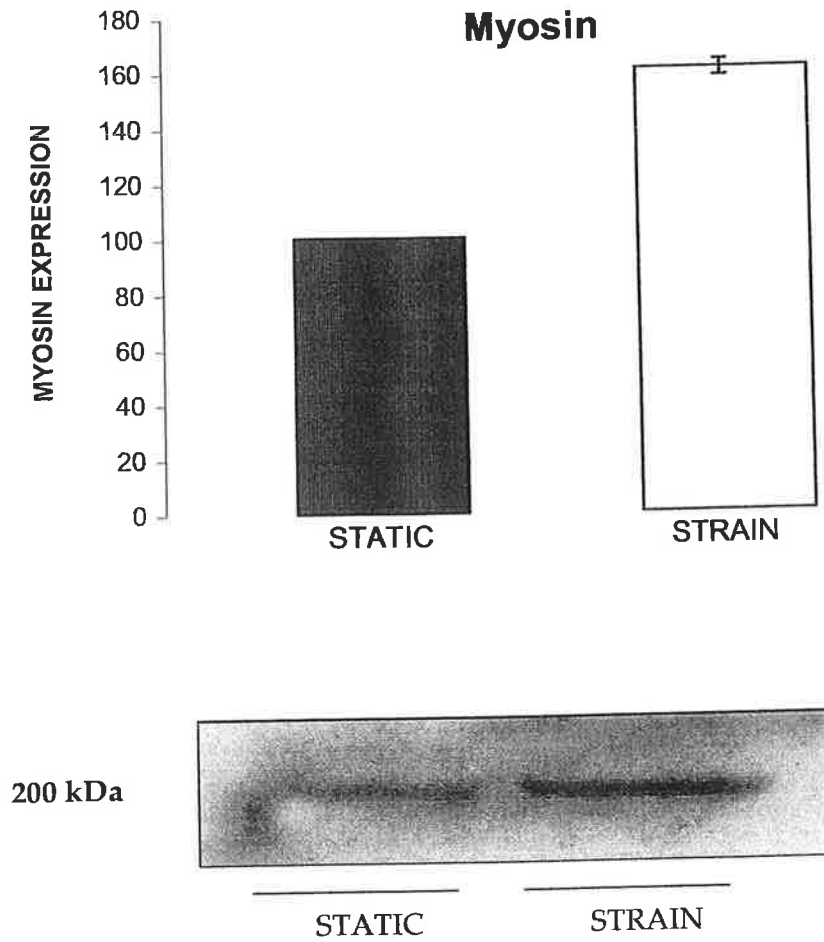


Fig. 52 Inset shows a representative graph displaying 60% up-regulation myosin protein expression in HVSMC in response to cyclic strain (10%, 24 hours) as compared to static cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

7.1.2 Immunocytochemical staining

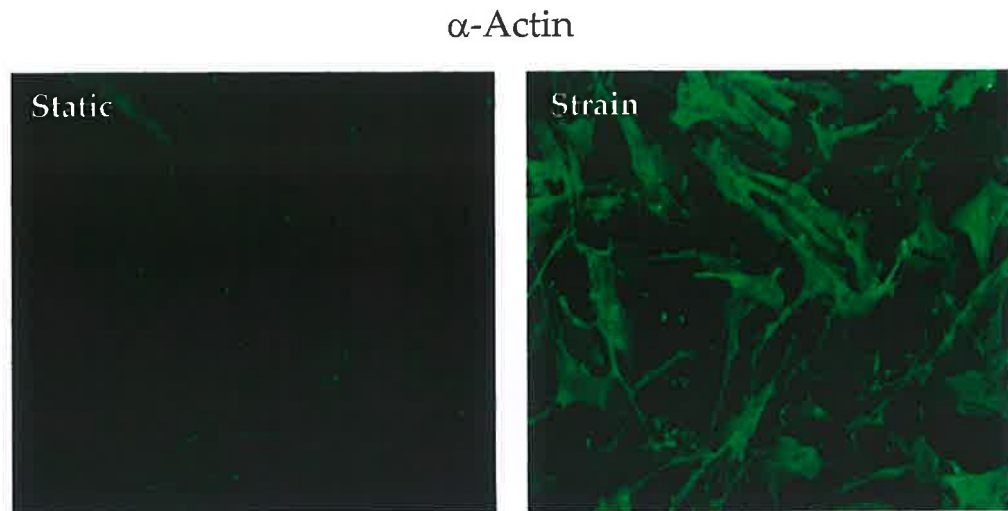


Fig. 53 Immunocytochemical staining of HVSMC's shows enhanced expression of α -actin protein in cells subjected to 10% cyclic strain as compared to static cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

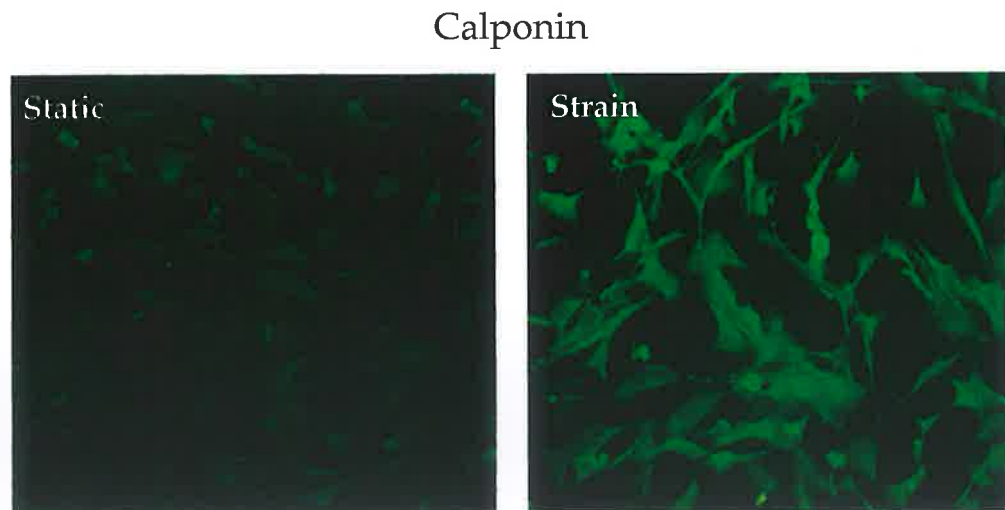


Fig. 54 Immunocytochemical staining of HVSMC's shows enhanced expression of calponin protein in cells subjected to 10% cyclic strain as compared to static cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

Myosin

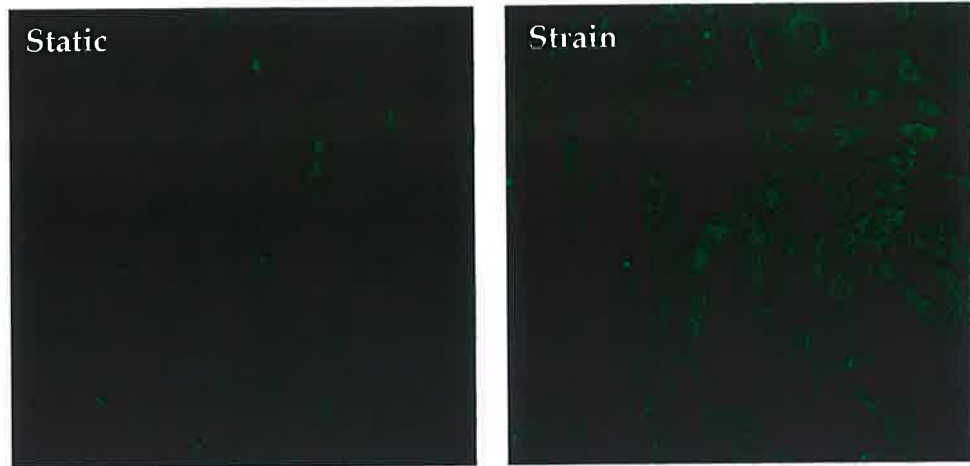


Fig. 55 Immunocytochemical staining of HVSMC's shows enhanced expression of myosin protein in cells subjected to 10% cyclic strain as compared to static cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

Smoothelin

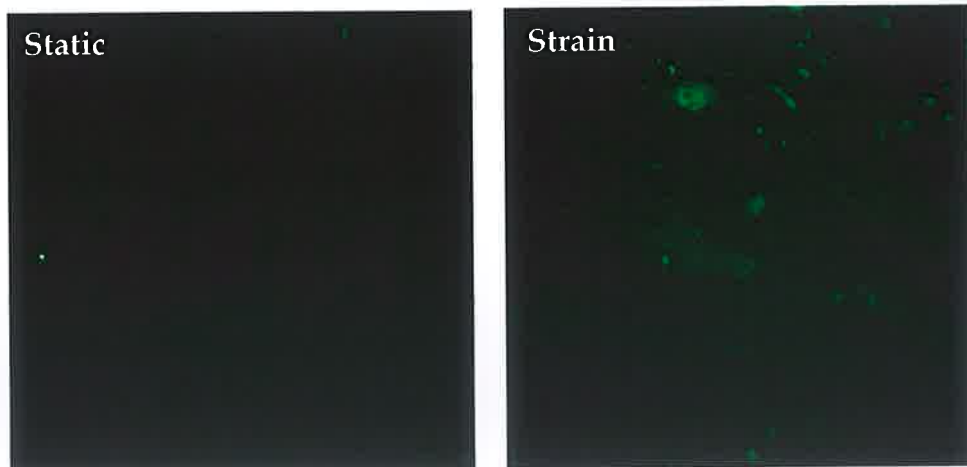


Fig. 56 Immunocytochemical staining of HVSMC's shows enhanced expression of smoothelin protein in cells subjected to 10% cyclic strain as compared to static cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

7.2 Cyclic strain induces changes in Notch 1 and Notch 3 IC protein expression

To determine the effect of physiological cyclic strain on Notch 1 and 3 IC protein expression, HVSMC were subjected to 10% cyclic strain for 24 hours and Notch 1 and 3 IC protein expression was analyzed using Western blot analysis and Immunocytochemical staining. 26% down-regulation of Notch 1 IC protein expression in cells subjected to cyclic strain was observed as compared to static cells (Fig. 57). These results were further confirmed by Immunocytochemical staining (Fig. 59).

A 23% down-regulation of Notch 3 IC protein expression was also observed in strained cells, as monitored by Western blot (Fig. 58). Immunocytochemistry confirmed these results as seen in Fig. 60.

Exposure of HVSMC to cyclic strain was described in section 5.1.2. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6.

The results are as follows:

7.2.1 Western blot analysis

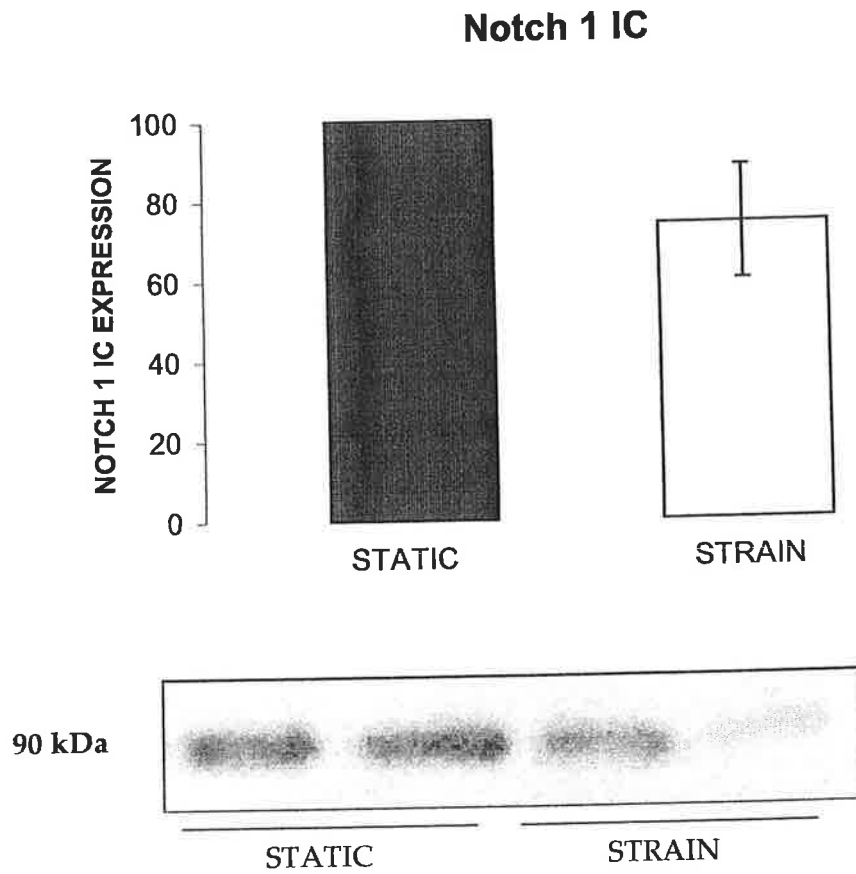


Fig. 57 Inset shows a representative graph displaying 26% down-regulation of Notch 1 IC protein expression in HVSMC in response to cyclic strain (10%, 24 hours) as compared to static cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

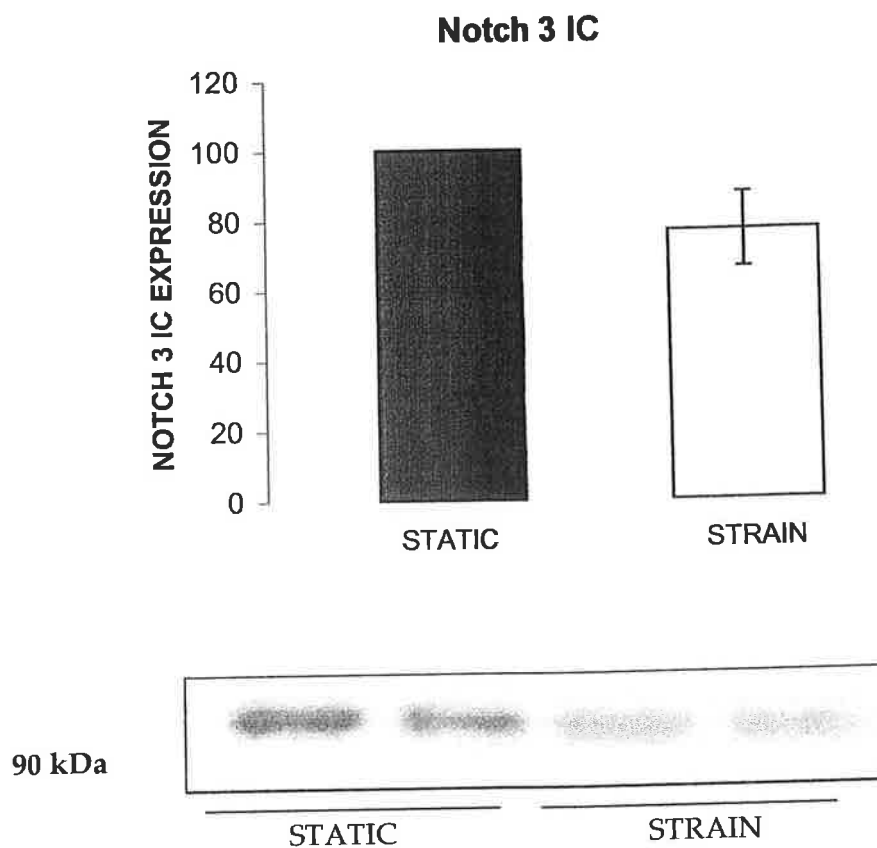


Fig. 58 Inset shows a representative graph displaying 23% down-regulation of Notch 3 IC protein expression in HVSMC in response to cyclic strain (10%, 24 hours) as compared to static cells. Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

7.2.2 Immunocytochemical staining

Notch 1 IC

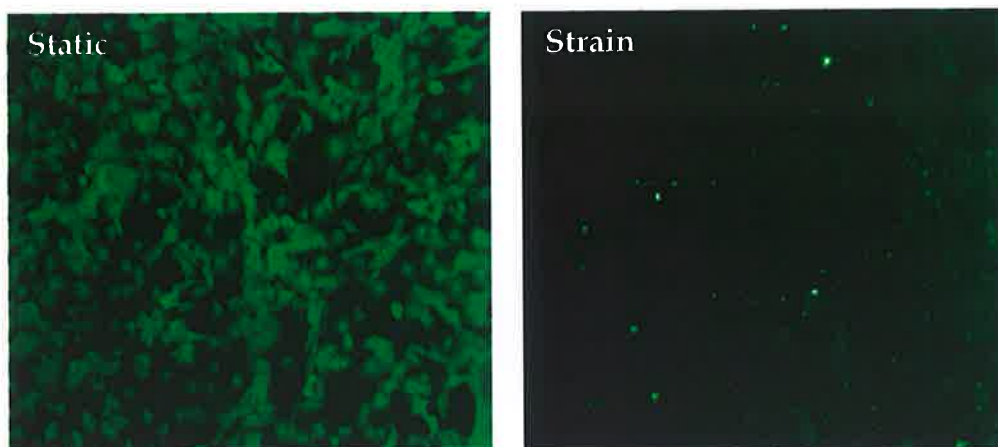


Fig. 59 Immunocytochemical staining of HVSMC's shows decreased expression of Notch 1 IC protein in cells subjected to 10% cyclic strain as compared to static cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

Notch 3 IC

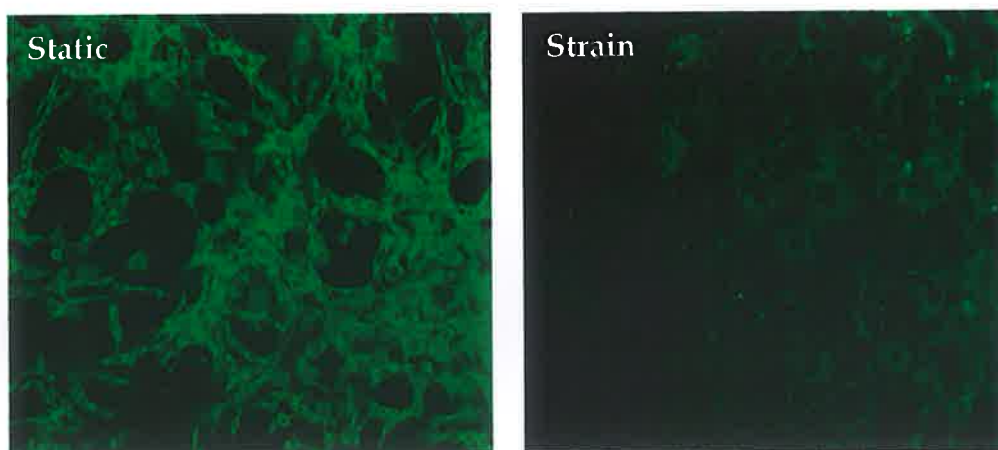


Fig. 60 Immunocytochemical staining of HVSMC's shows decreased expression of Notch 3 IC protein in cells subjected to 10% cyclic strain as compared to static cells. There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

7.3 Cyclic strain up-regulates differentiation of HVSMC by inhibition of Notch activity

To prove the hypothesis that Notch signalling dictates, in part, VSMC differentiation in response to mechanical stimuli, HVSMC were transfected with Notch inhibitor, RPMS-1, and subsequently, subjected to 10% cyclic strain for 24 hr. The protein expression of α -actin, calponin and myosin was then analyzed in these cells and compared to control cells.

Western blot analysis and Immunocytochemical staining revealed that the highest level of differentiation marker protein expression was in cells subjected to 10% cyclic strain and transfected with RPMS-1.

36% up-regulation of α -actin expression in RPMS-1-transfected strained cells (RPMS-1, strain) as compared to mock transfected, strained cells (mock, strain) and 96% up-regulation of this protein as compared to mock transfected unstrained cells (mock, static) (Fig.61).

Similar results were obtained for calponin and myosin. 61% up-regulation of calponin expression in RPMS-1-transfected strained cells (RPMS-1, strain) as compared to mock-transfected cells (MOCK, strain) and 138% up-regulation of this protein as compared to mock transfected unstrained cells (MOCK, static) was observed (Fig. 62).

There was a 34% up-regulation of expression of myosin protein in RPMS-1-transfected strained cells as compared to mock transfected cells (MOCK, 10%

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strained) and 135% up-regulation as compared to mock-transfected unstrained cells (MOCK, static) (Fig. 63). Results obtained by Western blot analysis were further confirmed by Immunocytochemical staining for each individual protein marker (Fig. 64-66). Exposure of HVSMC to cyclic strain was described in section 5.1.2. The whole cell lysates were prepared as described in section 5.4.1.1. SDS PAGE and Western blot analysis were carried out as described in section 5.4. Immunocytochemistry was carried out as described in section 5.6.

The results are as follows:

7.3.1 Western blot analysis

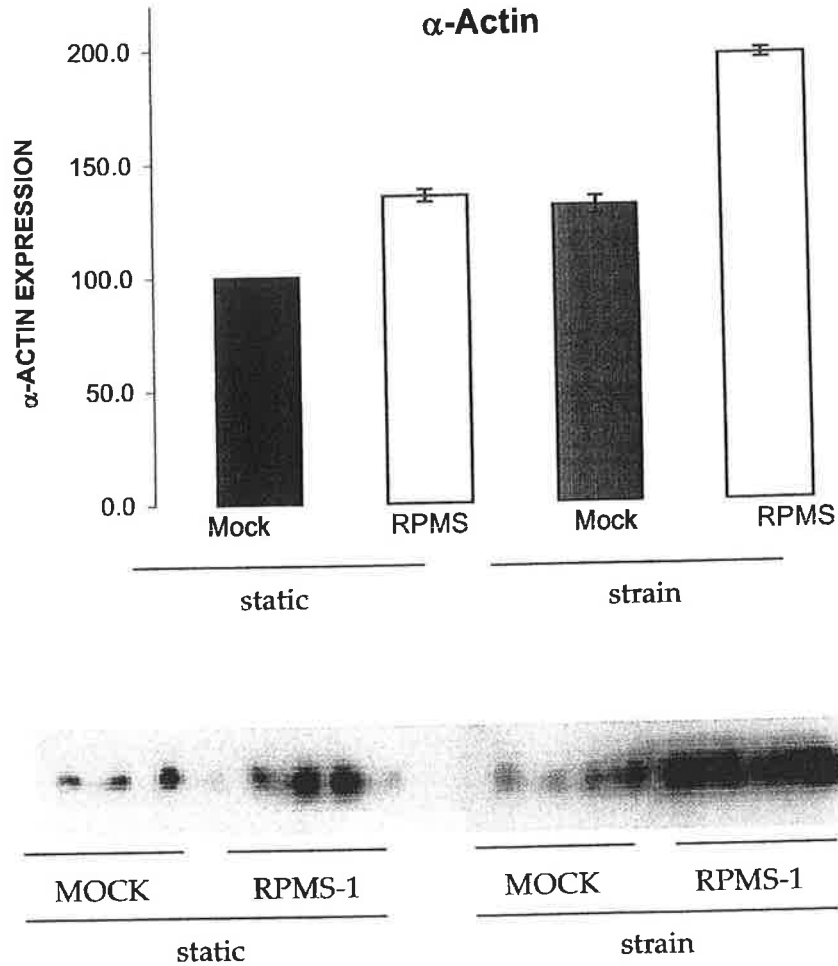


Fig. 61 Inset shows a representative graph displaying 36% up-regulation of α -actin expression in RPMS-1-transfected strained cells as compared to mock transfected strained cells (MOCK, 10% strained) and 96% up-regulation of this protein as compared to mock transfected, unstrained cells (MOCK, static). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

RESULTS

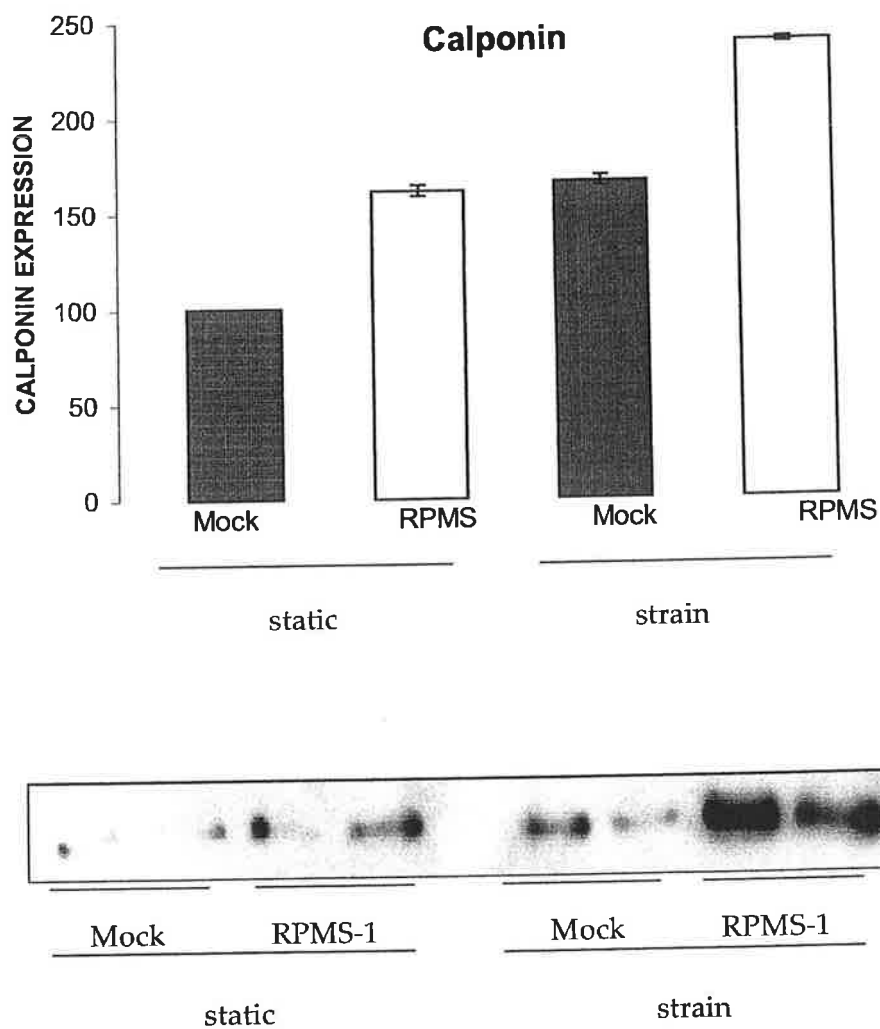


Fig. 62 Inset shows a representative graph displaying 61% up-regulation of calponin expression in RPMS-1-transfected strained cells as compared to mock transfected, strained cells (MOCK, 10% strained) and 138% up-regulation of this protein as compared to mock transfected, unstrained cells (MOCK, static). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

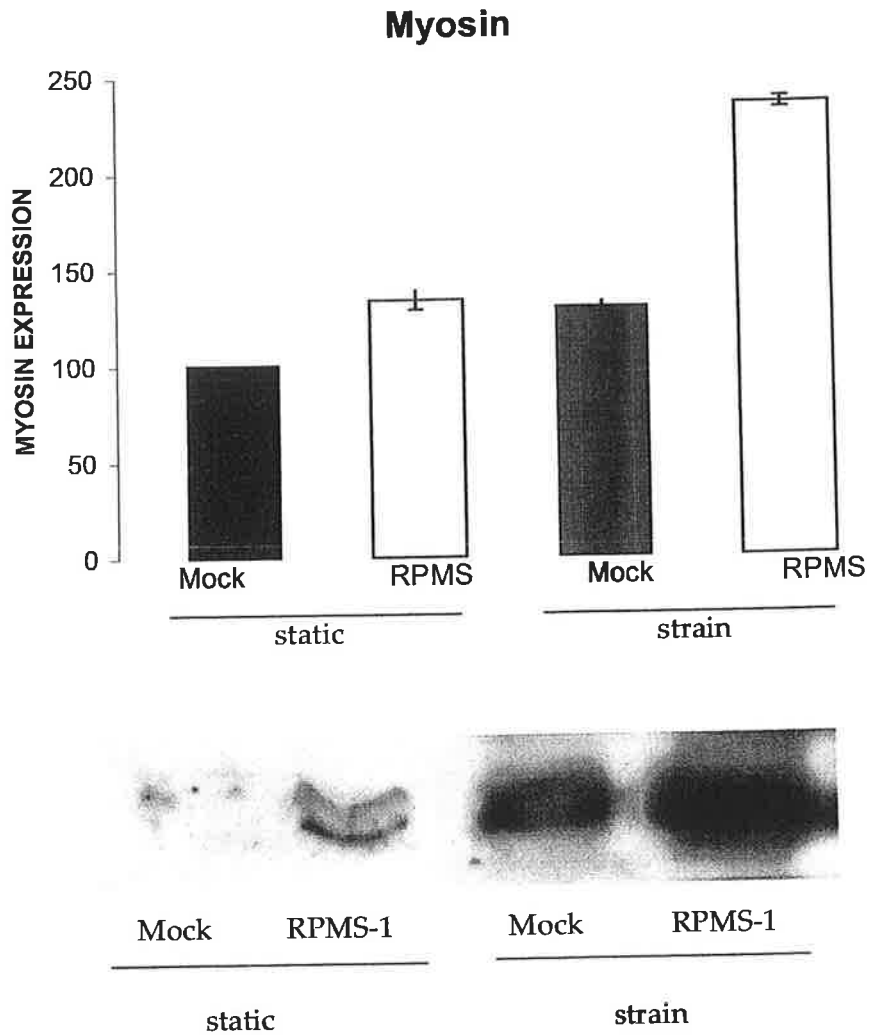


Fig. 63 Inset shows a representative graph displaying 34% up-regulation of myosin expression in RPMS-1-transfected strained cells as compared to mock transfected, strained cells (MOCK, 10% strained) and 135% up-regulation of this protein as compared to mock-transfected, unstrained cells (MOCK, static). Equal protein was loaded and confirmed by staining blot with Ponceau S stain. Data presented is representative of 3 individual experiments.

7.3.2 Immunocytochemical staining

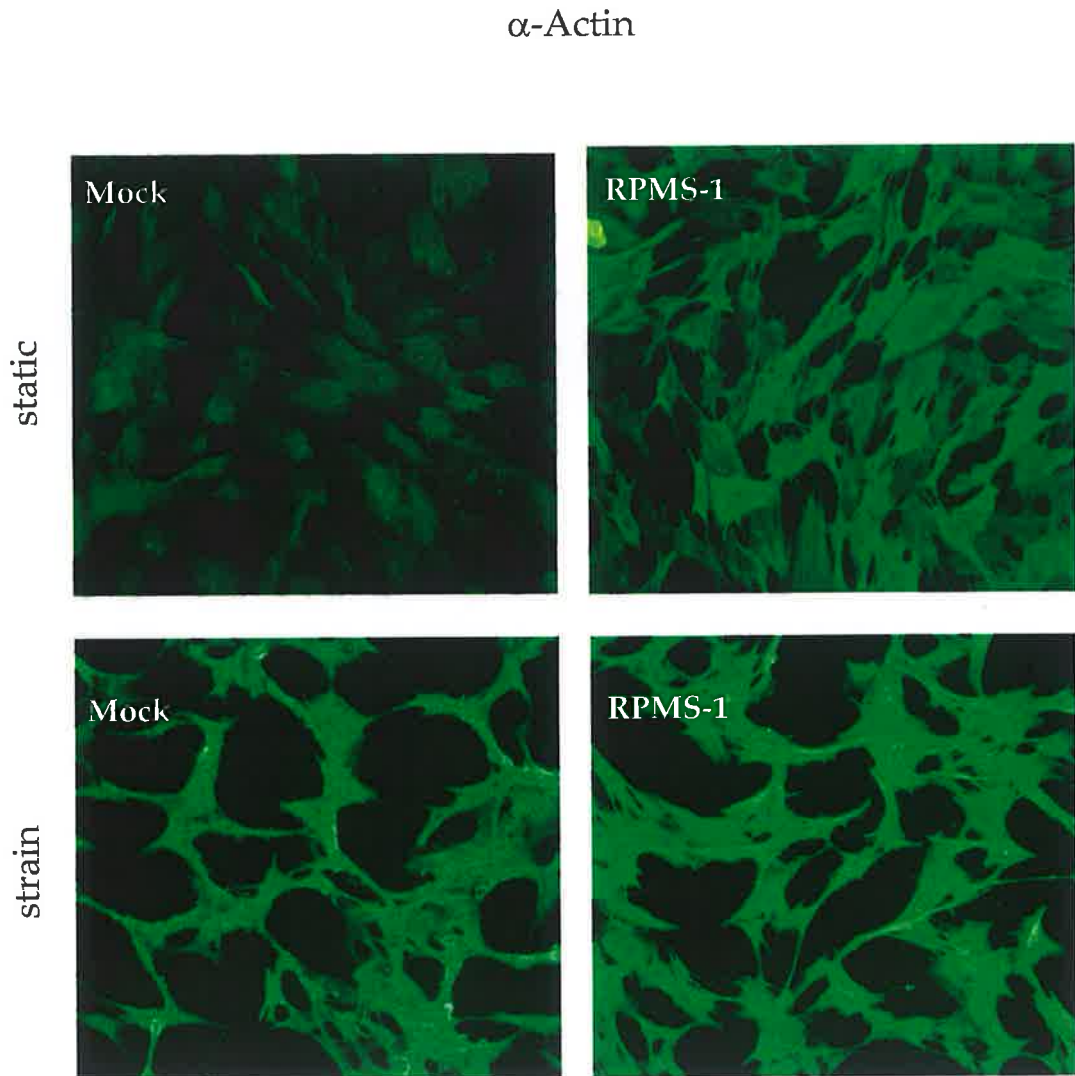


Fig. 64 Immunocytochemical staining of HVSMC's shows the highest expression of α -actin protein in cells transfected with RPMS-1 and subjected to 10% cyclic strain (RPMS-1, strain). α -Actin protein level in mock transfected, strained cells (MOCK, strain) is comparable to RPMS-1 transfected static cells (RPMS-1, static). There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

Calponin

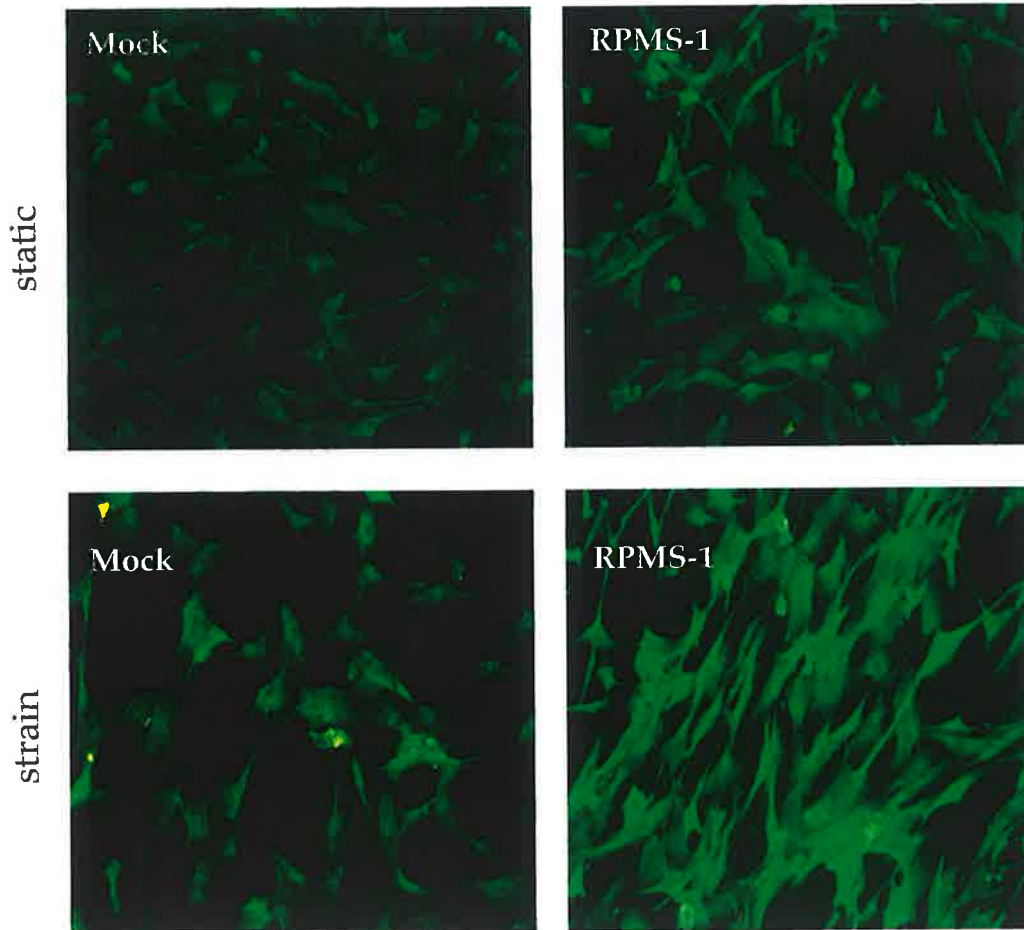


Fig. 65 Immunocytochemical staining of HVSMC's shows the highest expression of calponin protein in cells transfected with RPMS-1 and subjected to 10% cyclic strain (RPMS-1, strain). Calponin protein level in mock transfected, strained cells (MOCK, strain) is comparable to RPMS-1 transfected static cells (RPMS-1, static). There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

Myosin

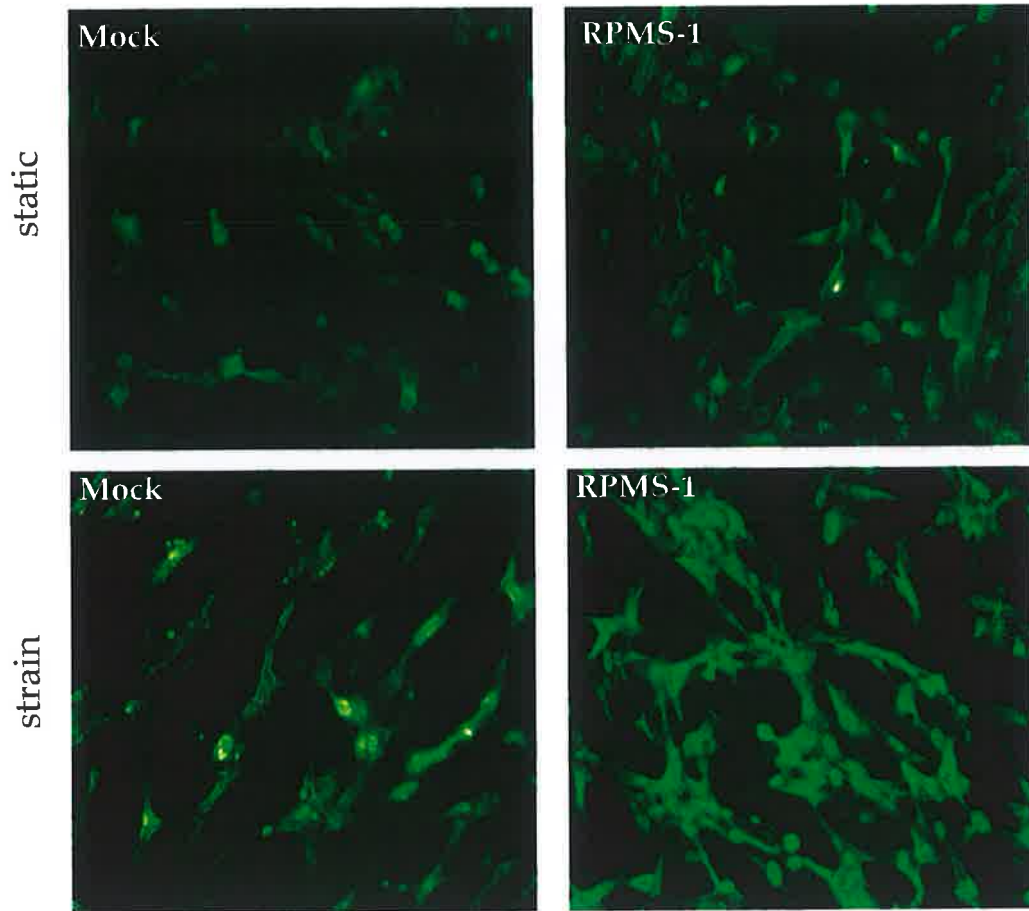


Fig. 66 Immunocytochemical staining of HVSMC's shows the highest expression of myosin protein in cells transfected with RPMS-1 and subjected to 10% cyclic strain (RPMS-1, strain). Myosin protein level in mock transfected, strained cells (MOCK, strain) is comparable to RPMS-1 transfected static cells (RPMS-1, static). There was no significant staining observed in cells treated with Alexa Fluor anti-mouse Ig alone (data not shown). Data presented is representative of 3 individual experiments.

7.4 DISCUSSION:

The above chapter includes data that evaluate the impact of cyclic mechanical strain on VSMC differentiation and its control via the Notch signalling pathway.

By using a Western blot technique it was demonstrated that application of mechanical strain dramatically increased protein expression of differentiation markers, α -actin, calponin and myosin. These results were further confirmed by Immunocytochemical staining of these cells. Smoothelin was undetectable by Western blot analysis but Immunocytochemical analysis revealed significant up-regulation of this protein expression in cells following mechanical strain as compared to static cells.

To determine the effect of mechanical stimuli on Notch 1 and 3 IC protein expression HVSMC were subjected to cyclic strain and the expression of both Notch receptors were measured. Western blot revealed marked down-regulation of Notch 1 and 3 IC protein expressions in strained cells as compared to static cells. These results were further confirmed by Immunocytochemical staining.

Subsequently, based on the above data that have shown the impact of cyclic mechanical strain on VSMC differentiation and Notch receptor proteins, it was demonstrated that cyclic strain up-regulated differentiation of HVSMC by inhibition of Notch activity. The Notch signaling pathway in HVSMC was inhibited by RPMS-1 and subjected them to cyclic strain. Western blot analysis

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revealed the increased level of α -actin, calponin and myosin proteins expression in cells subjected to cyclic strain and transfected with RPMS-1 as compared to control, untransfected, strained cells.

Taken together, this study shows for the first time the novel finding that the Notch signaling pathway dictates in part VSMC fate in response to mechanical stimuli following injury.

CONCLUSIONS

Vascular smooth muscle cell fate decisions play an important role in neointimal formation during the pathogenesis of hypertension, arteriosclerosis and arterial response to injury. The decision as to whether a VSMC differentiates, proliferates, migrates or undergoes apoptosis in response to mechanical stimuli is an obvious important determinant of vascular remodelling. However, how mechanical forces regulate these cell fate decisions remains poorly understood. The wall of blood vessels is an active organ formed by smooth muscle, endothelial and fibroblasts cells, which interact with each other and communicate in a "paracrine-autocrine" fashion [115]. The vasculature is able to sense changes within its environment, integrate these signals by intercellular communication, and change itself through the production of mediators influencing structure as well as function.

Vascular remodelling is an active process that occurs in response to long-term changes in hemodynamic conditions, but it may subsequently contribute to the pathophysiology of vascular diseases and circulatory disorders. It consists in important modifications in the vessel wall. Such modifications involve changes

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in at least four cellular processes - cell proliferation, cell differentiation, cell migration and apoptosis as well as production or degradation of extracellular matrix -- and is dependent on a dynamic interaction between locally generated growth factors, vasoactive substances, and hemodynamic stimuli [115].

Vascular cell fate decisions are hallmarks of phenotypically distinct SMC response to hemodynamic force-induced vascular remodelling following injury [131]. The cell fate decisions with regard to how a distinct VSMC phenotype responds to hemodynamic stimuli are unclear. Since these cell fate decisions are also apparent during vascular remodelling or morphogenesis, although in response to a different stimulus, it is believed that there is a similar arbiter that controls these cell fate decisions, namely the Notch signalling pathway [54]. Indeed, the Notch signalling pathway has been strongly implicated in vascular morphogenesis and remodelling of the embryonic vasculature [59, 111]. For example, alterations in Notch signalling have recently been reported in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) a condition that exhibits cerebral vascular remodelling [54, 60]. Notch receptor-ligand interaction is a highly conserved mechanism, originally described in *Drosophila*, that regulates inter-cell communication and directs individual cell fate decisions [110, 116]. Studies using constitutively activated Notch receptors missing their extracellular domains have shown that Notch signalling determines proliferation,

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differentiation and apoptosis in several cell types [54]. In addition, Notch receptor-ligand interactions are common during fibroblast growth factor induced angiogenesis suggesting a role of this pathway in regulating vascular development. For this reason, further analysis of the Notch signalling pathway following vascular injury may provide novel insights into the biology of VSMC. In this study the Flexercell™ Tension Strain Unit has been used to determine the effect of cyclic strain on VSMC fate and their control via the Notch signalling pathway.

Several previous reports have studied the expression of Notch components in adult vascular tissue and cells [60, 94, 95, 98, 99]. This study focused on the presence and over-expression of the intercellular domain (IC) of Notch 1 and Notch 3 receptors in human VSMC. The presence of both Notch 1 IC and 3 IC receptors has been detected in these cells, which provides evidence, in agreement with recent studies, of the presence of Notch in VSMC. The degree to which Notch IC was over-expressed following transfection with Notch 1 IC or Notch 3 IC has also been monitored. Moreover, the study revealed the cytoplasmic presence of Notch by using Immunocytochemistry. Notch seems to be localized mostly in cytoplasmatic area of human SMC's, which is contrary to the reports from other studies demonstrating nuclear localization of Notch [34, 67, 68].

CONCLUSIONS

Several reports demonstrated that constitutive expression of Notch 1 and 3 IC, also resulted in a significant increase in Notch target gene promoter activity in these cells [4, 34, 102]. It has been found that over-expression of Notch 1 IC and Notch 3 IC resulted in increased CBF-1/RBP-Jk-dependent promoter activity and Notch target gene mRNA levels [34]. Moreover, our lab have previously suggested that Notch 3 acts as an agonist of the CBF-1/RBP-Jk-dependent Notch signaling pathway [34], although other reports suggested that Notch 3 might act as an antagonist of the CBF-1/RBP-Jk-dependent Notch signaling pathway as they found Notch 3 IC repressing Notch 1-mediated activation through HES promoters [127]. Recent studies also demonstrated that Notch 1 IC and Notch 3 IC over-expression activate HRT-1 and Hes-5 promoter activity in VSMC through the CBF-1/RBP-Jk-dependent pathway [4, 34, 113] and that HRT-1, HRT-2 and Hes-5 are direct downstream target genes of Notch 1 and Notch 3 in VSMC.

Colleagues from the lab have also demonstrated that inhibition of CBF-1/RBP-Jk-dependent signaling using both pharmacological and molecular inhibitors resulted in a significant inhibition of Notch 1 and 3 IC-dependent Notch target gene promoter activity and receptor mRNA levels [34]. In normal conditions, after cleavage of full length Notch, the Notch IC domain is translocated to the Golgi apparatus and later to the nucleus. Brefeldin A inhibits translocation of Notch to the nucleus by disassembling the Golgi apparatus and Monensin prevents transfer of proteins from the Golgi to the plasma membrane [62].

CONCLUSIONS

These data suggest that expression of constitutively active Notch 1 and 3 IC, despite that it is already cleaved, require the Golgi apparatus to translocate to the nucleus since brefeldin A and monensin both inhibit Notch IC signaling events [34]. On the contrary, EBNA-2 (Epstein-Barr virus nuclear antigen 2)-induced activation of CBF-1/RBP-Jk-dependent promoter activity was unchanged by pretreatment with pharmacological inhibitors but was inhibited by co-expression with RPMS-1 (RPMS-1 has previously been shown to bind directly to the CBF-1 nuclear complex and inhibit Notch IC CBF-1/RBP-Jk-dependent promoter activity). These data suggest that the inhibition of Notch 1 and 3 IC activation of CBF-1/RBP-Jk-dependent promoters with brefeldin A was not due to impairment of nuclear transcription, but indicates an inhibition of Notch IC translocation to the nucleus [34]

Although many studies have shown the effect of Notch signaling on cell differentiation little is known about the influence of the Notch signaling pathway on differentiation of VSMC.

By using standard *in vitro* technologies, Western blot and immunocytochemical staining, VSMC differentiation following transfection with Notch 1 IC and 3 IC was assessed. Human VSMC expressed differentiated cell markers associated with contractile function (α -actin, calponin, myosin and smoothelin). Further analysis of the effect of Notch 1 IC and Notch 3 IC overexpression in modulating cell differentiation revealed that Notch significantly down-

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regulates the expression of α -actin, calponin, myosin and smoothelin in VSMC. Smoothelin was very difficult to detect by Western blot, thus the determination of the down-regulation of this marker following overexpression of Notch IC was very problematic and didn't bring expected results. However we successfully demonstrated the presence and down-regulation of smoothelin protein by immunocytochemical analysis as well as the presence of mRNA in cells by using QRT-PCR. Problems with detection of this particular marker were probably associated with incompatibility of the antibodies used in this study with Western blot technique but the same antibodies worked relatively well with immunocytochemistry. Another possible explanation is that as smoothelin is a poorly expressed protein in the cell it could be easily lost during the multi-step procedure of Western blot analysis or during protein lysate preparation. However, immunocytochemical analysis, although not as quantitative as Western blot analysis, clearly demonstrated the negative effect of the Notch signalling pathway on the expression of smoothelin, appearing as a down-regulation of this marker expression in cells following transfection with Notch IC.

In a parallel study, overexpression of Notch 1 IC promoted clonal proliferation in the same cells. In addition, the expression of proliferating cell nuclear antigen (pCNA), a marker for cell proliferation in SMC, was enhanced in cells following overexpression of Notch 1 IC compared with the mock control.

CONCLUSIONS

Previous reports have demonstrated that Notch signaling acts downstream of VEGF during arterial differentiation [123] and that HAND1 (the basic helix-loop-helix transcription factor) functions in part by regulating the Notch pathway during differentiation. *Hand1* may regulate the Notch pathway through enhanced expression of VEGF or by direct regulation of the Notch genes [124]. HRT-1, -2, and -3 are CBF-1/RBP-Jk-dependent Notch target genes downstream of Notch 1 and Notch 3 activation [125] and Notch 3 receptor expression is enhanced in neointimal formation in the rat balloon injury [4, 97]. However, there is no evidence suggesting that Notch, acting through CBF-1/RBP-Jk-dependent mechanisms, was responsible for changes in differentiation of SMC. This finding is important because of other published data suggesting additional signaling pathway(s) and that Notch acts independently of CBF-1/RBP-Jk, with evidence that proteolysis of the Notch receptor is not necessary [126]. This study presents significant new evidence that activation of Notch IC modulates SMC differentiation through CBF-1/RBP-Jk-dependent mechanisms. Western blot analysis, immunocytochemistry and QRT-PCR have been used in this study to determine the effects of inhibiting CBF-1/RBP-Jk-regulated promoter activity on the expression of differentiation marker protein and mRNA in VSMC. Overexpression of Notch 1 IC or Notch 3 IC revealed a significant decrease in differentiation marker protein and mRNA level, an effect that was significantly

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inhibited following co-expression of RPMS-1 or delta RAM deleted Notch (ED4), a selective inhibitor of CBF-1/SKIP interaction.

Interestingly, RPMS-1 or ED4 alone doesn't seem to significantly inhibit endogenous Notch as comparable protein levels of differentiation markers in RPMS-1, ED4 and mock transfected cells were observed. However, QRTPCR data for actin mRNA showed a significant inhibition of native Notch and resulted in enhanced expression of actin in RPMS-1 transfected cells in comparison to mock-transfected cells. Such a differences between mRNA level and protein expression can be explained easily. It needs to be remembered that eexpression data have their own limitations. mRNA levels may not always reflect protein levels and expression of a protein may not always have a physiological consequence. mRNA production precedes that of protein and if they are examined at the same time they do not always mirror each other as not always all the mRNA is to be transcribed into protein.

Surprisingly, inhibition of CBF-1/RBP-Jk-dependent signaling using pharmacological intervention (brefeldin A) had no effect on the expression of Notch 1 and 3 IC or on the expression of differentiation markers. Notch-induced inhibition of differentiation seemed to be unaffected by pre-treatment with brefeldin A. These data would suggest that inhibition of Notch-induced changes in differentiation markers were due to impairment of nuclear transcriptional machinery but was not dependent on Notch IC translocation to the nucleus. In contrast, data recently obtained by our lab demonstrated that

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expression of Notch 1 and 3 IC requires the Golgi trafficking mechanisms to translocate to the nucleus since brefeldin A inhibited Notch IC signalling [34]. It is noteworthy that in that study rat VSMC were utilised, while this current study use human VSMC, which may account for the conflicting results, however, concrete verification of the role of trafficking inhibitors in Notch IC translocation in human SMC needs to be further examined. Despite this, the data obtained above describes for the first time that regulation of Notch receptor signaling following inhibition of CBF-1/RBP-Jk-dependent Notch signaling or constitutive expression of functionally active Notch IC resulted in fundamental changes in VSMC differentiation.

Similar results for proliferation, migration and apoptosis have been demonstrated by colleagues in a parallel study [34].

Several experimental models have been developed to study effects of mechanical forces on cultured VSMC. Models such as the Flexercell Strain Unit allow the examination of the effects of mechanical strain on cultured VSMC, both in isolation and in combination with other important factors such as individual growth factors and extracellular matrices [31]

The current study evaluates the impact of cyclic mechanical strain on the expression of smooth muscle differentiation markers in cultured adult human VSMC.

CONCLUSIONS

This study found that application of mechanical strain increased differentiation marker protein expression in these cells, which is in agreement with other studies, which documented an increase of both α -actin protein expression and its promoter activity following mechanical strain [31].

Hautmann *et al.* demonstrated that induction of SM- α -actin expression by mechanical strain in adult VSMC is mediated through activation of JNK and p38, members of the MA kinase [128]. It appears that the physiologic response of cells requires collaboration between specific signaling pathways and mechanical forces.

There is a speculation that Notch, as one of the potential signalling pathway mediating the numerous cell fates during development, may play a role in the transduction of mechanical stimuli in VSMC. Therefore this study investigated the putative role of the Notch signaling pathway in mediating the strain-dependent increase in differentiation marker activities observed in VSMC.

The goal of this study was to determine the role of Notch signaling in controlling cyclic strain-induced VSMC differentiation. This study shows for the first time the novel finding that the Notch signaling pathway dictates in part VSMC fate in response to mechanical stimuli following injury. Vascular injury has been modeled *in vitro* by examining the effect of cyclic strain on VSMC.

This study demonstrates substantial decrease in the expression of endogenous Notch 1 IC and Notch 3 IC in VSMC in response to mechanical strain, which is

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associated with a considerable increase in differentiation marker protein expression. How Notch signaling controls mechanical strain-induced VSMC differentiation is not yet clearly identified. Downstream target genes of Notch signaling may directly or indirectly stimulate promoter activity of differentiation marker genes. Discovery of common sequence elements between differentiation marker promoters and Notch target genes could bring this hypothesis closer to the truth.

Several recent data demonstrated that myocardin plays a critical role in regulating the expression of genes encoding SMC contractile proteins [129] and that it is stimulated by angiotensin II, a known α -actin-stimulating factor [31]. Moreover, Wang *et al.* suggested that increased expression of myocardin by Ang II might enhance interaction of myocardin with SRF dimers bound to the two 5' CArG elements of the SM α -actin promoter (ie, CArGs B and A), and thereby stimulate SM α -actin gene transcription [129].

Whether the Notch signaling pathway, with or without other signaling pathways, contributes to myocardin or other factor-induced stimulation of SM α -actin still remains unknown. However, implications of Notch signalling in the cascade of behavioral changes the cell undergoes during development or following vascular injury, keep this signaling pathway in a center of interest and as a potential arbiter of vascular cell fate decisions.

CONCLUSIONS

In conclusion, this study focused on the presence and the over-expression of the IC portion of the receptor, CBF-1/RBP-Jk dependent signaling and investigated the cytoplasmatic localization of Notch IC in HVSMC. In addition, this study presents the novel finding that both Notch 1 IC and Notch 3 IC signal via a CBF-1/RBP-Jk dependent pathway in HVSMC and that Notch signaling dictates in part vascular smooth muscle cell differentiation in response to mechanical stimuli following injury. Although it was not possible to address many besetting questions, future studies will shed new light on this undoubtedly exciting theory.

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