MECHANISMS OF NOTCH4-INDUCED INHIBITION OF ENDOTHELIAL SPROUTING

by

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Abstract

Notch proteins comprise a family of transmembrane receptors. Ligand activation of Notch releases an intracellular region of the receptor that translocates to the nucleus and regulates transcription through interaction with the DNA-binding protein CBF1. CBF1-independent Notch signalling has also been described. Previously, the human Notch4 intracellular region (N4IC) was shown to inhibit endothelial sprouting *in vitro* and angiogenesis *in vivo*. The objective of this study was to determine the N4IC domains required for inhibition of endothelial sprouting and to resolve whether this inhibition involves CBF1-dependent signalling.

N4IC contains a RAM domain and six ankyrin repeats for protein binding and a C-terminal region (CT) that has poorly defined function. The necessity of each domain for Notch4 activity in endothelial cells was analysed using human dermal microvascular endothelial cells (HMEC-1) expressing N4IC or N4IC deletion constructs. As shown by immunofluorescent staining, mutants lacking the RAM domain had reduced nuclear localisation. Nuclear targeting was restored by fusion of a viral nuclear localisation signal. The angiogenic effect of the N4IC mutants was quantitated using an *in vitro* endothelial sprouting assay. Deletion of the ankyrin domain, but not the RAM or CT, abrogated the inhibition of FGF-2- and VEGF-induced sprouting, while the ankyrin repeats alone partially blocked sprouting. N4IC decreased VEGF Receptor 2 (VEGFR2) mRNA expression but did not alter FGF Receptor 1 (FGFR1) levels. Conversely, constructs lacking the RAM or CT increased VEGFR2 mRNA compared to controls. Therefore, Notch4 appears to block endothelial sprouting through mechanisms other than mere downregulation of VEGFR2 and FGFR1. The ankyrin repeats, but not the RAM or CT, were also required for upregulation of CBF1-dependent gene expression as shown by luciferase reporter assays and RT-PCR for endogenous CBF1 targets. The ankyrin domain alone

was sufficient to upregulate some, but not all, CBF1-dependent genes. Fusion of CBF1 to a viral transactivation domain created a construct that induced expression of target genes independently of N4IC. This constitutively-active CBF1 significantly inhibited HMEC-1 sprouting, but not as strongly as N4IC. Therefore, the inhibition of endothelial sprouting by N4IC requires the ankyrin repeats and appears to involve CBF1-dependent and -independent signalling.

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List of Abbreviations

Ang	Angiopoietin
bHLH	basic helix-loop-helix
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF1	C promoter binding factor 1
CBP/p300	CREB-binding protein
CIR	CBF1-interacting corepressor
CSL	CBF1/Su(H)/Lag-1
CT	Notch C-terminal region
Dll	Delta-like
DSL	Delta/Serrate/Lag-2
EBNA2	Epstein-Barr virus nuclear antigen 2
EGF-like	Epidermal growth factor-like
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
E(spl)	Enhancer of split
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN5	General control of amino acid synthesis 5
HA	Hemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HES	Hairy and enhancer of split
HIAEC	Human iliac aortic endothelial cells
HMEC-1	Human dermal microvascular endothelial cells
HRT	Hairy-related transcription factor
HUVEC	Human umbilical vein endothelial cells
KSHV	Kaposi's sarcoma-associated herpesvirus
MAML	Mastermind-like
MASH1	Mammalian achaete-scute homologue 1
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
N1IC	Notch1 intracellular region
N4IC	Notch4 intracellular region
NCOR	Nuclear receptor corepressor
NIC	Notch intracellular region
NLS	Nuclear localisation signal
PCAF	p300/CBP-associated factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
PEST	proline-glutamate-serine-threonine-rich
RAM	RBP-Jκ associated module

RBP-Jκ	Recombination signal binding protein at the Jκ site
RTA	Replication and transcription activator protein
SAP30	Sin3-associated polypeptide
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SKIP	Ski-interacting protein
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
Su(H)	Suppressor of hairless
TACE	TNF-alpha converting enzyme
TAD	Transactivation domain
T-ALL	T cell acute lymphoblastic leukemia
TAN-1	Translocation-associated notch homologue-1
Tie	Tyrosine kinase with immunoglobulin and EGF factor homology domains
TLE	Transducin-like enhancer of split
TSA	Trichostatin A
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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Chapter 1

Introduction

1.1.1 The Notch Family of Receptors

1.1.1 Metazoan Notch Receptors

Notch proteins are a highly conserved family of transmembrane receptors involved in intercellular signalling that controls a broad range of cellular functions (Artavanis-Tsakonas et al., 1999). Generally, these receptors relay signals that regulate the progression of cells into a differentiated state. Notch was originally identified in *Drosophila melanogaster* when partial loss of its function resulted in flies with notches at their wing margin (Artavanis-Tsakonas et al., 1999; Kidd et al., 1986; Wharton et al., 1985). The receptor was shown to play a role in inhibiting cell differentiation into the neuronal lineage as Notch-null mutants were embryonic lethal due to an expansion of neuroblasts at the expense of epidermal precursors. Since then, Notch has proven essential for the development of organs from all three primary germ layers (Hartenstein et al., 1992) and appears to function in all metazoans, with extensive research done in mammals, *Xenopus* (frogs), zebrafish, *Drosophila* (flies), and *Caernorhabditus elegans* (worms) (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Greenwald, 1998; Gridley, 1997).

1.1.2 Mammalian Notch Receptors

Four members of the mammalian Notch family have been identified and are most commonly referred to as Notch1 (del Amo et al., 1993; Ellisen et al., 1991), Notch2 (Weinmaster et al., 1992), Notch3 (Lardelli et al., 1994), and Notch4 (Li et al., 1998; Uyttendaele et al., 1996). Of the four, Notch1 was the first identified and has been the most extensively studied. Two additional receptors, Notch5 and Notch6, have been discovered in zebrafish (Sawada et al.,

2000), although corresponding mammalian homologues have not been identified. In mammals, the Notch signalling pathway functions in tissue development and maintenance while regulating a multitude of cellular processes including differentiation (Kato et al., 1997; Kopan et al., 1994; Nye et al., 1994; Pui et al., 1999; Radtke et al., 1999), survival/apoptosis (Deftos et al., 1998; Liu et al., 2003; Pear et al., 1996; Shelly et al., 1999), proliferation (Liu et al., 2003; Redmond et al., 2000; Taylor et al., 2002), adhesion/migration (Leong et al., 2002), and angiogenesis (Krebs et al., 2000; Leong et al., 2002; Uyttendaele et al., 2001).

1.1.3 The Notch Pathway and Disease

Notch function has been implicated in a variety of diseases. In particular, the mammalian Notch1 and Notch4 loci were originally identified as oncogenes. The Notch1 gene was discovered at the breakpoint of a chromosomal translocation commonly present in T-cell acute lymphoblastic leukemias (T-ALL) (Ellisen et al., 1991). These translocations produced a truncated, constitutively active form of the protein named TAN-1 (Translocation-associated notch homologue-1). Similarly, the Notch4 gene locus was discovered as a frequent insertion site for the mouse mammary tumour virus (MMTV) in mouse mammary carcinomas (Gallahan and Callahan, 1987; Uyttendaele et al., 1996). These MMTV insertions created a truncated gene encoding an activated intracellular form of Notch4 known as Int-3. Thus, Notch4 was originally termed Notch4/Int-3; however, Int-3 is now reserved specifically for the truncated form created by the insertion of MMTV. Truncated, constitutively active Notch4 was subsequently proven to cause tumours in transgenic mice. Notch1 and Notch2 have also been confirmed to induce neoplastic transformations *in vitro* and *in vivo* (Capobianco et al., 1997; Pear et al., 1996; Rohn et al., 1996). More recently, Notch1 has been identified as a tumour suppressor in skin. This was demonstrated when inactivation of Notch1 in the epidermis of mice led to the development

of skin tumours and facilitated chemical-induced skin carcinogenesis (Nicolas et al., 2003; Rangarajan et al., 2001).

The Notch pathway also plays a role in diseases other than cancer. Notch3 mutations are associated with a human familial syndrome known as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Joutel et al., 1996). This disease is characterised by vascular degeneration that causes recurrent strokes and dementia. The developmental disorder Alagille Syndrome is caused by loss-of-function mutations in the Notch ligand Jagged1 and is characterised by abnormalities of the liver, heart, eye, skeleton, and kidney (Li et al., 1997; Oda et al., 1997).

1.2 Notch Structure, Activation and Regulation

1.2.1 The Structures of Notch Receptors

Functional Notch receptors are heterodimers formed by an extracellular subunit and a plasma membrane spanning subunit that includes a short extracellular portion and a longer intracellular region (Weinmaster, 1997). These two subunits are linked by an extracellular, non-covalent bond that is dependent upon the presence of calcium (Rand et al., 2000). The extracellular subunit of mammalian Notch members contains 29 - 36 epidermal growth factor (EGF)-like repeats that are involved in ligand binding (Weinmaster, 1997). C-terminal to the EGF-like repeats are three cysteine-rich Lin-12/Notch repeats, where Lin-12 refers to a Notch receptor in *C. elegans*, whose two Notch homologues are known as Lin-12 and Glp-1. The Lin-12/Notch repeat motif is unique to Notch family members and is required for binding calcium to maintain interaction with the transmembrane chain (Rand et al., 2000). A pair of conserved cysteine residues on the extracellular side of the membrane subunit is also important in this regard (Lieber et al., 1993).

The Notch intracellular region (NIC) has an N-terminal RAM (RBP-Jκ associated module, sometimes referred to as RAM23) domain that is unique to the Notch family and is defined by its high affinity binding to the transcriptional regulator RBP-Jκ, also known as CBF1 (Tamura et al., 1995). Downstream of the RAM domain are six tandem ankyrin/cdc10 repeats (hereafter referred to collectively as the ankyrin repeats or the ankyrin domain) that also function in mediating protein-protein interactions (Kurooka and Honjo, 2000; Wu et al., 2000; Zhou et al., 2000a; Zhou et al., 2000b). It has been proposed that Notch receptors have a seventh ankyrin motif immediately downstream of the six established tandem repeats (Jeffries et al., 2002). Ankyrin repeat motifs have been identified in over 400 proteins having diverse functions and are found in organisms ranging from viruses to humans (Sedgwick and Smerdon, 1999). These motifs are tandemly repeated with each repeat typically about 33 amino acids long. Individual ankyrin repeats fold into a defined β-hairpin-helix-loop-helix structure but do not recognise a consensus sequence or structural motif (Sedgwick and Smerdon, 1999). Near the C-terminus of NIC is a PEST (proline-glutamate-serine-threonine-rich) domain that is involved in protein turnover (Oberg et al., 2001; Wu et al., 2001). Although the mechanisms for PEST-mediated degradation are not fully understood, the serine and threonine residues within these domains are often targets for phosphorylation. Once phosphorylated, these PEST sequences can be bound by ubiquitin ligases which subsequently ubiquitinate other regions of the target protein, directing it for proteolytic degradation (Hershko and Ciechanover, 1998).

There are notable structural differences between the members of the mammalian Notch family (Figure 1). Notch4 is the smallest member, having an abbreviated extracellular subunit and intracellular C-terminus. It contains 29 EGF-like repeats compared to 34 for Notch3 and 36

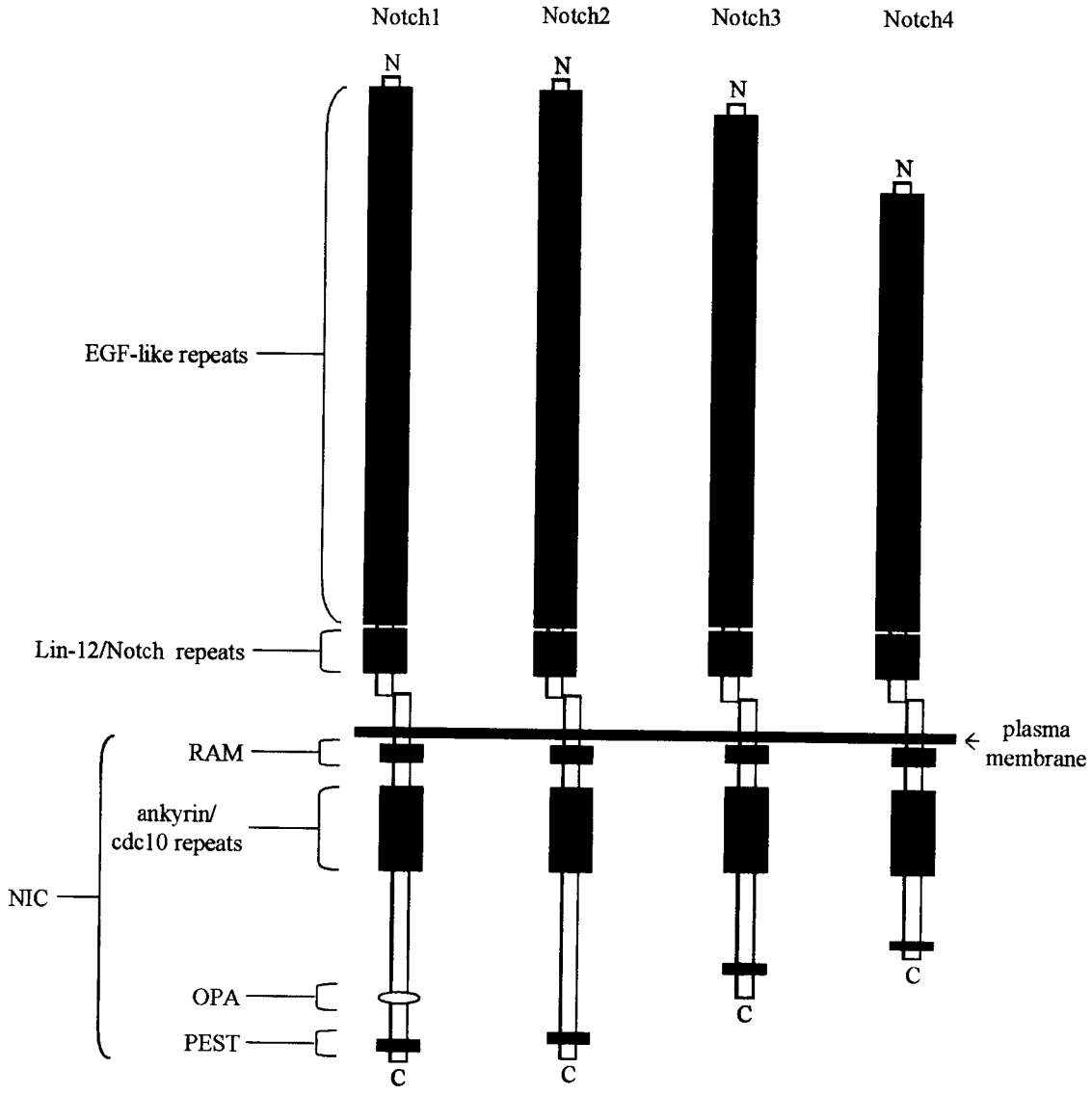


FIGURE 1. Protein structure diagrams of the mammalian Notch receptors. Protein structure diagrams of mammalian Notch1, Notch2, Notch3, and Notch4 are depicted. The full-length receptors are shown in their heterodimeric state at the plasma membrane before engagement by ligand. The N-terminus (N) and C-terminus (C) of each receptor are labelled. The major functional domains are identified and include 29-36 EGF-like repeats, 3 Lin-12/Notch repeats, the RBP-Jk Associated Module (RAM), 6 ankyrin/cdc10 repeats, the OPA (glutamine-rich), and the PEST (proline-glutamate-serine-threonine-rich) motifs. Notch1 and Notch2 have 36 EGF-like repeats, Notch3 has 34, and Notch4 has 29. Notch1 is the only mammalian member with an OPA domain. NIC denotes the intracellular region of each receptor.

for each of Notch1, Notch2, and *Drosophila* Notch. Furthermore, based on sequence identity, the Notch4 protein is the most divergent among family members (Li et al., 1998). Figure 2 illustrates an amino acid sequence alignment of the intracellular domains of the human Notch receptors. All four homologues share greatest sequence similarity in their ankyrin repeats and are most divergent in the region C-terminal to the ankyrin repeats (CT) (Li et al., 1998). The glutamine-rich OPA domain found in *Drosophila* Notch is conserved in Notch1, but not the other mammalian members. The OPA domain is located between the ankyrin repeats and PEST domain and has undetermined function.

1.2.2 The Structures of Notch Ligands

Notch ligands belong to the Delta/Serrate/Lag-2 (DSL) family of proteins and include Delta-like1 (Dll1) (Bettenhausen et al., 1995; Han et al., 2000), Dll3 (Bulman et al., 2000; Dunwoodie et al., 1997), Dll4 (Shutter et al., 2000), Jagged1 (Lindsell et al., 1995; Oda et al., 1997), and Jagged2 (Deng et al., 2000) in mammals. These ligands are divided into Delta family and Serrate family subclasses, of which Jagged belongs to the latter class (Fleming, 1998). Members of both subclasses share structural features including a transmembrane domain, EGF-like repeats, and a DSL domain that is unique to Notch ligands and is required for binding to Notch (Fleming, 1998; Muskavitch, 1994). The Serrate family is distinguished from the Delta family by its cysteine-rich domain in the extracellular region and sequence insertions that interrupt some of its EGF-like repeats.

1.2.3 Ligand Activation of Notch

Notch receptors are activated by binding ligands on the surface of neighbouring cells (Muskavitch, 1994; Shimizu et al., 2000). In a process known as lateral specification or lateral

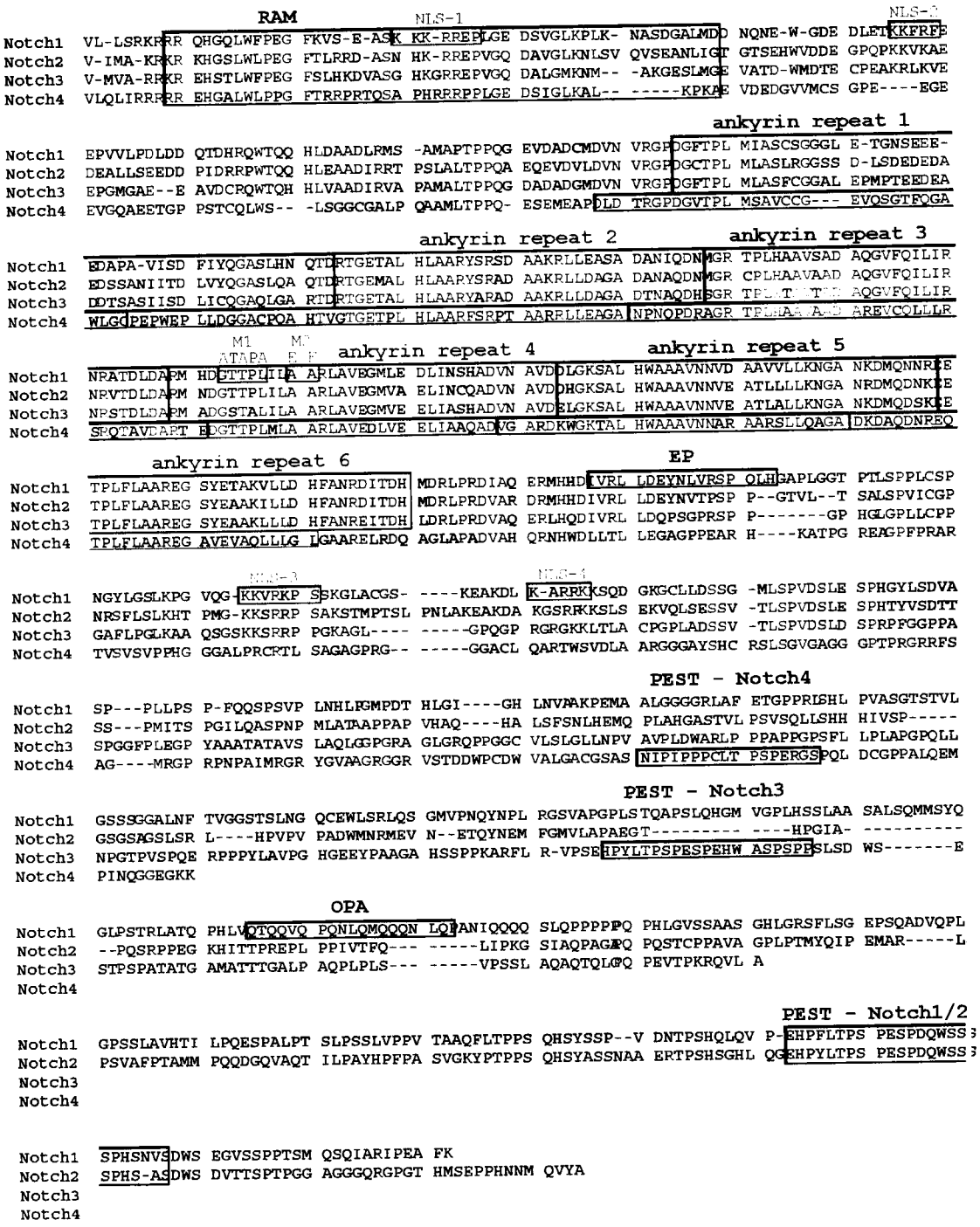


FIGURE 2. Sequence alignment of the intracellular regions of the human Notch receptors. The reported amino acid sequences for the intracellular regions of the four identified members of the human Notch family are aligned and annotated. Amino acid residues are identified by their one letter code. Aligned residues are red when similar for all four homologues. Blue letters denote consensus residues that are not similar for all four homologues. Amino acids that form structural domains are captured in boxes and labelled above. The putative nuclear localisation signals (NLS), M1 and M2 mutations, and EP domain are discussed later in the Introduction. For this thesis, the NLS are numbered NLS-1 through NLS-4 in the N- to C-terminal direction.

inhibition, the Notch pathway is able to control the differentiation of adjacent cells (Artavanis-Tsakonas et al., 1999). In lateral specification, a cell expresses Notch ligands after receiving a stimulus to differentiate into a particular fate. These ligands are then able to activate Notch receptors on adjacent cells, instructing these neighbours to remain undifferentiated or to assume a fate distinct from that of the ligand expressing cell. Receptor-ligand interaction triggers release of the Notch intracellular region (NIC), an activated form of the protein that can translocate to the nucleus and upregulate transcription of target genes (Schroeter et al., 1998; Struhl and Adachi, 1998). Due to this signalling mechanism, enforced expression of a truncated receptor consisting of only the intracellular domain provides constitutive Notch activity (Greenwald, 1994; Rebay et al., 1993).

Notch is synthesised as single chain polypeptide with an N-terminal signal peptide that directs the receptor to the secretory pathway for plasma membrane targeting (Egan et al., 1998). Notch is cleaved in the trans-Golgi network by a furin-like convertase (Blaumueller et al., 1997; Logeat et al., 1998). This cleavage event is named S1 and produces a heterodimeric protein that is transported to the cell surface. Ligand binding induces a proteolytic cleavage in the extracellular region of the membrane spanning subunit of Notch (Figure 3). This cleavage, referred to as S2, is mediated by a disintegrin/metalloproteinase, thought to be TNF-alpha converting enzyme (TACE) in vertebrates (Brou et al., 2000), and releases the majority of extracellular Notch. S2 is automatically followed by another cleavage, known as S3, which digests the remaining membrane-bound Notch and releases activated NIC. This intramembranous digestion is mediated by a multimeric γ-secretase complex that includes the functional components Presenilin-1 or Presenilin-2, putative aspartyl proteases, and Nicastrin

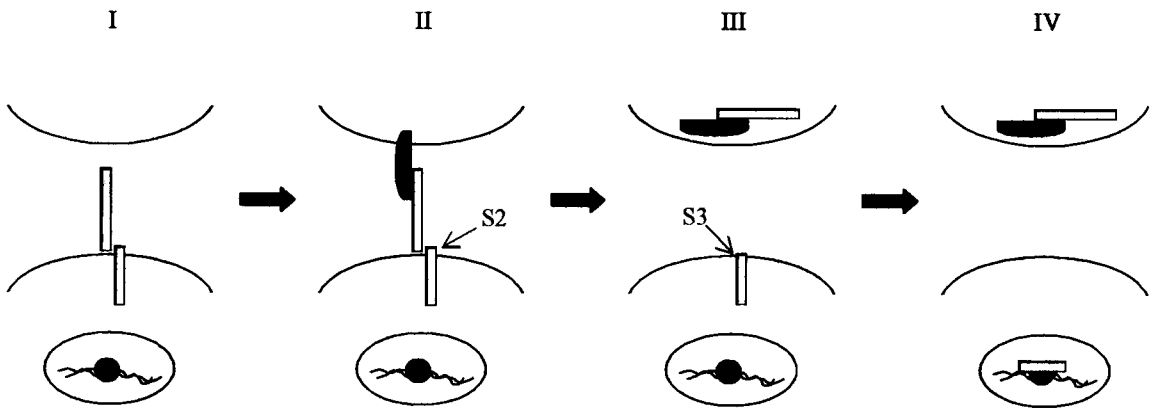


FIGURE 3. A model of Notch activation. Notch receptors (yellow) are transmembrane heterodimers composed of an extracellular subunit and a membrane spanning subunit (I). These receptors interact with transmembrane ligands (red) presented on the surface of neighbouring cells (II). Receptor-ligand binding induces an enzyme-mediated cleavage in the extracellular portion of the membrane spanning subunit of Notch. This cleavage event is designated S2 and releases the extracellular subunit to the ligand expressing cell (III). The remaining membrane spanning subunit is then automatically cleaved in its transmembrane domain by another enzyme. This cleavage event is named S3 and releases the Notch intracellular region (NIC) which can then translocate to the nucleus and interact with other factors (blue) to regulate gene transcription (IV).

(Chen et al., 2001; Li et al., 2003; Mizutani et al., 2001; Yu et al., 2000). This same complex is involved in β-amyloid precursor protein processing, an event associated with the formation of cytotoxic plaques found in patients with Alzheimer's disease (Kopan and Goate, 2000). Four putative nuclear localisation signals (NLS) have been identified in the Notch1 intracellular region (N1IC) (Aster et al., 1994; Aster et al., 1997; Kopan et al., 1994). The two most C-terminal NLS sequences are often considered to be a single bipartite NLS. In this thesis, the four NLS sequences are numbered 1 - 4 in the N- to C-terminal direction (Figure 2). All four are partially conserved in Notch2 and Notch3. However, only NLS-1 has notable amino acid sequence similarity in Notch4. This putative NLS is located in the RAM domain, deletion of which greatly reduced N4IC nuclear staining in a mouse mammary epithelial cell line (Lee et al., 1999).

1.2.4 Regulation of Notch Signalling

Notch signalling is regulated by several mechanisms. The Fringe family of proteins can positively or negatively regulate the binding of ligands to Notch receptors. Fringe proteins are thought to act as glycosyltransferases in the Golgi apparatus, where they can modify the ligand-binding EGF-like repeats of Notch before the receptor is transported to the cell membrane (Hicks et al., 2000; Munro and Freeman, 2000; Shao et al., 2003).

The ubiquitin ligase Itch acts in the cytoplasm to bind and ubiquitinate the intracellular domain of Notch upstream of the CT (Qiu et al., 2000). Numb binds Itch and cooperatively enhances this ubiquitination, which targets Notch for degradation by the proteosome (McGill and McGlade, 2003). In *Drosophila*, Numb has also been shown to bind the RAM and CT domains of Notch (Guo et al., 1996). Both membrane-bound and free cytoplasmic forms of NIC are ubiquitinated by Itch and Numb, however, it is the free intracellular form that is degraded.

Membrane-bound Notch receptors appear to have a basal level of phosphorylation, while activation and nuclear localisation can result in hyperphosphorylation (Foltz and Nye, 2001; Kidd et al., 1998; Redmond et al., 2000; Schroeter et al., 1998; Shimizu et al., 2000). This hyperphosphorylation is not required for Notch activation of its downstream partner CBF1. However, hyperphosphorylated forms of NIC are preferentially bound and ubiquitinated by SEL-10 in the nucleus, again targeting the receptor for degradation by the proteasome (Wu et al., 2001). The Notch PEST domain is thought to be important for SEL-10-mediated turnover, and SEL-10 has been show to specifically bind and ubiquitinate the PEST-containing CT region of Notch4 (Wu et al., 2001).

The Notch pathway also exhibits autoregulation, as activation can increase receptor expression while decreasing Notch ligand levels (Artavanis-Tsakonas et al., 1999; Huppert et al., 1997). Such inverse coordination of receptor and ligand levels may seem counterproductive, however, research indicates that coexpression of receptors and ligands within a given cell can inhibit Notch signalling. This is due, at least in part, to receptor-ligand interactions that can occur within the cell and prevent transportation to the membrane (Sakamoto et al., 2002). Notch also augments expression of transcription factors that ultimately downregulate their own expression (Nakagawa et al., 2000; Takebayashi et al., 1994).

1.3 Notch Signalling Pathways

1.3.1 The Transcriptional Regulator CBF1

Once in the nucleus, NIC positively regulates gene transcription through association with a DNA-binding protein known in mammals as CBF1 (C promoter binding factor 1), RBP-J κ (Recombination signal binding protein at the J κ site), or KBF2 (H-2K binding factor-2). Across species, this factor is referred to as CSL (CBF1/Su(H)/Lag-1) for CBF1 in mammals, Suppressor

of hairless (Su(H)) in *Drosophila* and *Xenopus*, and Lag-1 in *C. elegans*. Notch itself has no DNA-binding activity and is guided to the transcriptional regulatory sites bound by CBF1, which recognises a core consensus sequence of GTGGGAA (Ling et al., 1994; Tun et al., 1994). In mammals, CBF1 is ubiquitously expressed (Hamaguchi et al., 1992; Ling et al., 1994) and is essential for development, as CBF1-null mice show severe growth retardation by 8.5 days post coitum (dpc) and death before day 10.5 (Oka et al., 1995).

In addition to NIC, viral factors are also known to target CBF1, further indicating a key regulatory role for this pathway in cellular function. For example, the Epstein-Barr virus nuclear antigen2 (EBNA2) binds and activates CBF1 as an essential step in the immortalization of human B-cells by Epstein-Barr virus (EBV) (Grossman et al., 1994; Henkel et al., 1994; Hsieh and Hayward, 1995). CBF1 is also bound and activated by the RTA (Replication and transcription activator) protein of the Kaposi's sarcoma (KS)-associated herpes virus (KSHV) (Liang et al., 2002) and the adenoviral oncoprotein 13SE1A (Ansieau et al., 2001).

1.3.2 CBF1-Mediated Transcriptional Repression

In the absence of NIC or viral activators, CBF1 actively represses transcription of downstream genes via recruitment of a corepressor complex that includes SKIP, CIR, SMRT, SAP30, and HDAC1 or HDAC2 (Figure 4) (Lai, 2002). SKIP (Ski-interacting protein) binds to CBF1, SMRT, and CIR and appears to function as an adaptor that stabilises the overall complex (Zhou and Hayward, 2001). CIR (CBF1-interacting corepressor) and SMRT (Silencing mediator of retinoid and thyroid hormone receptors) also bind to CBF1 and to SAP30 and are important in recruiting the histone deacetylases HDAC1 or HDAC2 (Hsieh et al., 1999; Kao et al., 1998; Zhou and Hayward, 2001). NCOR (Nuclear receptor corepressor) proteins are related to SMRT

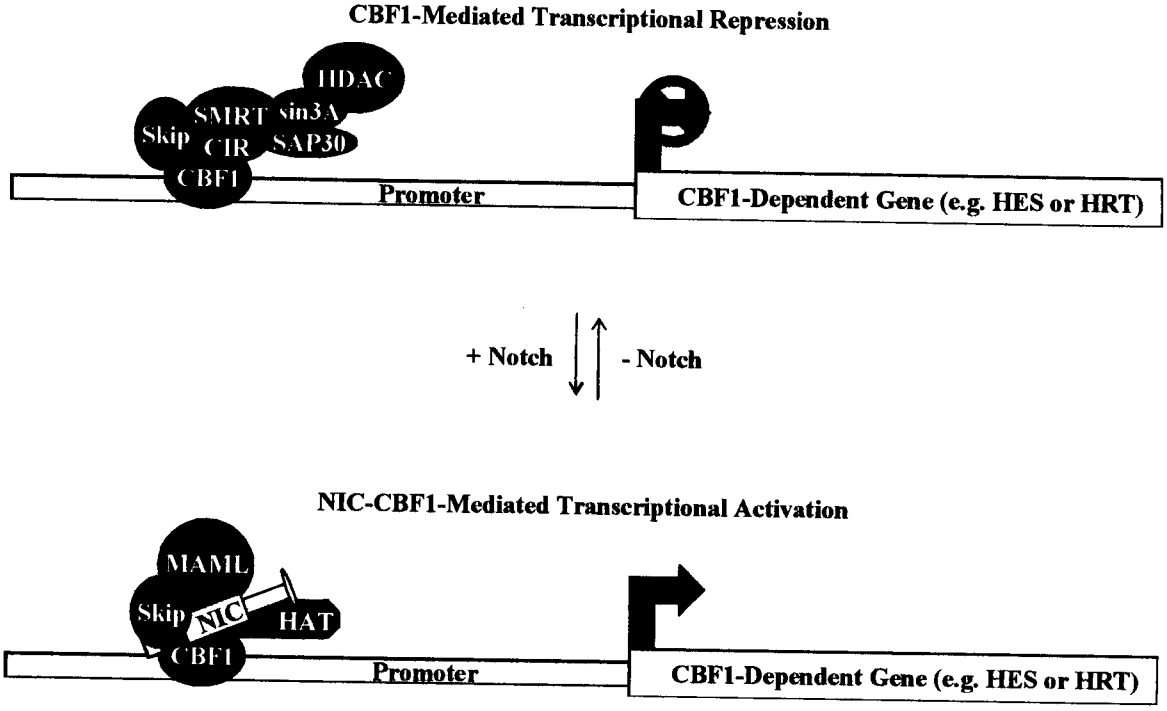


FIGURE 4. A model of CBF1-dependent gene regulation. In the absence of NIC in the nucleus, the DNA-binding protein CBF1 recruits a corepressor complex that negatively regulates transcription of nearby genes, including those belonging to the HES and HRT families. Members of the corepressor complex that have been identified include CBF1, SKIP, CIR, SMRT, SAP30, Sin3a, and the histone deacetylases HDAC1 and HDAC2. In the presence of NIC, the corepressor complex is disbanded and a coactivator complex formed that positively regulates transcription. Identified members of the coactivator complex include NIC, CBF1, SKIP, MAML, and the histone acetyltransferases (HATs) p300/CBP, GCN-5, and PCAF.

and may alternatively serve in the corepressor complex (Hsieh et al., 1999). SAP30 (Sin3-associated polypeptide) usually functions as a linker between SMRT and Sin3, which ultimately associates with HDAC. Therefore, it is likely that Sin3 is also a member of the CBF1 corepressor complex (Hsieh et al., 1999).

The nature of the corepressor complex indicates that chromatin remodelling by deacetylation is a major factor in CBF1-mediated repression. This is supported by studies with trichostatin A (TSA), an HDAC-specific inhibitor shown to alleviate CBF1-mediated repression of transcription (Kao et al., 1998; Kurooka and Honjo, 2000). Furthermore, CBF1 may suppress transcription independently of corepressor recruitment, as it can directly bind and perturb optimal interactions between the basal transcription coactivators TFIIA and TFIID (Olave et al., 1998). However, members of the corepressor complex also function in nuclear targeting of CBF1. In particular, SMRT and SKIP appear to facilitate the nuclear translocation of CBF1 and mutations that impair SMRT-CBF1 binding result in cytoplasmic targeting of CBF1 (Zhou et al., 2000a; Zhou et al., 2000b; Zhou and Hayward, 2001).

1.3.3 Notch-CBF1-Mediated Transcriptional Activation

Nuclear translocation of NIC leads to dissociation of repressor proteins from CBF1 and formation of a coactivator complex that potentiates gene expression (Hsieh et al., 1996; Kato et al., 1997; Tamura et al., 1995) (Figure 4). Activated forms of Notch1-4 have each been shown to transactivate CBF1-dependent reporters to varying degrees depending on the promoter and cell line (Mizutani et al., 2001; Saxena et al., 2001; Shimizu et al., 2002). However, it appears that in some contexts Notch3 is a poor transactivator and can repress Notch1 signalling by competing for binding of CBF1 and/or transcriptional coactivators (Beatus et al., 1999). There is also evidence of an inverse correlation between the expression of Notch3 and CBF1-dependent genes

in vivo (Beatus et al., 1999). Similarly, Notch2 can mitigate transactivation by Notch1 and Notch3 in cell lines where Notch2 is a poor activator of transcription (Shimizu et al., 2002).

NIC-induced transactivation can be blocked by KyoT2, which competes for CBF1 binding and acts to dislodge CBF1 from DNA (Taniguchi et al., 1998). SKIP, like CBF1, is part of both the corepressor and coactivator complexes. Similar to its role in repression, SKIP seems to stabilise the NIC-CBF1 interaction and all three proteins are capable of directly binding one another (Zhou et al., 2000a; Zhou et al., 2000b). The N-terminal and C-terminal regions of CBF1 provide contact to NIC (Tani et al., 2001), while SKIP binds to the fourth ankyrin repeat of Notch (Zhou et al., 2000a; Zhou et al., 2000b). The Notch RAM domain provides strongest interaction with CBF1 (Hsieh et al., 1996; Tamura et al., 1995), although the ankyrin repeats also bind but with lower affinity (Aster et al., 1997; Kato et al., 1997; Tani et al., 2001).

Other members of the NIC-CBF1-SKIP transactivation complex include MAML and the histone acetyltransferases (HATs) PCAF (p300/CBP-associated factor), GCN5 (General control of amino acid synthesis 5), and CBP/p300 (CREB-binding protein). Histone acetylation is central to transcriptional activation by NIC-CBF1, just as chromatin remodelling by deacetylation is important in CBF1-mediated repression. Accordingly, HAT inhibitors have been shown to repress Notch signalling (Kurooka and Honjo, 2000). MAML (Mastermind-like, also human Mastermind (hMAM)) proteins are the human homologues of *Drosophila* Mastermind (Lin et al., 2002; Wu et al., 2000). Three MAML proteins have been identified, MAML1-3, and shown to function in potentiating signalling by all four Notch receptors (Lin et al., 2002; Oswald et al., 2001; Wu et al., 2000). MAML1 directly binds the ankyrin repeats of Notch1-4 and may specifically recognise NIC-CBF1 complexes (Kitagawa et al., 2001; Lin et al., 2002; Wu et al., 2000). While MAML proteins seem to augment transcription by stabilising

NIC-CBF1, other mechanisms such as direct binding of CBP/p300 have also been demonstrated (Wallberg et al., 2002). Furthermore, *Drosophila* Mastermind has been shown to promote CBP/p300-mediated acetylation as well as enhance phosphorylation of both NIC and CBP/p300 (Fryer et al., 2002). NIC itself has been shown to interact with HATs, including direct binding of CBP/p300 through an EP domain as shown by *in vitro* binding assays and coimmunoprecipitation (Oswald et al., 2001). The ankyrin repeats and C-terminal region (CT) were required for NIC interaction with the HATs PCAF and GCN5 in a mammalian two-hybrid assay, although direct binding was not confirmed (Kurooka and Honjo, 2000). Therefore, the CBF1 corepressor complex and the NIC-CBF1 coactivator complex are both intricate arrays of proteins (Figure 4). The molecules within these groups are recruited and stabilised by cross binding between multiple factors.

The model of CBF1-mediated gene regulation implies that NIC can induce transcription through both derepression and coactivation of CBF1. This is supported by research in *Drosophila*, where certain gene enhancers appear to be NIC-instructive while others are NIC-permissive (Bray and Furriols, 2001). In regulating instructive enhancers, NIC induces transcription by alleviating Su(H)-mediated repression as well as recruiting transcriptional coactivators. However, NIC activation of permissive enhancers only requires derepression of Su(H).

1.3.4 Functional Roles of the Notch Intracellular Domains

Despite strong association between the RAM domain and CBF1, it is the ankyrin repeats that appear most essential for transactivation, as shown through numerous reporter assay experiments with Notch mutants (Aster et al., 1997; Jarriault et al., 1995; Kato et al., 1997; Kurooka et al., 1998; Zhou et al., 2000b). Accordingly, ankyrin repeat mutations have been

associated with the loss of activated Notch phenotypes such as inhibited myogenesis (Kato et al., 1997; Kopan et al., 1994), neoplastic transformation of kidney cells (Dumont et al., 2000), and induction of T-ALL (Aster et al., 1997). The necessity of this motif for Notch function probably owes to the crucial interactions between the ankyrin repeats and many factors in the coactivator complex, including MAML, SKIP, PCAF, and GCN5, in addition to CBF1. In particular, targeted mutations in the fourth ankyrin repeat 4 of Notch1 have been shown to abolish its activity (Jarriault et al., 1995; Kato et al., 1997; Kopan et al., 1994; Kurooka et al., 1998; Zhou et al., 2000b). These substitutions include a GTTPL to ATAPA mutation known as M1 and an AA to EF mutation known as M2 (Figure 2). The effects of these substitutions have only been tested in Notch1; however, all of the mutated residues in M1 and M2 are perfectly conserved across mammalian Notch receptors. Interestingly, the M2 mutation abolishes NIC binding to SKIP, but not CBF1, demonstrating the importance of SKIP in Notch signalling (Zhou et al., 2000b). While the ankyrin repeats are required for Notch activity, they are generally not sufficient, although examples where they do confer activity have been shown (Dumont et al., 2000; Nofziger et al., 1999; Shawber et al., 1996).

The RAM domain appears to enhance transactivation by strengthening NIC-CBF1 contacts and by competing with corepressors for CBF1 binding sites. However, whether or not this domain is required for NIC activity appears to depend largely on the specific promoter and cell type analysed. Most often, RAM mutation or deletion significantly reduces, but does not abolish, transactivation of CBF1-dependent reporters (Jarriault et al., 1995; Kato et al., 1997; Kurooka et al., 1998; Oswald et al., 2001). However, some studies indicate the RAM domain is absolutely required for transactivation (Nofziger et al., 1999; Redmond et al., 2000; Shawber et al., 1996), while others show it is entirely dispensable (Aster et al., 1997).

While all mammalian Notch proteins depend on CBF1 for DNA-targeting, they appear to use slightly different mechanisms for actual transactivation. When fused to the yeast GAL4 DNA binding domain, the CT of Notch1 and Notch2, but not Notch3 or Notch4, strongly activated reporter constructs containing GAL4 binding sites in their promoter (Kurooka et al., 1998). In fact, the CT of N1IC provided stronger activation than the full N1IC. The majority of this activity was narrowed to the 200 amino acid residues downstream of the most C-terminal NLS (NLS-4), and includes the OPA domain but not the PEST. As such, this region is sometimes referred to as the transactivation domain (TAD). Interestingly, the TAD of Notch1 does not upregulate genes when fused to CBF1, possibly due to an inability to displace dominant repressor proteins (Kurooka et al., 1998).

More recently, a group has identified a 16 amino acid sequence within the CT of Notch1 that is necessary for activation of a CBF1-dependent reporter in HeLa cells (Oswald et al., 2001). This region was named the EP domain and was found to contain a three amino acid sequence, LDE, crucial for transactivation (Figure 2). The LDE sequence is identical among human Notch1 and Notch2, as well as *Drosophila* Notch, proteins. However, human Notch3 and Notch4 have alterations in the LDE sequence that may, at least partially, explain why they have no apparent TAD downstream of their ankyrin repeats. Figure 5 summarises the NIC-interacting proteins that are discussed in this thesis and illustrates which domains of Notch are important for the binding or function of these various factors.

1.3.5 CBF1-Dependent Gene Targets of Notch

The primary genes targeted by CBF1-dependent Notch signalling are the HES (Hairy and enhancer of split) and HRT (Hairy-related transcription factor) families of basic helix-loop-helix

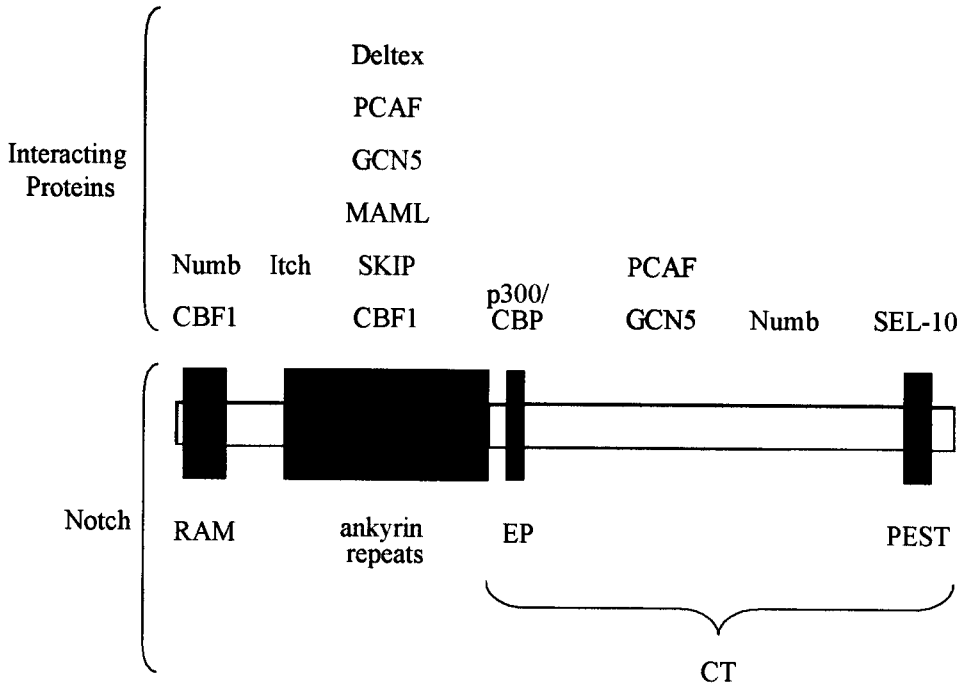


FIGURE 5. Proteins that interact with the Notch intracellular region. The protein structure of the Notch intracellular region (NIC) is depicted. Included in the diagram are the NIC domains thought to be important for protein binding. The Notch-interacting proteins discussed in this thesis are listed above the domains thought to be relevant for their binding or function (see text for details). Deltex is discussed later in the Introduction.

(bHLH) transcription factors (Iso et al., 2003). Seven mammalian HES genes, known as HES1-7, have been identified and are homologues of *Drosophila* E(spl) (Enhancer of split) (Akazawa et al., 1992; Bessho et al., 2001; Feder et al., 1994; Nishimura et al., 1998; Sakagami et al., 1994; Takebayashi et al., 1995; Takebayashi et al., 1994; Vasiliauskas and Stern, 2000). HES proteins typically act to repress transcription of target genes such as tissue-specific transcription factors. HES6 is the lone exception as it functions to derepress transcription by antagonising HES1 (Bae et al., 2000; Koyano-Nakagawa et al., 2000).

HES proteins have a conserved C-terminal WRPW motif, also found in *Drosophila* E(spl), for recruiting corepressors of the TLE (Transducin-like enhancer of split) family. Four members of the human TLE family have been identified to date and they are homologues of *Drosophila* Groucho (Chen and Courey, 2000). Little is known about TLE proteins, although their repression of transcription may involve chromatin remodelling since Groucho has been shown to recruit the histone deacetylase Rpd3 in *Drosophila* (Chen et al., 1999). HES proteins also contain an Orange domain, a putative protein-protein interaction motif that enhances their inhibition of transcription. The activity of the Orange domain may stem from binding of unknown corepressors or by stabilisation of WRPW-mediated activity (Castella et al., 2000; Iso et al., 2003). HES proteins also passively repress transcription by binding and sequestering activators (Castella et al., 2000; Iso et al., 2003).

Recently, a family of bHLH transcription factors known as HRT, and related to HES, was discovered. Three members of the mammalian HRT family have been identified to date and are referred to as HRT1 (also HERP2, Hesr1, Hey1, or CHF2), HRT2 (also HERP1, Hesr2, Hey2, CHF1, or Gridlock), and HRT3 (also HERP3, Hesr3, and HeyL) (Chin et al., 2000; Iso et al., 2003; Iso et al., 2001a; Kokubo et al., 1999; Nakagawa et al., 1999; Steidl et al., 2000). The

WRPW tetrapeptide found in the HES family is not conserved among HRTs, as this sequence is YRPW in HRT1, YQPW in HRT2, and YHSW in HRT3 (Nakagawa et al., 1999; Steidl et al., 2000). Furthermore, experiments indicate that the bHLH domain, rather than the C-terminal tetrapeptide, is responsible for the majority of HRT repression (Iso et al., 2001b; Nakagawa et al., 2000). This indicates that recruitment of TLE may not play a primary role in HRT activity. HRTs also have an Orange domain and may repress transcription by sequestering activators. In addition, since bHLH transcription factors function as homodimers or heterodimers, there is potential for transcriptional repression by HES-HRT complexes, possibly adding to the diversity of Notch signalling.

The HES and HRT genes have CBF1 recognition sequences in their promoters, marking them as putative targets for Notch signalling. However, Notch has only been confirmed to upregulate HES1, HES5, HES7, and HRT1-3 (Bessho et al., 2001; Iso et al., 2003; Iso et al., 2001a; Nakagawa et al., 2000; Ohtsuka et al., 1999). As expected, Notch appears to require CBF1 for this transactivation, as CBF1-null cells fail to upregulate HES and HRT mRNA in response to Notch (Iso et al., 2003). Induction can be restored by addition of exogenous CBF1. Interestingly, CBF1-null cells have reduced, but not abolished, HES and HRT levels (Iso et al., 2001a). Therefore, CBF1-independent pathways appear to exist for maintaining HRT and HES expression, although none have been specifically identified and it is unknown if these pathways would involve Notch signalling.

While considerable attention has been given to Notch regulation of HES and HRT, relatively few genes have been identified as targets for these transcription factors (Iso et al., 2003). The classic example in mammals is HES1 downregulation of MASH1 (Mammalian achaete-scute homologue 1), a neural-specific transcription factor. Notch can signal the

suppression of neurogenesis by upregulating the repressor HES1, which in turn downregulates the activator MASH1 (Chen et al., 1997a; Ishibashi et al., 1995). HES1 also relays Notch signals during T cell development, where it represses the CD4 promoter in $CD4^+$ $CD8^-$ T_H cells (Kim and Siu, 1998). HRT1 negatively regulates VEGFR2 (Vascular endothelial growth factor receptor 2) gene expression in endothelial cells as a means of inhibiting capillary-like network formation (Henderson et al., 2001; Taylor et al., 2002). HRT2 inhibits activation of a VEGF (Vascular endothelial growth factor) promoter-driven luciferase construct in NIH3T3 cells (Chin et al., 2000). HES1 can downregulate its own gene (Takebayashi et al., 1994), while HRT1 and HRT2 both repress HRT2 expression (Nakagawa et al., 2000).

CBF1-binding sites have been identified in the promoters of genes outside the HES and HRT families. Some of these promoters appear to be targets for Notch signalling as identified by CBF1 binding in electrophoretic mobility shift assays (EMSA) and by NIC upregulation of reporter constructs or mRNA (Iso et al., 2003). These targets include genes for CD23 (Ling et al., 1994), cyclin D1 (Ronchini and Capobianco, 2001), erbB-2 (Chen et al., 1997b), β-globin (Lam and Bresnick, 1998), interleukin-6 (Kannabiran et al., 1997), MHC class I (Shirakata et al., 1996), and NF-κB2 (Oswald et al., 1998).

1.3.6 CBF1-Independent Notch Signalling

While signalling through CBF1 remains the only well defined pathway mediating Notch action, evidence for CBF1-independent Notch signalling also exists. Studies on loss-of-function mutants indicate that the activity of Su(H) and Lag-1, CBF1 homologues in *Drosophila* and *C. elegans*, respectively, do not account for all observed Notch functions (Christensen et al., 1996; Lambie and Kimble, 1991; Lecourtois and Schweisguth, 1995; Ligoxygakis et al., 1998; Wang et al., 1997). There is also support for the existence of CBF1-independent pathways in mammals.

Some studies indicate that Notch-induced phenotypes result from the integration of both CBF1-dependent and -independent signals. For example, a constitutively-active CBF1 was shown to reproduce some, but not all, Notch effects on neurite cell outgrowth (Levy et al., 2002). As well, a dominant-negative form of CBF1 failed to block Notch-induced inhibition of myoblast cell differentiation, even though CBF1-mediated transactivation is well established in this process (Kato et al., 1997; Nofziger et al., 1999; Shawber et al., 1996).

While initial research gave no clues as to what factors may mediate alternative Notch pathways, recent reports show Deltex may play a key role. Deltex was originally identified in *Drosophila* as a cytoplasmic protein that can bind to the Notch ankyrin repeats and positively modulate Notch function (Diederich et al., 1994; Gorman and Girton, 1992; Matsuno et al., 1995; Xu and Artavanis-Tsakonas, 1990). Three mammalian homologues of Deltex, Deltex1-3, have been cloned. Deltex1 has the highest degree of sequence similarity to *Drosophila* Deltex and is widely expressed (Kishi et al., 2001; Lee et al., 2000a). Deltex1 has been shown to interact with the ankyrin repeats of Notch1 and Notch2, while there are no published reports testing Deltex binding to Notch3 or Notch4 (Matsuno et al., 1998; Yamamoto et al., 2001).

There is confusion as to the exact nature of Deltex function in mammalian systems, as it has been shown to both promote and antagonise Notch activity. For example, Notch directs lymphoid progenitors to the T cell lineage while overexpression of Deltex can induce differentiation into B cells (Izon et al., 2002). Deltex also antagonises Notch-initiated inhibition of neurite outgrowth from neurons (Sestan et al., 1999). In both aforementioned studies, Deltex was shown to inhibit Notch upregulation of CBF1-dependent reporter constructs. Conversely, Deltex mimics Notch downregulation of myogenin in myoblasts (Kishi et al., 2001), Notch-induced blocking of E47-mediated transcription (Matsuno et al., 1998; Ordentlich et al., 1998),

and Notch-mediated inhibition of neural progenitor cell differentiation (Yamamoto et al., 2001). Human Deltex1 has also been shown to potentiate Notch activation of a CBF1-dependent reporter in *Drosophila* cells (Matsuno et al., 1998).

In the study by Yamamoto et al. (2001), the inhibition of neurogenesis induced by Deltex and Notch occurred independently of CBF1 activation. Deltex was shown to localise to the nucleus and block activity of the neural-specific bHLH transcription factor MASH1 by sequestering CBP/p300. Inhibition of transcriptional activators may be a common theme for Deltex-dependent Notch signalling since this pathway has also been shown to inhibit the activity of the bHLH transcription factor E47 (Matsuno et al., 1998; Ordentlich et al., 1998). The CBF1-dependent effector HES1 has also been shown to inhibit the activity of MASH1, E47, and other transcriptional activators by sequestering them through direct association (Sasai et al., 1992). However, as in the studies describing Deltex as a Notch antagonist, Yamamoto et al. (2001) showed that Deltex inhibited Notch activation of CBF1. This raises the interesting possibility that Notch activity is a balance between Deltex- and CBF1-mediated pathways that compete for Notch binding. If true, it appears that in the context of some cells these coexisting pathways work together towards a common goal, whereas in other environments they act as rivals that induce conflicting phenotypes.

1.4 Notch in Vascular Development and Angiogenesis

Notch signalling is required for metazoan development and typically functions to regulate cell differentiation (Artavanis-Tsakonas et al., 1999). Notch has also been implicated in regulating the morphogenesis of various cell types, including the branching of neurites (Sestan et al., 1999) and the formation of mammary epithelial ducts (Jhappan et al., 1992; Uyttendaele et al., 1998). Mounting evidence demonstrates that Notch also functions in blood vessel

development, a process that involves the morphogenesis of endothelial cells. Firstly, Notch receptors and ligands are expressed throughout the vasculature, which includes the endothelium and surrounding perivascular cells. Notch4 is of particular interest because it is primarily expressed in the vascular endothelium of embryonic and adult mammals (Krebs et al., 2000; Li et al., 1998; Loomes et al., 2002; Shirayoshi et al., 1997; Uyttendaele et al., 1996; Villa et al., 2001). Notch1, Notch2, Notch3 (Krebs et al., 2000; Loomes et al., 2002; Villa et al., 2001) and ligands Jagged1, Jagged2, and Dll4 (Krebs et al., 2000; Mailhos et al., 2001; Shutter et al., 2000; Villa et al., 2001) are also expressed in various components of the vasculature, as are the downstream Notch effectors HRT1, HRT2, and HRT3 (Chin et al., 2000; Leimeister et al., 2000; Nakagawa et al., 1999).

Functionally, Notch is necessary for vascular development as proven by loss-of-function studies in mice. Notch1-null mice are embryonic lethal due to defects in blood vessel development (Huppert et al., 2000), while Notch4-deficient mice are viable and fertile (Krebs et al., 2000). However, the Notch1/Notch4 double knockout mice show more severe defects in vascular development than the Notch1-null mice (Krebs et al., 2000). Paradoxically, similar abnormalities occur in the embryos of transgenic mice expressing activated Notch4 under control of the regulatory elements of the VEGFR2 (also *Flk1*) gene (Uyttendaele et al., 2001). Expression under this promoter targets activated Notch4 to the endothelium as well as the common precursors of the endothelial and haematopoietic lineages. The similarity between loss-of-function and gain-of-function mutant mice suggests an essential need for a correct balance of Notch signalling in blood vessel development. Other Notch pathway members are also involved in vascular regulation, as mutations in Notch2 cause defects in the development of the mouse heart and eye vasculature. Mice deficient for the Notch ligands Jagged1 and Delta1 are

embryonic lethal and exhibit vascular remodelling defects and haemorrhaging, respectively (Hrabe de Angelis et al., 1997; Xue et al., 1999). Haemorrhaging also occurs in mice deficient for Presenilin-1, a protein involved in the proteolytic activation of Notch (Shen et al., 1997; Wong et al., 1997). Gridlock, also known as HRT2 in mammals, is activated by Notch signalling and has proven essential for aorta development in zebrafish (Zhong et al., 2000). However, knockout studies show that HRT2 is not essential for aortic development in mice but is important for cardiac development (Donovan et al., 2002; Gessler et al., 2002; Sakata et al., 2002).

Blood vessel development in the embryo begins with vasculogenesis, a process that involves the differentiation of precursor cells, known as angioblasts, into endothelial cells that assemble into a vascular network (Flamme et al., 1997). This immature labyrinth is a poorly functional vasculature known as the primary capillary plexus. This plexus is then remodelled into a functional network through angiogenesis, the process of developing new blood vessels from the existing microvasculature (Frater-Schroder et al., 1987; Risau, 1997). Other processes defined in the development of new endothelium include arteriogenesis, the rapid expansion of new arteries from existing collateral arterioles (Schaper and Buschmann, 1999), and lymphangiogenesis, the development of new lymphatic vessels (Alitalo and Carmeliet, 2002). In the Notch1/Notch4 and Jagged1 knockout mice, as well as the activated Notch4 transgenics, the primary capillary plexus develops normally. However, there is a failure to remodel this immature network into a mature vasculature. Specifically, Notch transgenic and knockout mutant embryos are unable to properly develop small blood vessels and their vascular networks are disorganised (Krebs et al., 2000; Uyttendaele et al., 2001). These findings indicate that the Notch pathway has a specific role in regulating angiogenesis.

1.5 Mechanisms of Angiogenesis

1.5.1 Pathological Angiogenesis

Lessons from human disease show that the Notch pathway is critical for proper vascular maintenance in the adult, as Notch3 mutations are associated with the vascular degenerative disease CADASIL. This condition is thought to stem from defects in the vascular smooth muscle cells and its symptoms include recurrent strokes and dementia. In the healthy adult, the vasculature is largely quiescent (Hobson and Denekamp, 1984) with new vessel development typically reserved for the female reproductive cycle and the wound healing process. However, angiogenesis has proven to be a key regulator of many pathological conditions, including tumour growth and metastasis, diabetic retinopathy, psoriasis, atherosclerosis, and ischemic heart and limb disease (Carmeliet and Jain, 2000).

Angiogenesis-dependent diseases exhibit aberrant blood vessel development that contributes to their pathogenesis. In these conditions, blocking angiogenesis has become a therapeutic goal. The most prominent example of an angiogenesis-dependent disease is cancer, which requires new blood vessels for tumour growth and metastasis (Papetti and Herman, 2002). However, pathological angiogenesis frequently produces abnormal vessels that differ from the structurally mature and functional networks produced in physiological development. In particular, tumour vasculature often lacks functional perivascular cells and is highly disorganised leading to chaotic and erratic blood flow (Carmeliet and Jain, 2000).

In contrast to angiogenesis-dependent pathologies, stimulating blood vessel growth can prove beneficial in the treatment of conditions where tissues are damaged due to insufficient blood supply and/or failure of the angiogenic response. For example, therapeutic angiogenesis and arteriogenesis have become prominent areas of research in the study of ischemic heart

disease (Helisch and Ware, 2000). While the majority of microvascular remodelling that does occur in adults results from angiogenesis, evidence suggests vasculogenesis may also contribute through the mobilisation of bone marrow-derived endothelial precursors (Rafii, 2000). However, it is angiogenesis and arteriogenesis rather than vasculogenesis that produce mature vessels, making them the therapeutic goal in ischemic disease (Helisch and Ware, 2000). Ultimately, determining the cellular and molecular mechanisms that control angiogenesis has become paramount in the pursuit of treating many human diseases.

1.5.2 Cellular Events during Angiogenesis

Angiogenesis most commonly involves the sprouting of new vessels from existing structures, but also includes the division of vessels by either endothelial bridges or by replacement with matrix (intussusception) (Carmeliet, 2000; Risau, 1997). Figure 6 shows a schematic of the key events in the complex, multi-step process of sprouting angiogenesis. First, activation of the endothelium causes vasodilation and an increase in vascular permeability (Connolly et al., 1989; Senger et al., 1983). Fenestrations and endothelial vacuoles are formed and adhesion molecules are redistributed (Davis and Camarillo, 1996; Yang et al., 1999). This causes the destabilisation of interendothelial contacts as well as changes in the adhesions between endothelial cells and the surrounding matrix and mural cells (Brooks et al., 1994a; Brooks et al., 1994b; Dejana et al., 1999; Jackson, 2003). The local matrix is degraded to clear a path for a column of proliferating endothelial cells to migrate through (Rakic et al., 2003; Visse and Nagase, 2003; Vlodavsky et al., 2002). After continued proliferation and migration, the endothelial cells re-establish secure vessel adhesions, form a lumen, and construct a new basement membrane. Supporting pericytes and/or vascular smooth muscle cells are recruited to

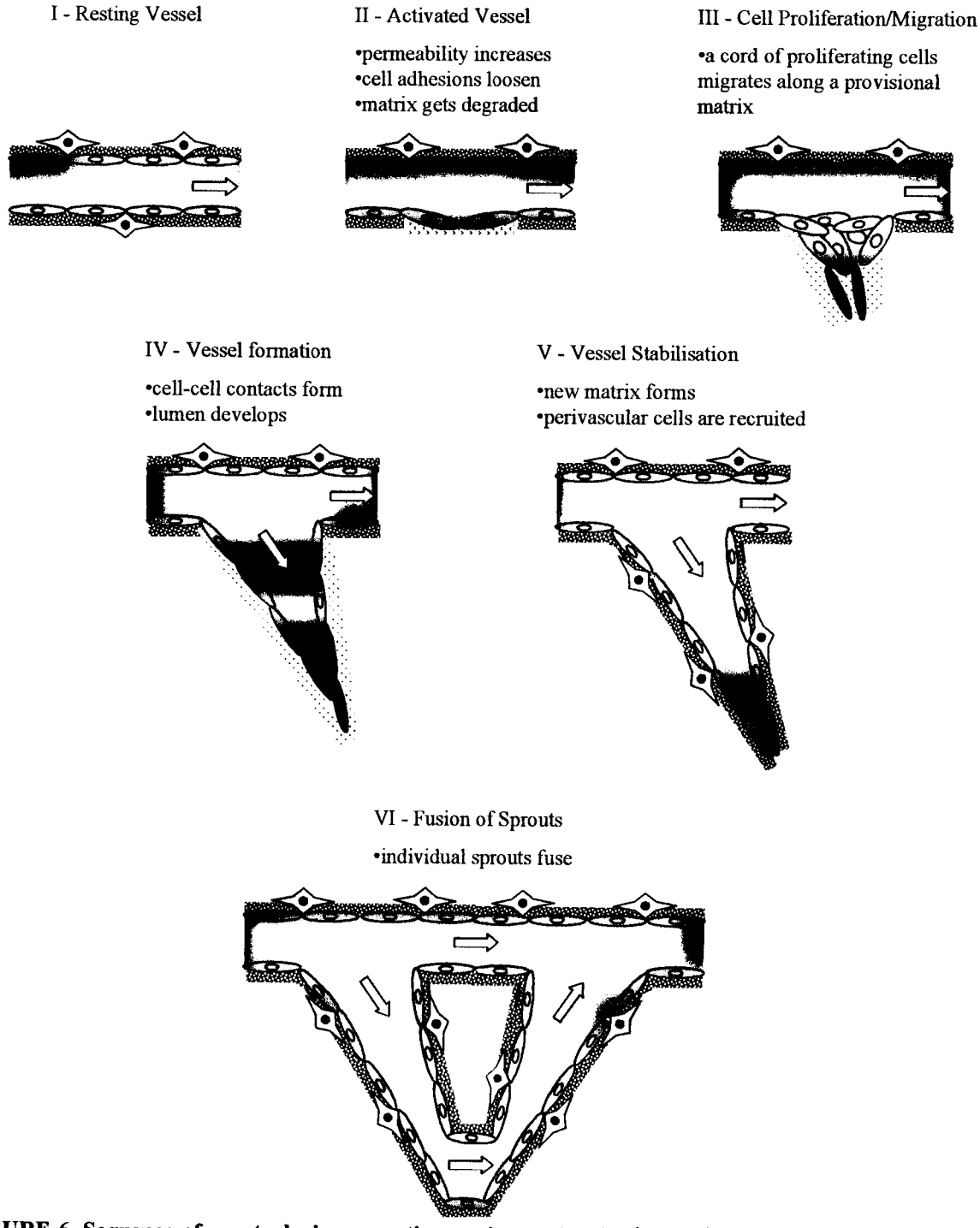


FIGURE 6. Sequence of events during sprouting angiogenesis. Angiogenesis is a multi-step process in which the resting endothelium (I) is activated, causing increased vessel permeability, loosening of cell-cell and cell-matrix contacts, and degradation of the local extracellular matrix (II). A provisional matrix is formed and provides a scaffold for a cord of proliferating endothelial cells to migrate along (III). Cells within the cord re-adhere and form a lumen, producing an immature vessel (IV). The vessel is stabilised by the formation of a new basement membrane and the recruitment of perivascular cells (V). Individual sprouts may fuse at their tips to form a loop capable of circulating blood (VI).

create a mature vessel. Furthermore, individual sprouts extending from a given vessel may fuse at their tips to form a capillary loop capable of circulating blood.

1.5.3 VEGF- and FGF-Induced Angiogenesis

While the general cellular events involved in angiogenesis have been described for some time, defining the molecular players and how they specifically regulate this process has proven more elusive. A balance of both pro- and anti-angiogenic factors act on the endothelium to determine its state. Changes in the level or availability of either positive or negative regulators can lead to a switch in angiogenic activity. Some of the anti-angiogenic molecules that have been identified include angiostatin, endostatin, antithrombin III, interferon-β, leukaemia inhibitory factor, and platelet factor 4 (Carmeliet, 2000).

Perhaps the most significant stimulator of angiogenesis is vascular endothelial growth factor (VEGF), which regulates both physiological and pathological vessel growth (Colville-Nash and Willoughby, 1997; Dvorak et al., 1995). VEGF, also known as VEGF-A, belongs to a family of growth factors that includes placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, as well as viral homologues known collectively as VEGF-E (Petrova et al., 1999; Zachary and Gliki, 2001). VEGF is a secreted glycoprotein that is largely specific for endothelial cells, although some precursors and haematopoietic cells also express VEGF receptors. VEGF is a ligand for the receptor-tyrosine kinases VEGF receptor 1 (VEGR1) and VEGF receptor 2 (VEGFR2) (Petrova et al., 1999; Zachary and Gliki, 2001). Neuropilin-1 and -2, transmembrane glycoproteins lacking protein-tyrosine kinase domains, also bind VEGF, although their precise function is unclear. VEGFR2 appears to be the most prominent receptor for mediating VEGF-induced signalling (Papetti and Herman, 2002). Loss-of-function studies have shown VEGFR2 is essential for vascular development in mouse embryos (Shalaby et al.,

1995). In contrast, mouse vascular development does not require VEGFR1 signalling (Hiratsuka et al., 1998). Furthermore, tissue culture studies show VEGFR1 possesses reduced kinase activity and may serve primarily to bind VEGF and modulate signalling through VEGFR2 (Petrova et al., 1999; Zachary and Gliki, 2001).

Hypoxia is a prominent stimulus for blood vessel growth. Hypoxia-inducible transcription factors specifically upregulate expression of VEGF, VEGFR1, VEGFR2, as well as a host of other angiogenic factors (Semenza, 1998). VEGF, which was originally known as vascular permeability factor, can then increase vessel permeability (Connolly et al., 1989; Senger et al., 1983). This allows extravasation of factors important for matrix degradation, cell migration, and proliferation. Degradation of the matrix also releases sequestered factors important for these processes. VEGF itself provides a strong mitogenic, chemotactic, and survival signal for endothelial cells.

Connections between the VEGF and Notch pathways have been demonstrated, although the exact nature of their interplay is unclear. VEGF stimulation of human arterial endothelial cells was shown to upregulate Notch1 and Dll4 mRNA (Liu et al., 2003). Using specific inhibitors as well as dominant-negative and constitutively-active constructs, this induction was shown to be dependent on VEGF activation of the phosphatidylinositol 3-kinase/Akt pathway, but not MAPK or Src signals. Notch1, Notch4, and HRT1 can suppress VEGFR2 expression in certain endothelial cells, as well as reduce the responsiveness of these cells to VEGF-induced proliferation and migration (Henderson et al., 2001; Taylor et al., 2002). HRT2 expression can inhibit induction of the VEGF promoter in NIH3T3 cells, apparently by sequestering relevant transcriptional activators (Chin et al., 2000).

Another highly prominent angiogenic factor is fibroblast growth factor (FGF). There are at least 20 proteins in the FGF family, with the classical factors FGF-1 (also acidic FGF or aFGF) and FGF-2 (also basic FGF or bFGF) being the most extensively studied to date. FGFs can induce proliferation and migration in a wide range of cell lineages, including endothelial cells, and play a role in embryonic vascular development (Flamme and Risau, 1992; Leconte et al., 1998). FGF-2, in particular, is a potent angiogenic factor (Bikfalvi et al., 1997; Friesel and Maciag, 1995) that has been implicated in both physiological and pathological angiogenesis (Auguste et al., 2001; Compagni et al., 2000; Giavazzi et al., 2001; Rak and Kerbel, 1997). FGF, like VEGF, signals through a family of receptor-tyrosine kinases, although expression of these receptors is more widespread than the VEGFR family. Of the six FGF receptors, denoted FGFR1-6, FGFR1 is the major receptor expressed in endothelial cells and its function is vital for embryonic vascular remodelling (Lee et al., 2000b).

1.5.4 Other Regulators of Angiogenesis

The Tie2 receptor-tyrosine kinase regulates vessel stability and is essential for vascular remodelling in the embryo (Papetti and Herman, 2002; Ward and Dumont, 2002). Tie2 is activated by angiopoietin1 (Ang1), which has also proven essential for the developing vasculature. Ang1 inhibits endothelial proliferation and appears to promote angiogenesis by increasing vessel stability and endothelial survival. Ang2 functions as a Tie2 antagonist in the vasculature and can destabilise vessels and promote VEGF-induced angiogenesis. Therefore, Ang2 appears to function in the early activation stages of angiogenesis while Ang1 is necessary for the later stages of angiogenesis involving vessel stabilisation.

A host of additional growth factors and cytokines are capable of inducing angiogenesis, including placenta growth factor, platelet-derived growth factor, erythropoietin, and hepatocyte

growth factor (Carmeliet, 2000). However, execution of this multi-step process requires many other factors with diverse functions. Proteolytic enzymes, including matrix metalloproteinase (MMP) (Visse and Nagase, 2003), plasminogen activator (Rakic et al., 2003), and heparanase (Vlodavsky et al., 2002) family members, are required to degrade the local basement membrane. Molecules such as fibrin form a provisional extracellular matrix that provides a scaffold to guide and promote cell migration, proliferation, and differentiation into vessels (Montesano et al., 1987; Nicosia and Ottinetti, 1990).

Adhesion molecules that mediate cell-cell and cell-matrix contacts are tightly regulated during neovascularization. Two prominent endothelial factors mediating cell-cell interactions are vascular endothelial (VE)-cadherin (CD144) and platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) (Dejana et al., 1999; Jackson, 2003). These molecules provide survival signals for endothelial cells and promote vascular maintenance. In particular, VE-cadherin forms a complex with VEGFR2 that is required for VEGF-mediated survival signals (Carmeliet et al., 1999). During vessel activation, VE-cadherin and PECAM-1 are redistributed from specific junctions at cell borders and aid in vacuole formation, cell elongation, and migration (Dejana et al., 1999; Jackson, 2003; Yang et al., 1999). Ultimately, these molecules mediate new cell-cell contacts in the quiescent vasculature. Other adhesion molecules such as integrins connect the cell and surrounding matrix. Increased integrin activity can inhibit angiogenesis by causing strong adherence to the matrix that prevents migration, as shown with β_1 integrins (Leong et al., 2002). However, other integrins, such as the $\alpha_v\beta_3$ isoform, are upregulated during sprout formation and antagonists to these molecules can inhibit angiogenesis (Brooks et al., 1994a; Brooks et al., 1994b).

1.6 Notch Angiogenic Activity *in vitro*

While Notch receptors are required for vascular remodelling, the exact role they play in angiogenesis remains undetermined. Numerous *in vitro* experiments focusing on Notch1 and Notch4 have been carried out in an attempt to characterise their involvement in regulating endothelial cell morphogenesis into capillary-like structures. Constitutively active Notch4 expressed in human dermal microvascular endothelial cells (HMEC-1) was shown to inhibit VEGF- and FGF-2-induced endothelial sprouting in fibrin gels (Leong et al., 2002). This anti-angiogenic activity was confirmed *in vivo* in the chick chorioallantoic membrane. Notch has also been shown to negatively regulate the morphogenesis of endothelial cells in collagen gel assays, where blocking Notch signals with anti-sense oligonucleotides against the ligand Jagged1 enhanced bovine microvascular endothelial cell invasion and cord formation (Zimrin et al., 1996). However, in this instance, the Notch pathway regulated morphogenesis induced by FGF-1 but not VEGF.

Conversely, other groups have shown that Notch activity in endothelial cells can stimulate morphogenesis. Overexpression of activated Notch4 induced the morphogenesis of rat brain endothelial cells into cord-like structures when grown in two-dimensions on collagen (Uyttendaele et al., 2000). This effect was also stimulated by ectopic expression of Jagged1. Additionally, overexpression of Notch1 or HES1 in human iliac aortic endothelial cells (HIAEC) enhanced VEGF-induced invasion and network formation in collagen (Liu et al., 2003). This effect appeared CBF1-dependent as overexpressing dominant negative CBF1 reduced network formation.

In yet another set of *in vitro* experiments, Notch signalling was shown to be both pro- and anti-angiogenic. Specifically, Notch activity, including upregulation of HRT1 expression, was

increased during endothelial cell morphogenesis into capillary-like structures in collagen (Henderson et al., 2001; Taylor et al., 2002). Accordingly, morphogenesis was inhibited by blocking HRT1 expression or preventing ligand activation of Notch. Paradoxically, HRT1 overexpression also diminished network formation. Presumably, this was due to the inhibition of cell migration and proliferation, since Notch signalling was shown to suppress VEGF-induction of these processes by downregulating VEGFR2. Thus, based on *in vivo* and *in vitro* research, Notch signalling in endothelial cells appears to play a complex role in regulating angiogenesis.

1.7 Objectives

In the present study, the role of activated Notch4 in inhibiting endothelial sprouting was investigated. Notch4 was analysed because it has an established association with angiogenesis and it is primarily expressed in the vascular endothelium. As an *in vitro* model of angiogenesis, human microvascular endothelial cells (HMEC-1) were seeded on gelatin-coated microcarrier beads, suspended in three-dimensional fibrin gels, and induced by FGF-2 or VEGF to form capillary-like sprouts. Using this quantitative method, overexpression of activated Notch4 was previously shown to inhibit sprouting in HMEC-1 and primary human umbilical vein endothelial cells (HUVEC) (Leong et al., 2002). *The objectives of this thesis were to determine which Notch4 intracellular (N4IC) domains were required for inhibition of sprouting and activation of CBF1 in HMEC-1, and to determine whether Notch regulation of sprouting was mediated by CBF1.* The results showed that the ankyrin repeats, but not the RAM domain or C-terminal region (CT), were required for N4IC inhibition of VEGF- and FGF-2-induced HMEC-1 sprouting. However, only partial inhibition was provided by the ankyrin repeats alone. Furthermore, deletion of the ankyrin repeats, but not the RAM domain or CT, abolished N4IC induction of CBF1-dependent gene expression as seen by luciferase reporter assays and RT-

PCR. The ankyrin repeats alone were capable of transactivating some, but not all, CBF1-dependent targets. Finally, constitutively active CBF1 significantly inhibited VEGF- and FGF-2-induced HMEC-1 sprouting, but not as strongly as activated Notch. In conclusion, N4IC-induced inhibition of HMEC-1 sprouting requires the Notch ankyrin repeats and may involve signalling through CBF1-dependent and -independent pathways.

Chapter 2

Materials and Methods

2.1 Cell Culture

HMEC-1 (hereafter referred to as HMEC), a simian virus 40 (SV40) large T antigen-transformed microvascular endothelial cell line (Ades et al., 1992), was provided by the Centers for Disease Control and Prevention (Atlanta, Ga.). Cells were cultured in MCDB medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, Utah), 10 ng/ml of epidermal growth factor (Sigma), and 50 U/ml of penicillin - 50 μg/ml streptomycin (Gibco, Grand Island, NY)) (HMEC medium). Cells were maintained at 37°C in 5% CO_2.

2.2 Plasmid Constructs

The cDNA for N4IC and its related deletion constructs (referred to collectively as the N4IC constructs or N4IC mutants) were created by PCR and each include a C-terminal hemagglutinin (HA)-tag having the amino acid (a.a.) sequence YPYDVPDYA. Mutant cDNAs were inserted into the LNCX retroviral vector, where expression is controlled by the cytomegalovirus (CMV) immediate early enhancer/promoter. The HA-tagged N4IC construct was previously prepared (Leong et al., 2002) and includes a.a. 1476-2003 of the 2003 a.a. full-length Notch4 (Li et al., 1998). The N4IC mutants (see Figure 6A) included constructs: (i) lacking the entire RAM domain (ΔRAM-N4IC; encodes a.a. 1518-2003); (ii) lacking the RAM and N-terminally fused with an SV40-derived NLS (NLS-ΔRAM-N4IC; encodes a.a. 1518-2003); (iii) lacking all six ankyrin repeats (ΔAnk-N4IC encodes a.a. 1476-1578 and 1801-2003); (iv) lacking the C-terminal region (ΔCT-N4IC; a.a. 1475-1789); (v) composed of only the six ankyrin repeats (Ank; encodes a.a. 1579-1789); and (iv) composed of the six ankyrin repeats plus

additional upstream sequence and fused with an N-terminal SV40 NLS (NLS-Ank; encodes a.a. 1518-1789). The NLS tags had the amino acid sequence DPKKKRKV. N4IC was also cloned into the MSCV-IRES-YFP (MIY) retroviral vector (gift of Dr. R. K. Humphries), as was CBF1-VP16, a constitutively-active CBF1. In the MIY vector, gene expression is controlled by the murine stem cell virus long terminal repeats (LTR). CBF1-VP16 has an N-terminal FLAG-tag and includes the transactivation domain of the herpes simplex virus transcription factor VP16 fused to the C-terminus of CBF1. CBF1-VP16 was constructed by PCR amplification of the 3' region of the mouse CBF1-VP16 cDNA (gift of E. Manet) (Waltzer et al., 1995). This PCR product, which includes the coding region for the VP16 transactivation domain, was digested with AflII and ligated to the corresponding AflII site of the cDNA for FLAG-CBF1, which itself was derived from the RBP-2N isoform of human CBF1 (gift of Dr. R. Schmid) (Oswald et al., 1998). The 4xCBF1 luciferase construct (gift of Dr. D. Hayward) (Hsieh et al., 1996) includes four copies of a CBF1 binding element (GATCTGGTGTAAACACGCCGTGGGAAAAAA TTTATG) cloned upstream of an SV40 promoter-driven firefly luciferase. The HRT2 luciferase construct (gift of Dr. E. N. Olsen) (Nakagawa et al., 2000) is comprised of a 10-kb fragment of the mouse HRT2 promoter cloned into a promoterless firefly luciferase vector.

2.3 Gene Transfer

HMEC lines used for the experiments with the N4IC mutants (Chapter 3) were transduced using the LNCX retroviral vector. Experiments involving N4IC and CBF1-VP16 (Chapter 4) used the MIY retroviral vector. In both instances, HMEC were transduced with empty vector control or vector with cDNA insert according to methods described previously (Karsan et al., 1996). Polyclonal HMEC lines were isolated by selection in 300 μg/ml of G418

(Gibco) for the LNCX constructs and by sorting for YFP expression using a FACS 440 (Becton Dickinson (BD), Franklin Lakes, NJ) for the MIY constructs.

2.4 Total Cellular Extract Preparation and Immunoblotting

Total cellular extracts were prepared from confluent cell monolayers. Cells were washed once with phosphate-buffered saline (PBS) and scraped into 1.5-ml Eppendorf tubes using 3.6 $\mu l/cm^2$ of pre-heated (100°C) lysis buffer containing 10 mM Tris-HCl pH 7.4 (Fisher Scientific, Fair Lawn, NJ), 1% sodium dodecyl sulfate (SDS) (Fisher Scientific), and a protease inhibitor cocktail (1:1000 dilution) (Sigma, catalogue number P 8340). The lysate was heated at 100°C for 3 min, sonicated for 15 seconds, and clarified at 21,000xg for 10 min at 4°C. Supernatants were transferred to new tubes and stored at -80°C until use.

Forty μg of total protein (Bio-Rad DC Protein Assay System; Bio-Rad Laboratories, Hercules, CA) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Bio-Rad Laboratories). Nonspecific binding was blocked by incubation with PBS, 0.1% Tween 20 (Fisher Scientific), and 5% skim milk. Primary and secondary antibody solutions were made in PBS, 0.1% Tween. The primary antibodies used included a mouse anti-HA-epitope monoclonal antibody (1:4000 dilution) (Sigma), the M5 mouse anti-FLAG-epitope monoclonal antibody (1:1000 dilution) (Sigma), and a mouse anti-alpha-tubulin monoclonal antibody (1:5000 dilution) (Sigma). In each case the secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (Bio-Rad Laboratories). Images were captured using the MulitiImage™ Light Cabinet with FluorChem™ FC Software (Alpha Innotech Corporation, San Leandro, CA).

2.5 Immunofluorescence

Transduced HMEC lines were cultured overnight on chamber slides (BD) and then fixed with 4% paraformaldehyde (Fisher Scientific) and permeabilised with cold methanol (Fisher Scientific) for 3 min. Nonspecific binding was blocked by incubation with PBS, 5% goat serum. Cells were stained with the mouse anti-HA monoclonal primary antibody (1:100 dilution) for 1 h and then for 30 min with an AlexaFluor 488-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution) (Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI for 5 min and coverslips mounted with 50% glycerol. Slides were viewed using a Zeiss Axioplan II Imaging inverted microscope (Carl Zeiss Canada Ltd, Toronto, ON) and images were captured with a 1350EX cooled CCD digital camera (QImaging, Burnaby, BC).

2.6 Endothelial Sprouting Assay

Transduced HMEC lines were grown on 10-cm culture plates (BD) until ~50% confluence, trypsinized, and then resuspended in 15-ml tubes (BD) with 5 ml of HMEC medium and 5 mg of gelatin-coated microcarrier beads (Cytodex 3; Sigma). Cells were allowed to adhere and grow to confluence on the beads while rocking at 37°C for 72 hr, changing the medium daily. For preparation of fibrin gels, type IV bovine fibrinogen (2.5 mg/ml) (Sigma) was dissolved in MCDB and supplemented with aprotinin (0.05 mg/ml) (Sigma). Fibrinogen solutions were further supplemented with either FGF-2 (15 ng/ml) (R&D Systems, Inc., Minneapolis, MN), the $VEGF_{165}$ isoform of VEGF (30 ng/ml) (R&D), or no additional angiogenic factor (basal medium). These solutions were added to 96-well plates at a volume of 100 µl per well. Cell-coated beads were washed once with MCDB and subsequently suspended in the fibrinogen solutions at an approximate concentration of 40 - 60 beads per well. Clotting into fibrin gels was induced by thrombin (1.2 U/ml) (Sigma) with subsequent incubation for 10

min at 37°C in 5% CO_2. Gels were then overlaid with 100 µl of MCDB-2% FCS per well and incubated for 60 min at 37°C in 5% CO_2. The overlay was then removed and replaced with 100 µl of MCDB-2% FCS containing either FGF-2 (15 ng/ml), $VEGF_{165}$ (30 ng/ml), or no additional angiogenic factor (basal medium). After three days of incubation at 37°C in 5% CO_2 with daily changes of the overlay, endothelial sprouting was quantitated. Continuous capillary-like structures extending from the cell-coated beads and reaching at least 150 µm in length (the diameter of an average microcarrier bead) qualified as sprouts. The total sprouts per bead were calculated for each well with each sample done in triplicate. Samples were viewed and counted using a Nikon Eclipse TS100 standard inverted microscope and images were captured with a Nikon Coolpix 990 digital camera (Nikon Corporation, Tokyo, Japan).

2.7 Transient Transfection and Luciferase Assays

Transient transfection of luciferase reporter plasmids was carried out by electroporation. Transduced HMEC lines were grown until ~80% confluence and then trypsinized and resuspended in HMEC medium. Cells (1.5×10^6/transfection) were pelleted at 1000 rpm for 5 min, washed with PBS, pelleted as previous, and then resuspended in 0.4 ml of electroporation buffer (20 mM HEPES, 137 mM sodium chloride, 5 mM potassium chloride, 0.7 mM sodium phosphate 6 mM D-glucose, pH 7.0) (Ear et al., 2001) containing luciferase reporter plasmid DNA. The cell-DNA mixture was transferred to a 4 mm-gap electroporation cuvette (Bio-Rad), left for 10 min at room temperature, and then electroporated at a fixed capacitance of 900 µF and 200 V using a Bio-Rad Gene Pulser II instrument. For each transfection, 2.5 µg of 4xCBF1-binding promoter luciferase plus 1 µg of RL-CMV or 5 µg of HRT2 promoter luciferase plus 1 µg of RL-CMV were used. The RL-CMV reporter (Promega Corporation, Madison, WI) contains the renilla luciferase cDNA expressed under control of the CMV immediate early

enhancer/promoter and serves as a normalisation control for transfection efficiency. After electroporation, the cells were left for 10 min at room temperature before plating in pre-warmed (37°C) HMEC medium. The cells received fresh medium 24 hr later and were harvested for assay 48 hr after transfection. Lysis and dual-luciferase reporter assays were performed according to the manufacturer's recommendations (Promega) with luminescence measured on a Tropix™ tube luminometer (BIO/CAN Scientific, Mississauga, ON). Luminescence values of mock transfections were subtracted from sample luminescence readings to give the net firefly and net renilla luciferase units. The net firefly units divided by the net renilla units determined the relative luciferase units (RLU).

2.8 RNA Isolation and RT-PCR

Total RNA was isolated from confluent cell monolayers using TRIzol Reagent (Invitrogen, Burlington, ON) and the yield quantitated by spectrophotometry (260 nm). First strand cDNA was synthesised using 50 µl reactions containing 2.5 µg of RNA and 200 U of SuperScript II reverse transcriptase (Invitrogen). Negative control reactions were performed without the reverse transcriptase. RNAse H (2 U/reaction) (Invitrogen) was added to each sample at the end of the reverse transcription. PCR reactions were performed on an Eppendorf Mastercycler Gradient thermal cycler (Brinkmann Instruments Inc., Westbury, NY) using 3 µl of cDNA and the primer sequences and cycle conditions listed in Table 1. Included in Table 1 are the annealing temperatures for the primers (T_A), the number of cycles run for each PCR reaction (# of Cycles), and the size of each PCR product (Size). PCR products were resolved on TAE agarose gels and images captured using the MulitiImage™ Light Cabinet with FluorChem™ FC Software (Alpha Innotech Corporation, San Leandro, CA). No PCR products were detected in the negative control reactions performed without reverse transcriptase.

Table 1. Primers and PCR conditions.

Target	Sense (5'-3')	Anti-Sense (5'-3')	T_A (°C)	# of Cycles	Size (bp)
VEGFR2	agccctgtgcgctcaactgtc	aagagaaacactaggcaaacc	55	30	220
FGFR1	agctccatattggacatc	tatgatgctccaggtggc	54	25	196
HRT1	ggagaggcgccgctgtagtta	caagggcgtgcgcgtcaaagta	57	28	456
HRT2	tgagcataggattccgagagtgc	gaaggacagagggaagctgtgtg	57	28	481
HRT3	cactggtgggacaggattctttg	gtaagcagccgaccctgtaggac	57	30	576
HES1	aggcggacattctggaaatg	cggtacttccccagcacactt	55	30	103
HES4	caccgcaagtcctccaag	tcacctccgccagacact	53	30	265
GAPDH	cccatcaccatcttccag	atgaccttgcccacagcc	55	22	445

2.9 – Sequence Alignment and Statistical Analysis

The multiple sequence alignment in Figure 2 was performed using the Mult Alin software (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). For statistical analysis of the thesis results, *P* values were determined by One-Way Analysis of Variance (ANOVA) with a Tukey test for multiple comparisons using the SPSS 11.0 software (SPSS Inc., Chicago, IL). For each set of endothelial sprouting data, a separate test was done for the different growth factor stimuli (Figures 7C and 11B). A single test was done for each luciferase assay data set (Figures 9A, B, and 10B).

Chapter 3

Notch4-Induced Inhibition of Endothelial Sprouting Requires the Ankyrin Repeats

3.1 Introduction

In vivo, Notch4 appears to play a role in endothelial cell signalling during vascular development. Notch4 is predominantly expressed in the vascular endothelium (Krebs et al., 2000; Li et al., 1998; Shirayoshi et al., 1997) and loss of Notch4 function in mice enhances the vascular remodelling defects caused by loss of Notch1 function (Krebs et al., 2000). Intriguingly, a similar failure in mouse vascular development occurs when constitutively-active Notch4 is targeted to the endothelium under control of the regulatory elements of the VEGF receptor 2 (VEGFR2) gene (Uyttendaele et al., 2001). Enforced expression of activated Notch4 also blocks angiogenesis *in vivo* in the chick chorioallantoic membrane and inhibits endothelial sprouting *in vitro* (Leong et al., 2002). However, the primary signalling mechanisms by which activated Notch4 regulates endothelial cell morphogenesis during vascular remodelling remain unknown. For this thesis, human Notch4 function was studied using an *in vitro* model of angiogenesis where enforced expression of the activated receptor inhibits endothelial sprouting. This anti-angiogenic activity of Notch4 was shown previously using human dermal microvascular endothelial cell (HMEC) lines and was verified with primary umbilical vein endothelial cells (HUVEC) (Leong et al., 2002). The objective of the research described in this chapter was to determine which domains of the Notch4 intracellular region (N4IC) are required for inhibition of endothelial sprouting. Receptor function was analysed by overexpressing N4IC or various N4IC deletion mutants in HMEC. While similar deletion strategies have been used extensively to study Notch1, there has been little research into the specific function of individual Notch4 domains. Nevertheless, the ankyrin repeats have been established as the most essential

intracellular domain for Notch function across species (Kato et al., 1997; Kurooka et al., 1998; Lieber et al., 1993; Rebay et al., 1993; Roehl et al., 1996). Therefore, it is hypothesised that Notch4-induced inhibition of HMEC sprouting requires the ankyrin repeats.

3.2 Results and Conclusions

3.2.1 Deletion of the RAM Domain Inhibits Nuclear Localisation of Notch4

The N4IC deletion mutants analysed included constructs missing either the RAM domain (ΔRAM-N4IC), all six ankyrin repeats (ΔAnk-N4IC), or the C-terminal region (ΔCT-N4IC), as well as a construct consisting of the ankyrin repeats alone (Ank) (Figure 7A). Immunoblotting of total cellular lysates for these HA-tagged N4IC mutants confirmed expression of each protein following introduction by retroviral transduction (Figure 7B).

In addition to verifying the expression of these constructs, it was important to ensure proper subcellular localisation of each mutant, since activated Notch predominantly functions by modulating transcription in the nucleus. Protein localisation was analysed by immunofluorescent staining of HMEC lines for the various HA-tagged N4IC mutants (Figure 7C). Cell nuclei were counterstained with DAPI. N4IC showed strong nuclear with weaker cytoplasmic staining while the ΔAnk-N4IC and ΔCT-N4IC proteins were predominantly nuclear. Conversely, both ΔRAM-N4IC and Ank, the two constructs lacking a RAM domain, appeared to be inhibited in their ability to enter the nucleus as both stained primarily in the cytoplasm. The importance of the RAM domain for Notch4 nuclear localisation has been reported previously in studies isolating fusion proteins of N4IC and the virus-derived intracisternal type A particle (IAP) from MMTV-induced mammary tumours (Lee et al., 1999). In that study, fusion proteins with an intact N4IC sequence localised primarily in the nucleus of mouse mammary epithelial cells while those lacking a RAM domain were mainly cytoplasmic.

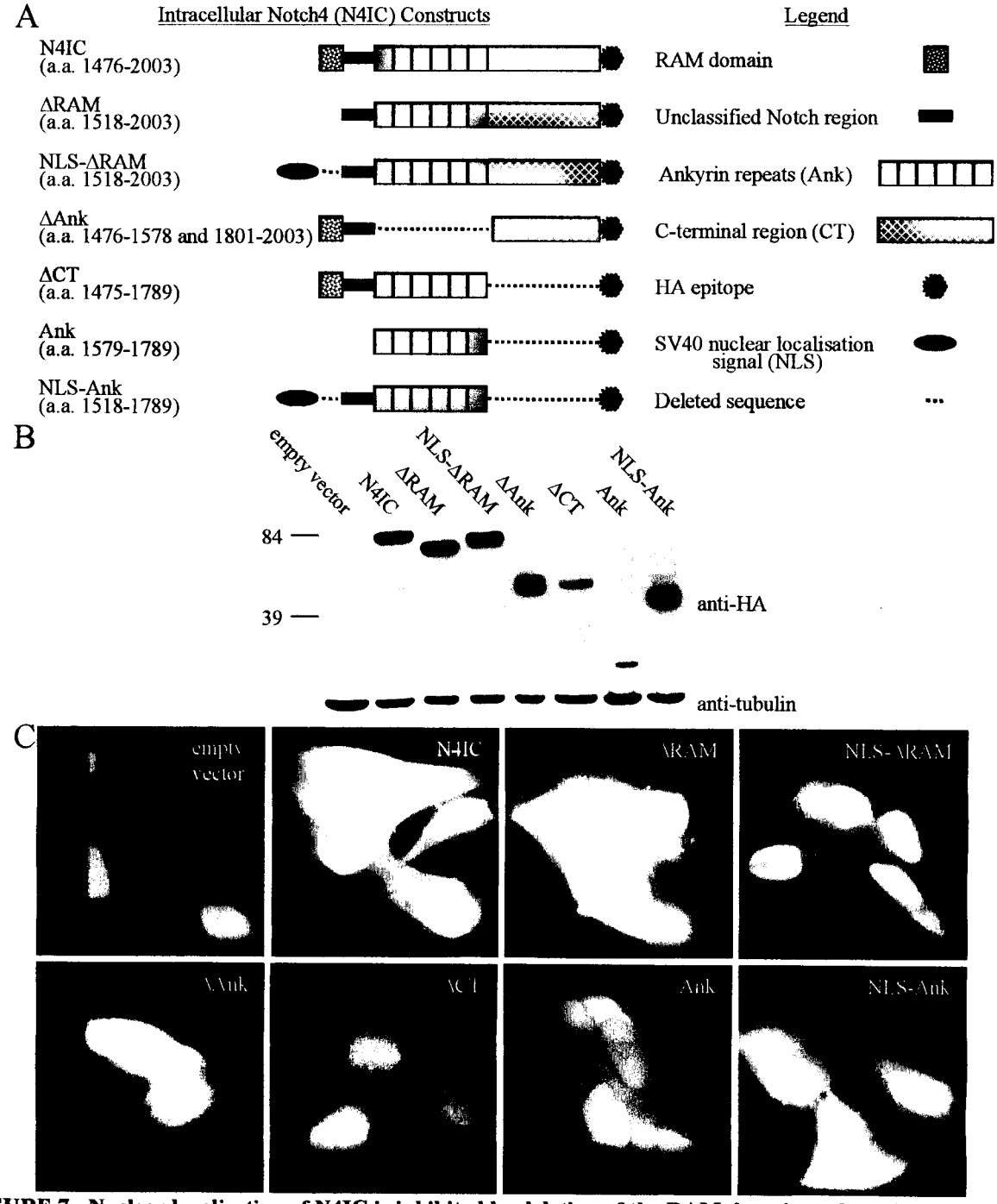


FIGURE 7. Nuclear localisation of N4IC is inhibited by deletion of the RAM domain and can be restored by fusion of an SV40 nuclear localisation signal. (A) Structure diagrams of HA epitope-tagged N4IC and its related deletion constructs. The amino acid (a.a.) numbers from the 2003 residue human Notch4 protein that are included in each construct are indicated in parentheses. (B) Expression of N4IC constructs in HMEC as detected by immunoblotting of total cellular lysates with a monoclonal anti-HA antibody. Protein loading was compared by stripping and re-probing with a monoclonal anti-tubulin antibody. (C) Expression and subcellular localisation of N4IC constructs as detected by immunofluorescent staining of transduced HMEC lines with a monoclonal anti-HA primary antibody and an Alexa Fluor 488-conjugated secondary antibody. Nuclei are counterstained with DAPI. Original magnification, 400x.

In an attempt to induce greater nuclear localisation of ΔRAM-N4IC and Ank, a simian virus 40 (SV40) nuclear localisation signal (NLS) sequence was attached to the N-terminus of each, creating NLS-ΔRAM-N4IC and NLS-Ank, respectively. The NLS-Ank protein also included amino acid residues found between the RAM domain and ankyrin repeats that were not included in the Ank construct. Expression of the NLS-ΔRAM-N4IC and NLS-Ank constructs was confirmed by Western blotting (Figure 7B). Immunofluorescent staining showed that fusion of the NLS sequence restored the ability of these mutants to localise to the nucleus (Figure 7C). The NLS-ΔRAM-N4IC construct was strongly nuclear with faint staining in the cytoplasm while the NLS-Ank protein stained evenly throughout the nucleus and cytoplasm. To focus on Notch signalling in the nucleus, all subsequent experiments were carried out with the NLS-ΔRAM-N4IC and NLS-Ank constructs rather than ΔRAM-N4IC and Ank.

The subcellular distributions for the N4IC mutants shown in Figure 7C are representative of the vast majority of cells from each population. However, the localisation of any particular construct did exhibit some variability (data not shown). For example, there were instances where N4IC appeared exclusively nuclear rather than throughout the nucleus and cytoplasm as seen in Figure 7C. Similar inconsistencies in Notch localisation within cells of a particular population have been reported before for Notch1 in NIH3T3 cells (Kopan et al., 1994). Therefore, it appears that some undefined variability in the cells of a given population can influence Notch subcellular localisation. Although purely speculative, this may be explained by differential expression of unknown factors that can modulate N4IC localisation. Such differences could arise from inherent heterogeneity in HMEC, which was not originally a monoclonal cell line (Ades et al., 1992). Alternatively, subtle alterations in the spatial arrangement of cellular clones may also induce differences in protein expression.

Further analysis of the immunoblotting and immunofluorescence data indicates that there were distinct differences in the regulation of the N4IC mutants when expressed in HMEC. In particular, ΔCT-N4IC and Ank, which both lack the CT domain, were expressed at significantly lower levels than the other constructs as noted by their weaker Western blot signals (Figure 7B). The immunofluorescence analysis confirmed reduced levels of ΔCT-N4IC and Ank, as seen by lower intensity staining on a per cell basis (Figure 7C). However, the proportion of cells expressing exogenous protein was similar for all of the N4IC mutant cell lines. These patterns of expression were consistent and observed for two separate Ank and NLS-Ank transductions as well as at least three separate transductions for the other N4IC mutants. Ironically, previous research indicates that the CT motif is important for SEL-10-directed degradation of Notch4 and therefore deletion of this domain would be expected to increase protein levels (Wu et al., 2001). Consequently, some unknown mechanism appears to differentially regulate the mRNA stability, translation efficiency, and/or protein stability of ΔCT-N4IC and Ank in HMEC as compared to the other mutants. Of note, the NLS-Ank mutant was expressed at considerably higher levels than the Ank construct. This does not appear to arise from differences in nuclear targeting alone, since NLS-ΔRAM-N4IC and ΔRAM-N4IC have comparable expression levels to one another despite their differences in nuclear/cytoplasmic distribution. Elevated NLS-Ank expression compared to Ank may signify a role for the region between the RAM domain and ankyrin repeats in regulating the mRNA stability, translation efficiency, and/or protein stability of Notch4.

3.2.2 Notch4-Induced Inhibition of Endothelial Sprouting Requires the Ankyrin Repeats

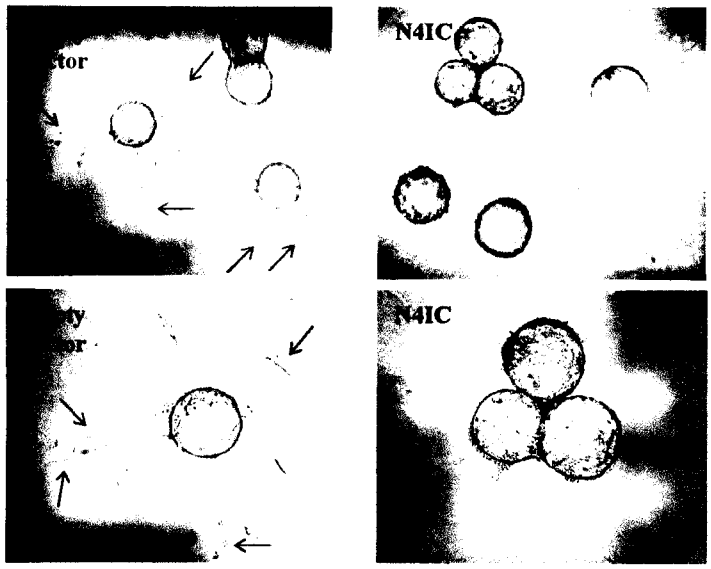
Activated Notch4 was previously shown to inhibit endothelial cell morphogenesis into tubules using an endothelial sprouting assay (Leong et al., 2002). In this *in vitro* model of

angiogenesis, endothelial cells coated on microcarrier beads are induced to form capillary-like tubes, or sprouts, within a three-dimensional fibrin gel (Nehls and Drenckhahn, 1995). These distinct cellular sprouts are easily counted to provide a quantitative measure of endothelial sprouting. This method represents a good mimic of blood vessel development *in vivo* and was chosen to determine which intracellular domains of Notch4 are required for its inhibition of *in vitro* angiogenesis.

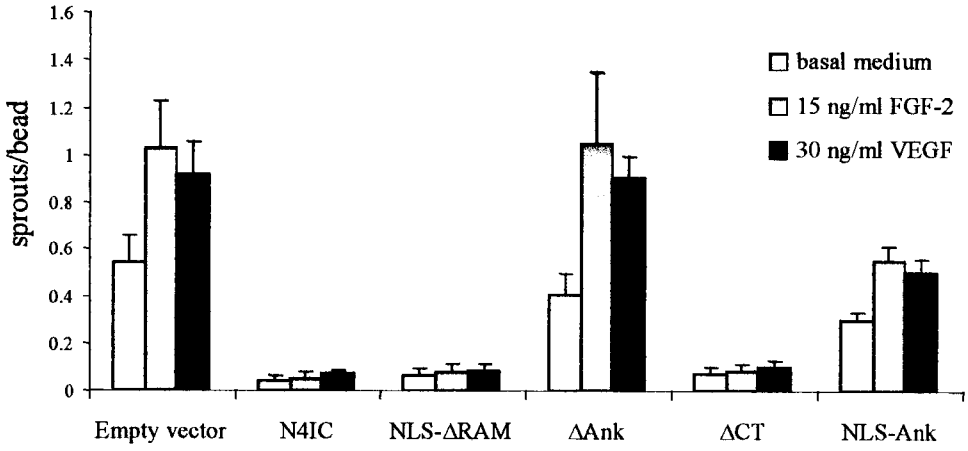
The endothelial sprouting assay was performed using HMEC lines transduced with the N4IC mutants or empty vector control. Fibrin-embedded cells were stimulated with basal medium containing 2% FCS or medium with added FGF-2 or VEGF. These growth factors were chosen because of their potent angiogenic effect and their known role in regulating both physiological and pathological vessel development (Auguste et al., 2001; Colville-Nash and Willoughby, 1997; Compagni et al., 2000; Dvorak et al., 1995; Giavazzi et al., 2001; Rak and Kerbel, 1997). The phase contrast micrographs in Figure 8A show beads coated with HMEC-N4IC or empty vector control cells and stimulated with FGF-2 for 72 hr. Populations of beads are shown in the upper panel. A section from each micrograph in the upper panel is enlarged and displayed in the lower panel for a closer view of the sprouting response. In the control cells, numerous endothelial tubes sprouted from the cell-coated beads as indicated by arrows. In contrast, no sprouting was detected in HMEC expressing N4IC.

The angiogenic response of each N4IC mutant cell line was analysed and quantitated as the number of sprouts per microcarrier bead (Figure 8B). Sprouts were counted if the tube formed was at least 150 μm in length after 72 hr in the gel. Results of the statistical analysis of the sprouting data in Figure 8B are shown in the table in Figure 8C. Sprouting in the control

A



B



C

P value ranges for multiple comparisons of different cell lines receiving a given growth factor stimulus						
	comparing each N4IC construct to empty vector control					Comparing N4IC and NLS-Ank
	N4IC	NLS-ΔRAM	ΔAnk	ΔCT	NLS-Ank	
basal medium	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$
FGF-2	$P < 0.001$	$P < 0.001$	$P > 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$
VEGF	$P < 0.001$	$P < 0.001$	$P > 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$

FIGURE 8. N4IC-induced inhibition of endothelial sprouting requires the ankyrin repeats. (A and B) Endothelial sprouting assay. (A) Phase-contrast micrographs of gelatin-coated microcarrier beads seeded with HMEC lines transduced with N4IC or empty vector control and stimulated with FGF-2 while suspended in a fibrin matrix. Images were taken after 72 hr of stimulation. Sections from each micrograph in the upper panel are enlarged and shown in the lower panel. Arrows indicate capillary-like sprouts of sufficient length to be counted. Continuous capillary-like structures extending from the cell-coated beads and reaching at least 150 μm in length (the diameter of an average bead) qualify as sprouts. Original magnification, 100x. (B) Quantitation of sprouting for HMEC-N4IC mutant, and empty vector control, cell lines after 3 days stimulation with basal medium or medium supplemented with FGF-2 or VEGF. Sprouts are counted as the number of tube-like structures formed per microcarrier bead (sprouts/bead) and graphed as means + standard deviations. Data are from a single experiment done in triplicate. The relative sprouting patterns are representative of at least four separate experiments. (C) Table of *P* value ranges for noteworthy comparisons using sprouting data from (B).

cells was induced by basal medium (0.55 sprouts/bead) and this activity was enhanced by the addition of either FGF-2 (1.0 sprouts/bead) or VEGF (0.91 sprouts/bead). Activated Notch4 almost completely blocked this angiogenic effect, reducing basal sprouting to 0.04 sprouts/bead, with little increase upon addition of FGF-2 (0.05 sprouts/bead) or VEGF (0.07 sprouts/bead). This inhibitory effect was retained in N4IC mutants lacking either the RAM domain or the CT. In all cases for these two mutants, mean sprouting was less than 0.10 sprouts per bead with little difference between cells maintained in basal medium and those stimulated with FGF-2 or VEGF. Conversely, deletion of the Notch4 ankyrin repeats abrogated the receptor's ability to block endothelial sprouting. The number of sprouts formed by the ΔAnk-N4IC cells in basal medium was 0.40 sprouts/bead and this sprouting was increased to 1.0 sprouts/bead by FGF-2 and to 0.90 sprouts/bead by VEGF. Hence, the sprouting of the N4IC and ΔAnk-N4IC cells was quantitatively similar in response to each stimulus.

Since the ankyrin repeats were essential for Notch4-mediated inhibition of endothelial sprouting, the next question was whether this domain alone was sufficient for the receptor's activity. As shown in Figure 8B, HMEC expressing only the ankyrin domain showed reduced sprouting in basal medium (0.29 sprouts/bead) ($P < 0.001$), FGF-2 medium (0.55 sprouts/bead) ($P < 0.001$), and VEGF medium (0.50 sprouts/bead) ($P < 0.001$) as compared to vector control cells. However, the quantity of tubes formed in the HMEC-NLS-Ank cells was significantly greater than that observed for the HMEC-N4IC cells for all stimuli ($P < 0.001$). Therefore, the ankyrin repeats alone provide a partial inhibition of serum-induced, as well FGF-2 and VEGF-induced, angiogenic sprouting. In summary, activated Notch4 strongly inhibits endothelial cell sprouting. This inhibition requires the ankyrin repeat domain, but not the RAM domain or CT,

of Notch4. The ankyrin repeats alone are able to inhibit sprout formation, but to a significantly lesser degree than N4IC.

3.2.3 Notch4 Downregulates VEGFR2 mRNA but not FGFR1 mRNA

The Notch signalling pathway has been shown to downregulate expression of VEGF Receptor 2 (VEGFR2) mRNA in endothelial cells and to reduce the responsiveness of these cells to VEGF-induced proliferation and tube formation in collagen (Taylor et al., 2000; Henderson et al., 1998). Therefore, it was important to determine whether the N4IC-induced inhibition of VEGF- and FGF-2-induced HMEC sprouting was simply a result of downregulation of the cognate growth factor receptors. VEGFR2 and FGFR1 were specifically analysed because they are known to be expressed in endothelial cells and they have established roles in mediating the angiogenic responses to VEGF and FGF-2, respectively (Colville-Nash and Willoughby, 1997; Dvorak et al., 1995; Rak and Kerbel, 1997).

The RT-PCR results in Figure 9 show the levels of VEGFR2 and FGFR1 mRNA expressed in each of the HMEC-N4IC mutant cell lines. In agreement with the results published for other endothelial cell types, enforced expression of activated Notch downregulated VEGFR2 mRNA expression in HMEC. Paradoxically, the other Notch4 mutants that strongly inhibited HMEC sprouting, NLS-ΔRAM-N4IC and ΔCT-N4IC, caused a considerable increase in expression of VEGFR2 transcripts (Figure 9). Similarly, the NLS-Ank construct, which partially inhibits cellular tube formation, induced a strong increase in VEGFR2 expression. In contrast, the HMEC-ΔAnk-N4IC cells had VEGFR2 mRNA levels similar to the vector control cells. FGFR1 mRNA was expressed at comparable levels in all of the cell lines (Figure 9).

In conclusion, downregulation of VEGFR2 does not appear to be the primary mechanism

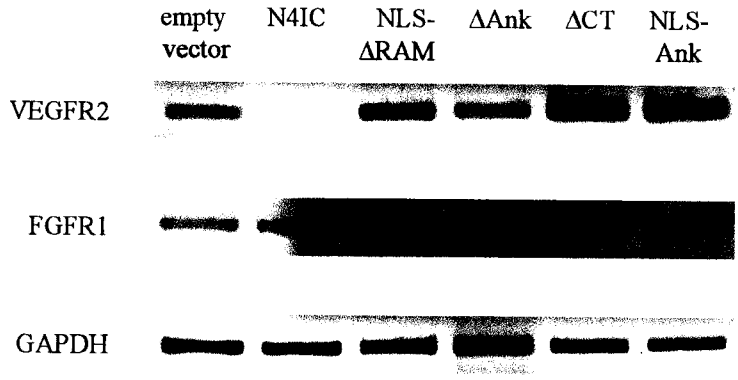


FIGURE 9. N4IC downregulates VEGFR2 mRNA but not FGFR1 mRNA. RT-PCR performed using single-stranded cDNA reverse transcribed from total RNA isolated from HMEC-N4IC mutant, and empty vector control, cell lines. PCR amplifications were done with primers specific for VEGFR2, FGFR1, and GAPDH cDNA sequences. Amplification of the GAPDH fragment was used as a control to demonstrate equivalent levels of cDNA input. The relative mRNA expression patterns are representative of at least two separate experiments.

for N4IC-induced inhibition of endothelial sprouting because the NLS-ΔRAM-N4IC, ΔCT-N4IC, and Ank mutants all strongly upregulate VEGFR2 transcripts while also inhibiting sprouting. In addition, the modulation of FGF-2-induced *in vitro* angiogenesis by N4IC cannot be explained by changes in expression of the FGFR1 gene since the mRNA levels for this target remained similar for the N4IC mutant and control cell lines.

Chapter 4

Notch4-Induced Inhibition of Endothelial Sprouting Involves CBF1-Dependent Signalling

4.1 Introduction

The canonical Notch signalling pathway involves DSL ligand interaction with Notch causing subsequent proteolytic cleavage of the receptor and release of NIC, an activated intracellular form of the protein (Fleming, 1998; Mizutani et al., 2001). NIC then translocates to the nucleus and activates CBF1, a ubiquitously expressed DNA-binding protein that regulates gene transcription (Ling et al., 1994; Schroeter et al., 1998). Activation of CBF1 by NIC induces expression of target genes, including those of the HES and HRT families (Iso et al., 2003). However, there is growing evidence that Notch may also signal through CBF1-independent pathways. In particular, Notch appears able to inhibit transcriptional activators such as MASH1 and E47 independently of CBF1 (Matsuno et al., 1998; Ordentlich et al., 1998; Yamamoto et al., 2001). Therefore, it was important to determine the significance of CBF1 activation during Notch4 signalling in HMEC. The objective of the research described in this chapter was to identify the N4IC domains required for activation of CBF1 and to determine whether N4IC-initiated inhibition of HMEC sprouting involves signalling through CBF1. It was hypothesised that Notch4 activation of CBF1 would require the ankyrin repeats and that receptor-independent activation of CBF1 would mimic the inhibition of *in vitro* angiogenesis by N4IC.

4.2 Results and Conclusions

4.2.1 Notch4 Upregulation of CBF1-Dependent Gene Expression Requires the Ankyrin Repeats

Activation of endogenous CBF1 by the ectopically expressed N4IC mutants was analysed using reporter plasmids containing promoters with CBF1-binding sites. First, each HMEC-N4IC

mutant cell line was transiently transfected with a reporter construct containing four copies of a CBF1 binding element upstream of an SV40 promoter-driven firefly luciferase gene (4xCBF1 luciferase) (Hsieh et al., 1996). The resultant firefly luciferase activities relative to an internal luciferase control are shown in Figure 10A. As expected, HMEC expressing N4IC had increased (5.1-fold) CBF1-driven luciferase activity as compared to vector control cells. N4IC activity was maintained by mutants lacking the RAM domain (5.9-fold) and the CT (5.5-fold), but not by the ΔAnk-N4IC construct. Expression of the NLS-tagged ankyrin repeats resulted in a mild 1.7-fold increase in luciferase activity that was not statistically significant ($P > 0.05$).

Next, a luciferase reporter driven by a 10-kb region of the HRT2 promoter (Nakagawa et al., 2000) was used as a more relevant indicator for Notch activation of natural CBF1 targets. As shown in Figure 10B, N4IC, NLS-ΔRAM-N4IC and ΔCT-N4IC were once again all capable of significantly activating the reporter. N4IC induced a 4.3-fold increase in luciferase activity while NLS-ΔRAM-N4IC and ΔCT-N4IC augmented luciferase production 4.0-fold and 2.9-fold, respectively. Deletion of the CT slightly reduced N4IC activation of the HRT2 promoter, indicating that this domain may be involved in Notch4-induced derepression and/or coactivation of CBF1 at specific promoters. Deletion of the ankyrin repeat domain once again completely abolished N4IC-induced derepression/coactivation of the CBF1-dependent promoter. Similar to its weak effects on the 4xCBF1 luciferase construct, the NLS-Ank mutant caused a 1.3-fold increase in HRT2 luciferase activity that was not statistically significant ($P > 0.05$).

HRT and HES mRNA levels were analysed to determine the effect of each Notch mutant on the expression of endogenous CBF1-dependent genes. Total RNA was isolated from each HMEC-N4IC mutant cell line and used to generate random hexamer-primed single-stranded

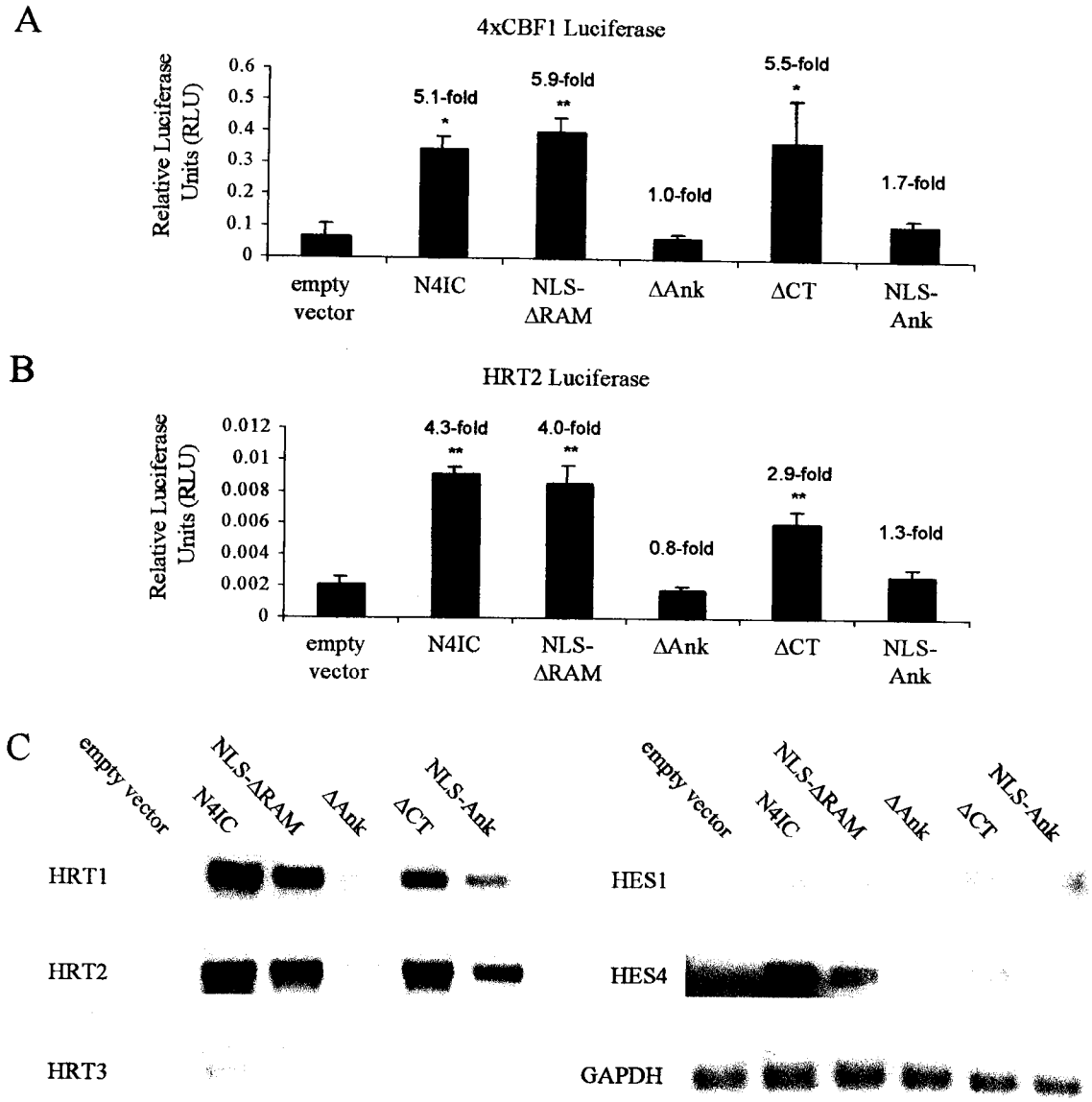


FIGURE 10. N4IC upregulation of CBF1-dependent gene expression requires the ankyrin repeats. (A and B) Luciferase assays using a reporter construct with four copies of a CBF1-binding element upstream of an SV40 promoter-driven firefly luciferase gene (4xCBF1 Luciferase) (A) or a HRT2 promoter-driven firefly luciferase gene (HRT2 Luciferase) (B). Reporter plasmids were electroporated into HMEC-N4IC mutant, or empty vector control, cell lines along with a CMV promoter-driven renilla luciferase plasmid used as a normalisation control for transfection efficiency. Cell lysates were harvested 48 hours after electroporation and the relative luciferase units (RLU) were determined as the ratio of firefly-derived luminescence over renilla-derived luminescence. Data are means + standard deviations for a single experiment done in triplicate. Fold increases are reported for each N4IC construct cell line as compared to the empty vector control cell line. A single asterisk (*) indicates a P value < 0.01 and a double asterisk (**) a P value < 0.001 for sample means compared to the empty vector control. The relative RLU patterns in (A) are representative of at least three separate experiments. (C) RT-PCR performed using single stranded cDNA reverse transcribed from total RNA isolated from HMEC-N4IC mutant, or empty vector control, cell lines. PCR amplifications were done with primers specific for HRT1-3, HES1, HES4, and GAPDH cDNA sequences. Amplification of the GAPDH fragment was used as a control to demonstrate equivalent levels of cDNA input. The relative patterns of mRNA expression are representative of at least four separate experiments except the HES4 result, which was observed twice.

cDNA. Figure 10C shows the RT-PCR results using primers targeted to cDNA for HRT1-3, HES1, and HES4. HMEC transduced with empty vector was used a baseline control and PCR for GAPDH provided a control for cDNA input. The HRT genes were the primary focus because their expression has been established in the mammalian vasculature (Chin et al., 2000; Leimeister et al., 2000; Nakagawa et al., 1999). As well, N4IC has been shown to induce HRT1 expression in cultured human endothelial cells (Taylor et al., 2002). HES1 was chosen because it has been the most extensively studied of all known CBF1-dependent genes (Iso et al., 2003). HES4 was examined in addition to HES1 and the HRTs to determine if there were any general differences in N4IC-mediated effects on HES family members compared to HRT family members.

N4IC upregulated expression of all of the HRT and HES transcripts analysed (Figure 10C). In particular, the HRT1 and HRT2 genes were strongly induced while modest increases in HRT3 and HES1 expression were detected. N4IC mutants lacking the RAM or CT still positively regulated HRT and HES gene expression. Each showed strong upregulation of HRT1 and HRT2, although induction was visibly lower than that stimulated by N4IC. However, ΔRAM-N4IC and ΔCT-N4IC induced similar levels of HRT3, HES1, and HES4 compared to N4IC. Conversely, the ΔAnk-N4IC mutant was not capable of inducing gene expression for any of the targets analysed. These findings are in concordance with the luciferase assay results and show that the ankyrin repeats are essential for Notch4-mediated transactivation of CBF1-dependent genes in HMEC.

On the one hand, luciferase reporter assays indicate that the ankyrin domain alone is not capable of significantly activating CBF1-dependent transcription. On the other hand, RT-PCR analysis of endogenous CBF1 targets reveals a more complex reality. The NLS-Ank mutant

stimulated a distinct, though partial, upregulation of HRT1 and HRT2 (Figure 10C). NLS-Ank weakly upregulated HES1 mRNA, although this induction was comparable to that induced by N4IC. As for the other CBF1-dependent genes, little to no induction of HRT3 or HES4 was detected in the HMEC-NLS-Ank cell line. The variations in the ability of the ankyrin domain alone to upregulate different HRT and HES transcripts imply that distinct mechanisms are involved in the N4IC-mediated transcriptional activation of various genes. The mechanism by which Notch potentiates transcription at a particular site may depend on whether derepression and coactivation of CBF1 is required for upregulation, or whether derepression alone is sufficient. However, the results in this chapter do not indicate that there are any consistent differences between the regulation of HRT family members and HES family members.

In summary, overexpression of activated Notch4 upregulates CBF1-dependent gene expression in HMEC as seen by luciferase reporter assays and RT-PCR. This activity is completely abolished by deletion of the ankyrin repeat domain, but not the RAM or CT motifs. The ability of the ankyrin repeats alone to positively regulate HRT and HES transcripts depends on the family member analysed.

4.2.2 CBF1-VP16 Upregulates CBF1-Dependent Gene Expression

There is a correlation between the ability of the various N4IC mutants to inhibit endothelial sprouting and to activate CBF1. In both instances, Notch4 function is dependent on the ankyrin repeats, although this domain alone is insufficient for full function. In addition, the RAM and the CT are largely dispensable for inhibition of angiogenesis and for activation of CBF1-dependent genes. To draw a stronger connection between these two functions of Notch, a constitutively-active form of CBF1, known as CBF1-VP16, was tested for its ability to mimic N4IC-induced inhibition of HMEC sprouting. CBF1-VP16 was cloned based on an established

construct that fuses the RBP3 isoform of CBF1 to the transactivation domain of the herpes simplex virus transcription factor VP16 (Waltzer et al., 1995). The C-terminally fused VP16 recruits transcriptional coactivators, thereby inducing expression of downstream genes independently of Notch.

Stably-transduced HMEC lines expressing CBF1-VP16 were selected along with control cell lines transduced with either N4IC or empty vector. The CBF1-VP16 construct was FLAG-tagged for detection of the exogenous protein. Expression of CBF1-VP16 and the HA-tagged N4IC protein were confirmed by Western blotting (Figure 11A). Tubulin levels demonstrated equal loading of protein. Of note, isolation of stably-transduced cell lines expressing detectable levels of CBF1-VP16 was difficult to achieve, presumably due to strong negative selection against long-term, constitutive expression of this protein. A similar, although seemingly less severe, effect can be seen when overexpressing N4IC in HMEC. HMEC expression of the HA-tagged N4IC decreases with passage even though the cell lines are stably-transduced (data not shown). However, transient transfection methods are impractical for most experiments due to their low efficiency in HMEC.

The first step in assessing CBF1-VP16 function was to verify its ability to transactivate target genes using a luciferase reporter assay and RT-PCR. Activation of the 4xCBF1 luciferase construct was confirmed (Figure 11B). CBF1-VP16 and N4IC induced similar levels of luciferase activity, generating 4.0-fold and 3.4-fold increases over vector control, respectively. Figure 11C shows the levels of HRT1-3 mRNA expression in the CBF1-VP16, N4IC, and empty vector control cell lines as determined by RT-PCR. The HRT genes were specifically analysed

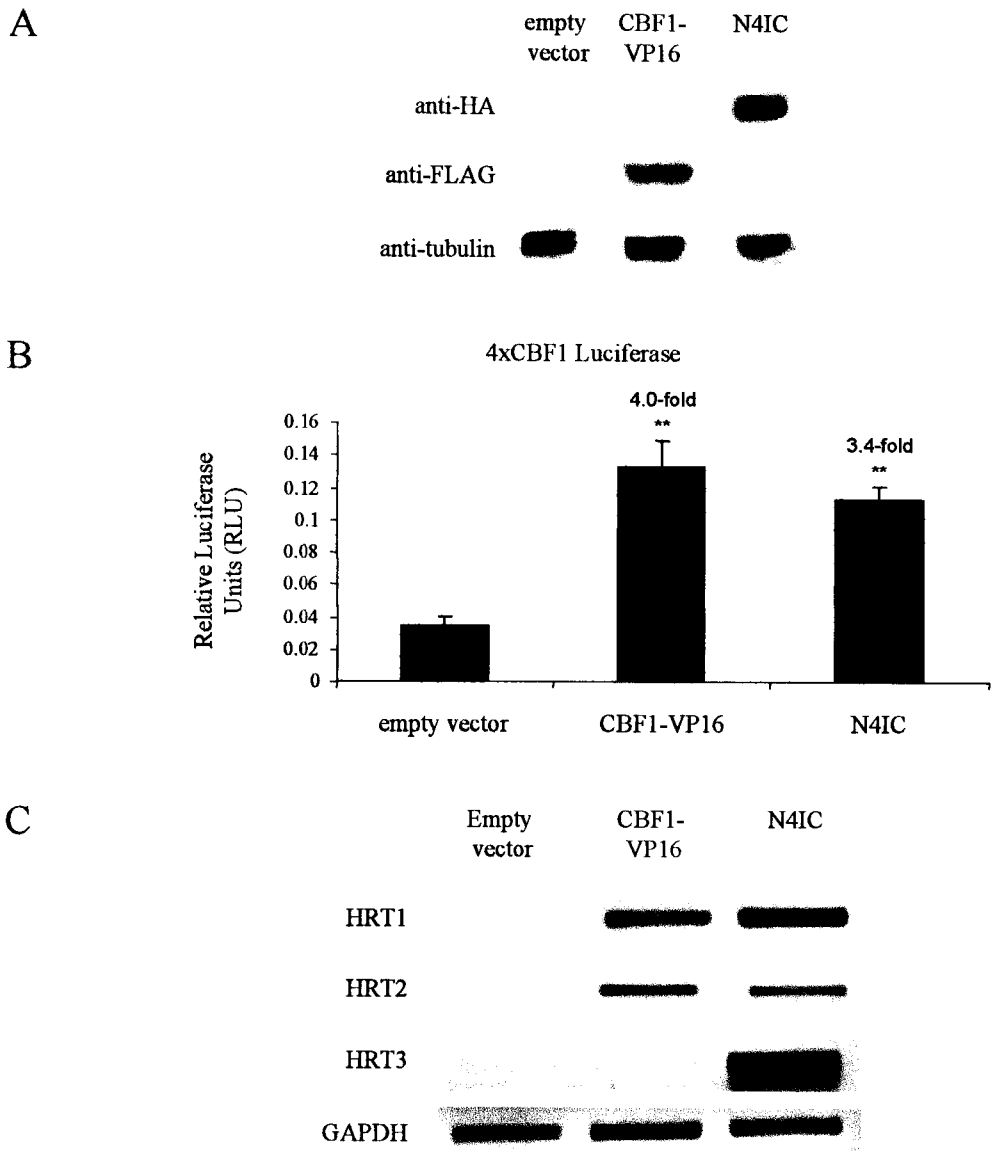


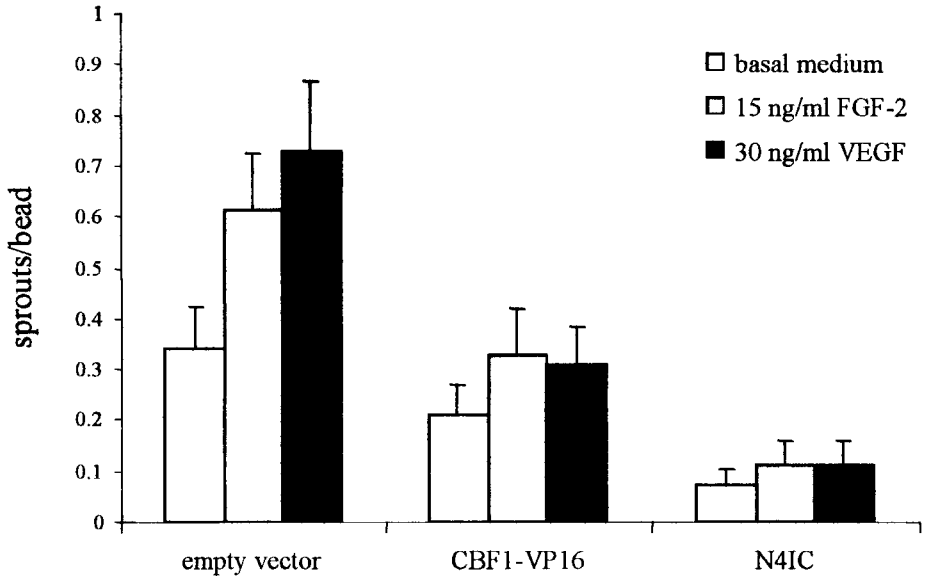
FIGURE 11. CBF1-VP16 upregulates CBF1-dependent gene expression in HMEC. (A) Expression of CBF1-VP16 and N4IC in HMEC as detected by immunoblotting of total cellular lysates with a monoclonal anti-FLAG antibody and a monoclonal anti-HA antibody, respectively. Protein loading was compared with a monoclonal anti-tubulin antibody. All three immunoblots were done in parallel with the same set of lysates. (B) Luciferase assay using the 4xCBF1 luciferase construct electroporated into HMEC-CBF1-VP16, -N4IC, or -empty vector control cell lines along with a CMV promoter-driven renilla luciferase plasmid. Cell lysates were harvested 48 hours after electroporation and the relative luciferase units were determined as the ratio of firefly-derived luminescence over renilla-derived luminescence. Data are means + standard deviations for a single experiment done in triplicate. Fold increases are reported for CBF1-VP16 and N4IC cell lines as compared to the empty vector control. A double asterisk (**) indicates a P value < 0.001 for sample means compared to the empty vector control. The relative RLU patterns are representative of at least three separate experiments. (C) RT-PCR performed using single stranded cDNA reverse transcribed from total RNA isolated from HMEC-CBF1-VP16, -N4IC, or -empty vector control cell lines. PCR amplifications were done with primers specific for HRT1-3 and GAPDH cDNA sequences. Amplification of the GAPDH fragment was used as a control to demonstrate equivalent levels of cDNA input. The relative patterns of mRNA expression are representative of at least three separate experiments.

because their expression has been established in the mammalian vasculature (Chin et al., 2000; Leimeister et al., 2000; Nakagawa et al., 1999) and HRT1 and HRT2 were the most strongly induced of all transcripts analysed in the HMEC-N4IC cell lines (see Chapter 4.2.1, Figure 10C). CBF1-VP16 signalling upregulated HRT1 and HRT2 transcripts but did not generate a detectable increase in HRT3 compared to the empty vector control. The failure to detect HRT3 upregulation in the HMEC-CBF1-VP16 cell lines may reflect an inherent inability of CBF1-VP16 to activate HRT3 transcription. Alternatively, it may stem from difficulties in isolating cell lines expressing high levels of CBF1-VP16. In conclusion, CBF1-VP16 is capable of transactivating CBF1-dependent genes as detected by a luciferase reporter assay and RT-PCR analysis of HRT1 and HRT2 expression. However, there was no detectable increase in HRT3 mRNA expression in the HMEC-CBF1-VP16 cell lines.

4.2.3 CBF1-VP16 Partially Mimics N4IC-Mediated Inhibition of Endothelial Sprouting

CBF1-VP16 was tested for its ability to regulate endothelial cell morphogenesis into tubules using the endothelial sprouting assay. HMEC-CBF1-VP16, -N4IC, or empty vector control cell lines were stimulated with either basal medium or medium supplemented with FGF-2 or VEGF, and the ensuing sprouting responses were quantitated (Figure 12A). Results of the statistical analysis of the sprouting data in Figure 12A are shown in the table in Figure 12C. In agreement with the results in Chapter 3 (Figure 8B), the sprouting of HMEC control cells in basal medium (0.32 sprouts/bead) was significantly enhanced by the addition of FGF-2 (0.74 sprouts/bead) and VEGF (0.65 sprouts/bead). Enforced expression of N4IC strongly inhibited basal sprouting (0.06 sprouts/bead) and also prevented enhancement of sprouting by FGF-2 (0.07 sprouts/bead) and VEGF (0.04 sprouts/bead). Compared to vector control, CBF1-VP16 also

A



B

P value ranges for multiple comparisons of different cell lines receiving a given growth factor stimulus			
	empty vector to CBF1-VP16	empty vector to N4IC	N4IC to CBF1-VP16
basal medium	$P < 0.05$	$P < 0.001$	$P < 0.05$
FGF-2	$P < 0.01$	$P < 0.001$	$P < 0.05$
VEGF	$P < 0.001$	$P < 0.001$	$P < 0.05$

FIGURE 12. CBF1-VP16 partially mimics N4IC-mediated inhibition of HMEC sprouting. (A) Endothelial sprouting assay for HMEC lines transduced with either CBF1-VP16, N4IC, or empty vector control. Cells were seeded on gelatin-coated beads, suspended in fibrin matrices, and stimulated for 3 days with basal medium or medium supplemented with FGF-2 or VEGF. Sprouts per bead were counted and graphed as means + standard errors for an average of four experiments, each done in triplicate. (B) Table of *P* value ranges for noteworthy comparisons using sprouting data from (A).

caused a significant reduction in endothelial cell sprouting in response to basal medium (0.12 sprouts/bead) and medium supplemented with FGF-2 (0.28 sprouts/bead) or VEGF (0.28 sprouts/bead). The increase in HMEC-CBF1-VP16 basal sprouting induced by FGF-2 and VEGF was not statistically significant ($P > 0.05$ for both). Moreover, the inhibition of sprouting by CBF1-VP16 was significantly lower than that induced by N4IC for all stimuli.

In conclusion, constitutively-active CBF1 inhibits HMEC sprouting, but not as severely as N4IC. This indicates that Notch4 blocks *in vitro* angiogenesis by triggering the induction of CBF1-dependent gene expression and may additionally activate CBF1-independent pathways to enhance this inhibition. As a result, future research will attempt to identify CBF1-independent pathways that are initiated by N4IC to block endothelial sprouting. It has not been determined whether Notch4-induced inhibition of endothelial sprouting is, to any degree, dependent upon CBF1. If Notch4 does indeed prevent sprouting through CBF1-independent pathways, it is possible that these signals are capable of full inhibition regardless of CBF1 activation. For that reason, CBF1 function must be blocked in the HMEC-N4IC cell lines to determine if Notch4-induced inhibition of endothelial sprouting requires CBF1-dependent pathways. Previous attempts were made to abrogate Notch activation of CBF1 using a dominant-negative human CBF1. This construct was cloned based on an established dominant-negative mouse CBF1 known as RBP-Jκ R218H (Chung et al., 1994). RBP-Jκ R218H can bind Notch but is unable to bind DNA. In theory, overexpression of RBP-Jκ R218H obstructs activation of wild-type CBF1 by sequestering available Notch. However, co-transducing the dominant-negative human CBF1 and N4IC into HMEC did not strongly inhibit the transactivation of CBF1-dependent genes, as seen by luciferase assays and RT-PCR (data not shown). It is believed that this is principally due to the inability of the dominant-negative CBF1 to completely soak up the exogenous N4IC,

which itself is highly overexpressed. Transient transfection methods where the dominant-negative CBF1 can be supplied in large excess to N4IC are not practical because of the low efficiency of these transfection methods in HMEC. Currently, the Karsan lab is focusing on the use RNA interference (Cullen, 2002; Paddison et al., 2002) to block the expression and activity of CBF1 to further elucidate its function during Notch4 signalling in HMEC.

Chapter 5

Summary and Discussion

5.1 Introduction

5.1.1 *In vitro* Modelling of Angiogenesis

Angiogenesis is the formation of new blood vessels from the existing vasculature and is important in mammalian development, wound healing, and pathogenesis (Carmeliet and Jain, 2000; Papetti and Herman, 2002; Risau, 1997). In particular, it is the essential role that angiogenesis plays in regulating many human diseases that has inspired considerable research into this complex process. Numerous *in vitro* models have been developed to help further our understanding of angiogenesis. These systems typically involve the formation of capillary-like tubes by endothelial cells layered on, or incorporated within, collagen or fibrin gels (Meyer et al., 1999; Nehls and Drenckhahn, 1995; Nehls et al., 1994; Yang et al., 1999). Alternatively, endothelial cord-like structures can be induced in Matrigel, an extracellular matrix component isolated from a sarcoma cell line (Yang et al., 1999). These tube formation or endothelial morphogenesis assays provide a valuable tool for a rapid, efficient, and quantitative assessment of the angiogenic properties of cells and molecular factors. However, these models can generate inconsistent and/or invalid conclusions if the results are not interpreted carefully (Auerbach et al., 2003). One concern with any *in vitro* system is whether the cultured cells offer a reasonable representation of their *in vivo* counterparts. Even when primary cells are used, brief culturing prior to experimentation may alter their phenotype. Of particular concern with cultured endothelial cells is that passage selects for proliferative populations, whereas the *in vivo* cells they are intended to model are largely quiescent. Furthermore, any given *in vitro* assay typically incorporates only one specific type of endothelial cell. In the organism, the endothelium is

diverse and even the microvessels of a given vascular bed can be a heterogeneous population (Risau, 1995). Tube formation assays most often study endothelial cells free of interaction with the many other cell types and molecules that contribute to vessel remodelling in organisms. *In vivo*, pericytes and vascular smooth muscle cells have strong influence on the stability and morphogenesis of vessels through contact with the endothelium (Nehls et al., 1992). Additionally, a given stimulus that does not directly activate the endothelium may stimulate perivascular cells, stromal cells, or immune cells, in particular macrophages, to release secondary factors that regulate angiogenesis (Crowther et al., 2001).

Ultimately, any given *in vitro* model can only reconstruct some aspects of the complex process of *in vivo* neovascularization. The particular events mimicked in one *in vitro* system may not be the same as those modelled by a different technique, even if the various assays all focus on endothelial cell morphogenesis. For example, the endothelial sprouting assay starts with endothelial cells coated on microcarrier beads suspended in a three-dimensional matrix. These cells have been maintained confluent on the beads for several days prior to incorporation into the matrix. In theory, this system represents a fair model of a resting blood vessel where endothelial cells also form a tightly interlocked structure in three dimensions. Experiments that entail layering cells on top of a matrix and inducing subsequent penetration differ in that the initial sheet of cells is in two dimensions. Other methods start with individual endothelial cells suspended within a matrix component. These are distinct in that morphogenesis begins with the stimulation of cells that are already free of intercellular adhesions. Interpreted correctly, different *in vitro* tube formation assays can help decipher how and when a molecule is functioning during the angiogenic process. Nevertheless, to be certain of the net angiogenic effect of a given molecule, *in vivo* assays should be employed to confirm any *in vitro* findings.

This is exemplified by work with tumour necrosis factor-α, which was thought to be anti-angiogenic based on *in vitro* assays but proved to be a potent angiogenic factor *in vivo* (Frater-Schroder et al., 1987). Conversely, there are instances when *in vivo* results are difficult to interpret and a return to simpler *in vitro* models is necessary to decipher what is happening at individual steps of a complex process. This is the case with Notch, which appears to have both pro- and anti-angiogenic activity based on loss-of-function and gain-of-function mutations in mice (Krebs et al., 2000; Uyttendaele et al., 2001).

5.1.2 Notch and Angiogenesis

Notch receptors have been implicated in regulating angiogenesis. The Notch4 isoform may primarily function in vessel development and homeostasis as it is predominantly expressed in the vascular endothelium (Krebs et al., 2000; Li et al., 1998; Loomes et al., 2002; Shirayoshi et al., 1997; Uyttendaele et al., 1996; Villa et al., 2001). In mouse embryos, Notch4-null mutations enhance the defects in vascular remodelling caused by Notch1 deficiency (Huppert et al., 2000; Krebs et al., 2000). Constitutive Notch4 activity in the endothelium causes a similar failure of the primary capillary plexus to undergo remodelling (Uyttendaele et al., 2001). Enforced expression of activated intracellular Notch4 (N4IC) also inhibits angiogenesis in the chick embryo (Leong et al., 2002). In tissue culture, N4IC inhibits human microvascular endothelial cell (HMEC) sprouting in three-dimensional fibrin (Leong et al., 2002) but enhances rat brain microvessel endothelial cell cord-formation on two-dimensional collagen (Uyttendaele et al., 2000). Therefore, based on *in vivo* and *in vitro* data, it appears that Notch4 signalling in endothelial cells plays a role in inhibiting as well as enhancing morphogenesis into vascular networks. This may signify that Notch has different functions in distinct endothelial populations.

Alternatively, Notch signalling may be inhibitory to certain phases of the angiogenic process while being required for other stages of vessel formation.

Angiogenesis requires a synchronised order of several cellular events involving a diverse range of factors (Risau, 1997). In the early stages, the targeted endothelium must be activated leading to local vessel destabilisation followed by endothelial proliferation and migration. Conversely, the later stages of the process require the activated endothelial cells to settle into a stable and quiescent vasculature. Notch potentially blocks angiogenesis on multiple levels by inhibiting proliferation and migration as well as promoting cell adhesion and stability. Not all of these responses are necessarily required and those that are actually employed may vary from one endothelial cell type to another. For example, published research and unpublished work from the Karsan lab show that Notch can inhibit proliferation in a variety of endothelial cell types (Liu et al., 2003; Taylor et al., 2002). In contrast, N4IC does not prevent cell cycle progression in HMEC (Leong et al., 2002), likely due to the effects of the SV40 large T antigen used to transform this cell line (Ades et al., 1992). The viral large T antigen stimulates progression through the cell cycle by binding and inactivating the cellular retinoblastoma tumour suppressor protein, Rb, which normally promotes cell cycle arrest (Lee and Cho, 2002). Although N4IC does not inhibit proliferation in HMEC, it does increase β_1-integrin-mediated adhesion (Leong et al., 2002). While this activity was shown to inhibit migration through collagen, such promotion of cell-matrix contacts may also serve to increase the survival, stability, and long-term maintenance of vessels.

Notch activity has been correlated with anti-apoptotic phenotypes in a variety of cell lineages, including endothelial cells (Deftos et al., 1998; Liu et al., 2003; Pear et al., 1996; Shelly et al., 1999). The promotion of endothelial survival and stability may explain why Notch is

apparently necessary for the angiogenic development of vessels. In one specific study, overexpression of N1IC and HES1 in aortic endothelial cells stabilised cellular cord formation on Matrigel and enhanced tube length and network formation in collagen gels (Liu et al., 2003). However, NIC and HES1 blocked [^{3}H]thymidine uptake, indicating cell cycle arrest. Notch also inhibited apoptosis due to serum starvation. Therefore, Notch enhancement of angiogenesis evidently stemmed from its activity in the later stages of the process involving vessel formation and stabilisation rather than earlier phases requiring proliferation. In other research, Notch signalling was shown to be dormant in proliferating and migrating endothelial cells, but activated during endothelial network formation in three-dimensional collagen (Henderson et al., 2001; Taylor et al., 2002). As a result, both enforced activation and blocking of the Notch pathway inhibited tube formation. Thus, Notch signalling can have both pro- and anti-angiogenic consequences within a given endothelial cell type and its activity is therefore temporally regulated during morphogenesis. In this thesis, the role of Notch4 in inhibiting the morphogenic process of endothelial cell sprouting was further examined. The effect of constitutively-active Notch4 signalling within endothelial cells was specifically focused on.

5.2 Summary of Thesis Results

β_1-integrin activation and VEGFR2 downregulation have been identified as two of the potential effectors of Notch4 during the inhibition of endothelial cell morphogenesis into capillary-like structures (Leong et al., 2002; Taylor et al., 2002). Still, the primary mechanisms and pathways important for the anti-angiogenic activity of Notch4 in endothelial cells remain undetermined. Here, N4IC deletion mutants were used to ascertain which of the receptor's intracellular domains were important for inhibiting endothelial sprouting. These deletion mutants included constructs lacking either the RAM domain (ΔRAM-N4IC), all six ankyrin

repeats (ΔAnk-N4IC), or the C-terminal region (ΔCT-N4IC), as well as a construct consisting of the ankyrin repeats alone (Ank) (Figure 7A). Since Notch is supposed to modulate gene transcription in the nucleus, it was important to ensure that each mutant was targeted to this organelle. All of the N4IC mutants were found largely in the nucleus in HMEC except ΔRAM-N4IC and Ank, which localised primarily to the cytoplasm (Figure 7C). Fusion of an NLS sequence to these constructs, creating NLS-ΔRAM-N4IC and NLS-Ank, induced greater nuclear localisation. All subsequent experiments were carried out with the NLS-ΔRAM-N4IC and NLS-Ank mutants in place of ΔRAM-N4IC and Ank.

Angiogenesis was modelled using a quantitative *in vitro* assay in which N4IC was previously shown to block HMEC and human umbilical vein endothelial cell (HUVEC) morphogenesis into capillary-like sprouts in fibrin (Leong et al., 2002). In the present research, N4IC was verified to strongly inhibit HMEC sprouting in response to basal medium or medium supplemented with FGF-2 or VEGF (Figure 8). This inhibition required the ankyrin repeats but not the RAM or CT domains. The ankyrin domain alone conferred only partial inhibition of endothelial cell morphogenesis. N4IC decreased VEGFR2 mRNA expression, but not FGFR1 expression, in confluent cultures of HMEC (Figure 9). Furthermore, suppression of VEGFR2, but not inhibition of endothelial sprouting, was abrogated by deletion of the RAM or CT domains. This suggests that the downregulation of growth factor receptors by N4IC is not the primary mechanism for its anti-angiogenic effect.

While Notch may act through multiple pathways, the majority of research to date has focused on CBF1-dependent signalling. NIC and CBF1 bind in the nucleus to form a transactivation complex that upregulates downstream genes belonging to the HRT and HES families. Due to the prominence of this pathway, it was studied as a first step in determining the

downstream molecules important for relaying Notch4 signals in HMEC. Constitutive N4IC expression was shown to upregulate CBF1-dependent genes by luciferase reporter assays (Figure 10A, B). As with its anti-angiogenic activity, Notch4 activation of CBF1 was dependent on the ankyrin repeats but not the RAM or CT. N4IC also required the ankyrin repeats, but not the RAM or CT, to augment endogenous HRT and HES mRNA as seen by RT-PCR (Figure 10C). Moreover, the ankyrin repeats alone fully or partially upregulated HRT1, HRT2, and HES1, but induced little to no increase in HRT3 or HES4 expression. It is believed that the induction of HRT and HES mRNA by N4IC is due to transcriptional activation. However, it remains possible that changes in the stability of these mRNA transcripts are responsible for the observed effect.

Fusion of CBF1 to the transactivation domain of the viral protein VP16 created CBF1-VP16, which potentiated target genes in HMEC independently of exogenous N4IC (Figure 11). CBF1-VP16 was capable of inhibiting HMEC sprouting in response to basal medium, FGF-2, or VEGF (Figure 12). However, the CBF1-VP16-mediated block was not as pronounced as that induced by N4IC. Collectively, the results indicate that Notch4-induced inhibition of endothelial sprouting requires the ankyrin repeats and involves CBF1-dependent signalling, although CBF1-independent pathways may enhance the effect.

5.3 Discussion of Thesis Results and Future Directions

5.3.1 Long-term Overexpression of Activated Notch4

Ligand activation of endogenous Notch provides a transient signal that requires only a small amount of NIC in the nucleus and is terminated by protein degradation (Schroeter et al., 1998; Wu et al., 2001). As a result, there is concern that the N4IC activity reported here is an artefact of long-term overexpression. To address this, the Karsan lab is working on inducible expression systems to see if transient N4IC induction can inhibit endothelial sprouting. An

inducible system could also prove valuable for investigating the possibility that Notch has varying angiogenic effects during different stages of the sprouting process. Whereas constitutive Notch activity prevents sprouting, induction of Notch at later time points after endothelial cell activation may stabilise sprout formation.

Unpublished research in the Karsan lab suggests that long-term N4IC expression can induce an endothelial to mesenchymal transformation in a variety of cultured endothelial cells, including HMEC. Obviously, such a transformation may itself preclude angiogenesis. However, the N4IC mutants missing the RAM or CT do not efficiently induce this phenotypic switch, while the CBF1-VP16 cell lines are completely incapable of causing the transformation. Since all of these constructs can block sprouting, it appears that N4IC has specific anti-angiogenic effects that are unrelated to signals that cause a switch in the endothelial phenotype.

To induce more physiologically relevant levels of transient N4IC signals, a model that relies on ligand activation of the endogenous transmembrane Notch receptors could be employed. This strategy has been used successfully in studies analysing the morphogenic activity of Notch *in vitro* (Taylor et al., 2002; Uyttendaele et al., 2000). However, there is ambiguity as to which Notch receptor is being activated by ligand if the receptor-presenting cells express more than one Notch homologue. To simply lower expression of the N4IC mutants, weaker gene promoters could be used in place of the strong CMV promoter in the LNCX retroviral vector. Using reduced levels of activated Notch could also help address the possibility that certain deletion mutants remain highly active because of forced interactions due to sheer overabundance of the protein. Interestingly, the activity of the ΔCT-N4IC mutant in HMEC remained equal, or nearly equal, to that of N4IC despite having much lower levels of protein. It is hypothesised that this is due to small amounts of N4IC being sufficient for activity, with

excessive levels providing no additional effects. This is supported by previous research demonstrating that blocking SEL-10-mediated degradation of Notch does not result in an increase in N4IC-induced reporter activity (Wu et al., 2001). However, the same blocking strategy did increase N1IC-induced reporter activity. Still, the possibility remains that ΔCT-N4IC is a more potent factor than the other mutants and this greater potency is responsible for its high activity at low expression.

5.3.2 FGF- and VEGF-Induced Angiogenesis

It is significant that the functional N4IC mutants blocked both FGF-2- and VEGF-induced sprouting (Figure 8) since these growth factors can signal through different pathways to induce angiogenesis. For example, Src-family kinases are required for angiogenesis induced by VEGF, but not FGF-2, in chick embryo and mouse *in vivo* models (Eliceiri et al., 1999). In other Notch studies, pathway activation inhibited HUVEC tube formation induced by a combination of VEGF and FGF-2 (Henderson et al., 2001; Taylor et al., 2002). Conversely, Notch moderated the morphogenesis of bovine microvascular endothelial cells into networks in response to FGF-1 but not VEGF (Zimrin et al., 1996). Taken together, these results reveal that Notch is capable of regulating multiple pathways of angiogenesis and that the particular pathways that are acted on in any one situation depend on cellular context.

Overexpression of N4IC induced downregulation of VEGFR2 mRNA in HMEC (Figure 9). Although the mechanism of this action is unclear, analysing VEGFR2 expression in the HMEC-CBF1-VP16 lines could settle whether this effect is CBF1-dependent. It is unlikely that downregulation of VEGFR2 is due to direct transcriptional repression by Notch and CBF1 since this complex is only known to activate bound regulatory sites. Alternatively, Notch has been shown to indirectly repress transcription by helping sequester molecules required for the

activation of promoters by other transcription factors such as MASH1 (Yamamoto et al., 2001). Intuitively, it would seem more likely that VEGFR2 downregulation is carried out through induction of HES or HRT proteins since these factors are transcriptional repressors. In particular, HRT1 overexpression is known to reduce VEGFR2 mRNA in human capillary endothelial cells (Henderson et al., 2001). However, the HRT and HES factors analysed in this thesis do not appear responsible for VEGFR2 reduction in HMEC since ΔRAM-N4IC and ΔCT-N4IC elevated VEGFR2 expression while also inducing HRT and HES mRNA. Still, it has not been confirmed if the increased HRT and HES mRNA corresponds with elevated levels of HRT and HES protein.

VEGFR2 mRNA expression was unexpectedly upegulated by the NLS-ΔRAM-N4IC, ΔCT-N4IC, and NLS-Ank mutants (Figure 9). Although the mechanism of this activity remains unknown, these mutants may have a dominant-negative effect on basal repression of the VEGFR2 promoter. Alternatively, NLS-ΔRAM-N4IC, ΔCT-N4IC, and NLS-Ank may upregulate VEGFR2 expression through a pathway distinct from that which downregulates VEGFR2. Intriguingly, the VEGFR2 promoter (Patterson et al., 1995) does contain a consensus CBF1 recognition sequence, although it has not been determined whether CBF1 actually functions at this site. It does raise the possibility that N4IC can induce VEGFR2 expression through direct action on the VEGFR2 promoter but also activates other signals that ultimately downregulate VEGFR2 expression.

Based on the analysis of VEGFR2 and FGFR1 mRNA levels, N4IC seems to inhibit endothelial sprouting via mechanisms other than mere repression of relevant growth factor receptor expression. The protein levels of VEGFR2 and FGFR1 could be analysed to further validate these conclusions. As well, other potentially significant receptors such as VEGFR1 and

FGFR2 could be investigated. Rather than analysing all possible FGF-2 and VEGF receptors, the responsiveness of HMEC to stimulation by these factors could be measured by Western blotting for the active phosphorylated forms of downstream signalling molecules such as ERK1, ERK2, or Akt1.

5.3.3 Functional Significance of the Notch4 Intracellular Domains

The RAM and CT domains of Notch4 were not required for full inhibition of endothelial sprouting (Figure 8) and were dispensable for detectable upregulation of CBF1-dependent gene expression (Figure 10). Still, removal of the either the RAM or CT from N4IC did result in slight decreases in the induction of HRT1 and HRT2 mRNA expression (Figure 10C). Furthermore, deletion of the CT motif, but not the RAM domain, resulted in decreased activation of the HRT2 luciferase reporter (Figure 10B). Consequently, the CT may be more relevant than the RAM for N4IC-induced transcription. Although the RAM domain provides strong binding to CBF1, this activity is not strictly required for the interaction between Notch and CBF1 (Tani et al., 2001). Moreover, the ankyrin repeats and CT are able to bind numerous components of the CBF1-coactivator complex, likely compensating for loss of the RAM (see Chapter 1.3.3 and 1.3.4 for more details). The Notch1 CT motif has been shown to contain an intrinsic transactivation domain when analysed in COS7, NIH3T3, and C2C12 cells (Kurooka et al., 1998). This was determined by fusion of the CT to the GAL4 DNA-binding domain and measuring their stimulation of a reporter plasmid containing GAL4 binding sites in its promoter. While the CT of Notch1 activated the GAL4 reporter, the Notch4 CT did not (Kurooka et al., 1998). The CT is the least conserved region among human Notch proteins (Figure 2) and this divergence may explain why this domain is more relevant for the upregulation of CBF1-dependent gene expression by Notch1 than Notch4.

Alone, the ankyrin repeats were incapable of matching many of the functions of the intact N4IC, while addition of the RAM domain or CT greatly enhanced their effectiveness. This may indicate that the RAM and CT directly potentiate the activity of the ankyrin repeats. Alternatively, the ankyrin repeats may only require the RAM or CT for proper protein folding following translation. The RAM and CT were also crucial for downregulation of VEGFR2 mRNA expression. This highlights the possibility that these domains play a noteworthy role in regulating other untested genes or in activating CBF1-independent pathways not significant for inhibition of sprouting.

Removal of the RAM domain inhibited Notch4 nuclear localisation (Figure 7C). The importance of the RAM motif for nuclear targeting may owe to a region containing three consecutive arginine residues that has some homology to a lysine-arginine rich region found within the RAM domain of Notch1, 2, and 3 (Figure 2). Lysine and arginine are basic residues known to be important for nuclear import (Jans et al., 2000), and the lysine-arginine rich region within the RAM domain of Notch1 has been proposed to provide a putative NLS (NLS-1 in Figure 2) (Aster et al., 1994; Aster et al., 1997). However, this Notch1 NLS does not appear as crucial for nuclear translocation as that contained in Notch4 since Notch1 has three other downstream NLS sequences. Previous research shows that N4IC fusion proteins caused by MMTV integrations have reduced nuclear localisation in mammary epithelial cells when the RAM domain is lost (Lee et al., 1999). Interestingly, this phenomenon was dependent on cell type since ΔRAM proteins were exclusively nuclear in HeLa cells. This may indicate that certain cells, such as HeLa, express an unknown cellular factor that can facilitate Notch nuclear localisation when its intrinsic NLS sequences are lost.

The necessity of the ankyrin repeats for Notch4 function in HMEC was not surprising given the established significance of this domain for the activity of mammalian Notch1 and other Notch proteins across species (Kato et al., 1997; Kurooka et al., 1998; Lieber et al., 1993; Rebay et al., 1993; Roehl et al., 1996). In particular, deletions or loss-of-function mutations of this motif invariably abolish transactivation of CBF1-dependent genes (Aster et al., 1997; Jarriault et al., 1995; Kato et al., 1997; Kurooka et al., 1998; Zhou et al., 2000b). The requirement of the ankyrin repeats for CBF1-dependent signalling probably owes to their crucial recruitment and stabilisation of multiple factors in the coactivator complex including CBF1, SKIP, MAML, and the HATs PCAF and GCN5 (Kurooka and Honjo, 2000; Tamura et al., 1995; Wu et al., 2000; Zhou et al., 2000b). Studies have also shown that the ankyrin repeats are essential for CBF1-independent Notch functions (Dumont et al., 2000; Nofziger et al., 1999; Yamamoto et al., 2001). Again, the necessity of these tandem repeats is probably due to their importance in binding relevant factors such as Deltex, which requires the first three ankyrin repeats of Notch1 for interaction (Yamamoto et al., 2001).

While the ankyrin repeats are necessary for Notch4 function in HMEC, they only provide partial activity on their own. Numerous studies have shown that the Notch1 ankyrin repeats are insufficient for transactivation of reporter plasmids containing CBF1 regulatory sites (Dumont et al., 2000; Nofziger et al., 1999; Redmond et al., 2000; Shawber et al., 1996). As shown in this thesis, the Notch4 ankyrin repeats were unable to significantly activate the 4xCBF1 luciferase or HRT2 luciferase constructs (Figure 10A, B). However, the ankyrin repeats appear sufficient for augmenting the expression of certain endogenous CBF1-dependent genes since elevated levels of some, but not all, HRT and HES transcripts were detected in the HMEC-NLS-Ank cell line (Figure 10C). Of particular interest, the ankyrin repeats upregulated HRT2 mRNA expression

but did not activate the HRT2 luciferase in HMEC. There are several possible explanations for the discrepancy between these results. First, HMEC is a human cell line while the HRT2 promoter sequence contained in the luciferase construct is derived from mouse (Nakagawa et al., 2000). As well, the luciferase promoter fragment may not include all of the regulatory sequences important for transcription of the endogenous gene. Furthermore, the local genomic environment of the endogenous HRT2 promoter and its position within the nucleosome likely influence its expression in ways that the HRT2 promoter in the luciferase plasmid is not affected. Although plasmid DNA is known to associate with histones upon entering the nucleus, the nucleosome-like particles that form are often abnormal in structure (Jeong and Stein, 1994a; Jeong and Stein, 1994b). As for other possibilities, the ankyrin repeats may augment HRT2 mRNA expression by specifically increasing the stability of its transcript rather than inducing transcription. Thus, it would not be expected that the luciferase mRNA transcribed from the reporter plasmid would be similarly stabilised. Indeed, increased mRNA stability may also be responsible for the elevated HRT and HES mRNA levels observed in the HMEC-N4IC, -NLS-ΔRAM-N4IC, and -ΔCT-N4IC cell lines. However, this is not the most plausible explanation given the established role that Notch plays in the transcriptional regulation of these genes and the fact the N4IC, NLS-ΔRAM-N4IC, and ΔCT-N4IC constructs activate CBF1-dependent luciferase reporters in addition to increasing HRT and HES mRNA expression (Figure 10).

The ankyrin repeats have been reported to be sufficient for Notch-induced inhibition of C2C12 myoblast differentiation and Notch-induced neoplastic transformation of RK3E rat kidney cells (Dumont et al., 2000; Nofziger et al., 1999). In both instances, the ankyrin domain-mediated effects were proposed to be CBF1-independent based largely on the fact that this motif could not activate luciferase reporters driven by promoters containing multimerized CBF1-

binding elements. As shown here, it cannot be assumed that a given Notch mutant is unable to regulate all endogenous CBF1-dependent genes based solely on the fact that the mutant does not activate a reporter construct with CBF1-binding sites in its promoter.

The varying ability of the ankyrin domain alone to activate distinct CBF1-dependent promoters indicates that different genes require varying degrees or aspects of Notch function for their transcription. This may depend on the local availability of repressors and/or activators as well as the position of the target gene within the nucleosome. Previous work has also established that a given Notch homologue can regulate the same gene to varying degrees depending on cell type. For example, HES1 is a well-established target of Notch1 signalling in mammalian cells (Iso et al., 2003). However, HES1 mRNA expression is not elevated by transient overexpression of N1IC in certain cell lines (Iso et al., 2001a). Genetic studies in *Drosophila* support a theory that the transcription of some genes only requires Notch to relieve the repression mediated by CBF1, while other targets require both depression and the recruitment of coactivators for transcription (Bray and Furriols, 2001). Alleviation of CBF1-mediated repression may be sufficient for transcriptional activation when Notch-independent transcriptional activators are available or already in place, but are inhibited by the CBF1 corepressor complex. It is possible that in such instances the Notch ankyrin domain alone may be sufficient to disrupt the corepressor complex and allow transcription. However, the ankyrin repeats may not be sufficient to activate regulatory sites that require Notch-mediated derepression and coactivation.

5.3.4 CBF1-Dependent and -Independent Notch Signalling

CBF1-VP16 is a constitutively-active CBF1 construct that induced expression of multiple target genes in HMEC (Figure 11) and partially mimicked N4IC-induced inhibition of endothelial sprouting (Figure 12). Thus, it appears likely that Notch can prevent sprouting

through CBF1-dependent and -independent pathways. However, while N4IC elevated HRT3 mRNA expression in HMEC, the constitutively-active CBF1 did not (Figure 11C). Consequently, it remains possible that differences in HRT3 expression were responsible for the disparity between the extent to which N4IC and CBF1-VP16 inhibited sprouting. In the future, this could be investigated by testing the effects of HRT3 overexpression on HMEC sprouting. The failure of CBF1-VP16 to augment HRT3 transcription may reflect an inherent inability of this construct to modulate certain CBF1-dependent promoters normally activated by N4IC. This could result from failure of the VP16 transactivation domain to alleviate any transcriptional repression activity still exhibited by the CBF1 portion of the CBF1-VP16 construct. Previous research confirms that CBF1-mediated repression does mitigate the transactivation of downstream genes by CBF1-VP16 (Waltzer et al., 1995). It is also possible that VP16 does not recruit the proper coactivators to the HRT3 promoter. Alternatively, Notch may regulate HRT3 independently of CBF1 in HMEC. While it was assumed that N4IC-induced transactivation of the HRT and HES genes was mediated by endogenous CBF1, it was not verified. One way to confirm this would be to ensure that abrogating CBF1 signalling also blocks N4IC-mediated activation of HES and HRT promoters in HMEC. Obstructing CBF1 would also prove valuable in resolving whether this factor is at all required for the anti-angiogenic activity of N4IC. While CBF1 activation does inhibit sprouting, it is possible that alternative pathways are dominant to the CBF1-mediated signals and that blocking CBF1 will have no effect on sprouting. RNA interference is one potential method that can be used to prevent CBF1 signalling (Cullen, 2002; Paddison et al., 2002). In theory, RNA interference can block gene expression using double-stranded RNA designed to selectively degrade targeted mRNA.

The immediate downstream targets that propagate CBF1-mediated inhibition of sprouting remain undetermined. Other than HRT3, HRT1 and HRT2 are logical factors to pursue since they undergo the greatest rise in expression of all the targets analysed and were also activated by both N4IC and CBF1-VP16. In spite of this, preliminary studies in the Karsan lab indicate that overexpression of HRT1 or HRT2 does not mimic the Notch4 anti-angiogenic effect. However, overexpression of single factors may prove futile if inhibition of sprouting requires the upregulation of multiple primary targets.

It is proposed here that N4IC inhibits endothelial sprouting through CBF1-dependent and –independent signalling. To validate this proposal, CBF1-independent pathways that are induced by Notch4 must be identified and confirmed to inhibit endothelial tube formation. In pursuit of identifying such pathways, future experiments will focus on the function of Deltex in HMEC. The exact role of this protein is not known, although it directly binds the ankyrin repeats of Notch (Yamamoto et al., 2001). In some instances, Notch and Deltex appear to cooperate in negatively regulating the activity of transcriptional activators such as MASH1 and E47 independently of CBF1 (Matsuno et al., 1998; Ordentlich et al., 1998; Yamamoto et al., 2001). A specific role for Deltex in endothelial cell biology has not been determined, but it is strongly expressed in blood vessels (Matsuno et al., 1998; Mitsiadis et al., 2001). Therefore, Deltex will be overexpressed in HMEC to determine the effects of this protein on endothelial sprouting.

5.4 Summary and Conclusions

Enforced expression of activated Notch4 inhibits HMEC morphogenesis into capillary-like tubes, or sprouts, in three-dimensional fibrin gels. Future experiments will also focus on the use of an inducible expression system to confirm that transient Notch4 signalling can do the

same. The ankyrin repeats are required for Notch4-induced inhibition of endothelial sprouting, while overexpression of this domain alone partially reduces tube formation. The block of endothelial cell morphogenesis by Notch4 is also partially mimicked by CBF1-VP16, an activated form of CBF1 that can positively regulate target gene expression in HMEC. Interpreting these results, Notch4 seemingly prevents the sprouting of endothelial cells through CBF1-dependent and –independent pathways. This must be confirmed by identifying CBF1-independent pathways that contribute to Notch4-induced inhibition of endothelial sprouting. In pursuit of this, Deltex function in HMEC will be analysed. As well, RNA interference will be used to block CBF1 expression to determine whether Notch4-induced inhibition of endothelial cell sprouting requires CBF1-dependent signalling or if alternative pathways are sufficient for full inhibition.

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