NOTCH ACTIVATION INDUCES AN ENDOTHELIAL-TO-MESENCHYMAL TRANSFORMATION

By

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ABSTRACT

The Notch signalling pathway is an evolutionarily conserved intercellular signalling mechanism, and mutations in its components disrupt cell fate specification and embryonic development in organisms from nematodes to mammals. Various studies have demonstrated a critical role for Notch signalling in cardiovascular development. Patients with mutations of the Notch ligand, Jagged (JAG)1, demonstrate cardiac anomalies that are consistent with defects in endothelial-to-mesenchymal transformation that is essential for endocardial cushion formation. We demonstrate herein that constitutive activation of Notch in endothelial cells results in morphological and phenotypic changes consistent with this mesenchymal transformation. Specifically, we observe the downregulation of endothelial markers (such as vascular endothelial (VE)-cadherin) and the upregulation of mesenchymal proteins (such as alpha-smooth muscle actin (SMA)) in Notch-transformed endothelial cells. Moreover, we show that endothelial cells expressing activated Notch undergo a functional change characteristic of mesenchymal cells, in that they are capable of migrating towards platelet-derived growth factor (PDGF), but are no longer chemotactic towards vascular endothelial growth factor (VEGF). Our studies also reveal that the Notch-induced endothelial-to-mesenchymal transformation is cell autonomous, and independent of transforming growth factor (TGF)β signalling, which is an important regulator of this transformation *in vivo*. Furthermore, JAG1 stimulation of endothelial cells induces a similar mesenchymal transformation. Finally, we show that JAG1, Notch1 and Notch4 are expressed in the ventricular outflow tract at the commencement of endocardial cushion formation. This is the first direct evidence that JAG1-Notch interactions induce endothelial-to-mesenchymal transformation, and our findings suggest that the cardiovascular defects seen in patients with JAG1 mutations may be due to disruption of this process.

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ABBREVIATIONS

ADP	adenosine diphosphate
AGS	Alagille Syndrome
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
CAM	cell-cell adhesion molecule
CBF1	C-promoter binding factor 1
C. elegans	*Caenorhabditis elegans*
CSL	CBF1/Suppressor of Hairless/Lag-1
Dll	Delta-like ligand
DSL	Delta/Serrate/Lag-2
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transformation
eNOS	endothelial nitric oxide synthase
FCS	fetal calf serum
HA	hemagglutinin
HAEC	human aortic endothelial cells
HAT	histone acetyltransferase
HDAC	histone deacetylase
HES	hairy and Enhancer of split
HMEC	human microvascular endothelial cells
HRT	Hairy related transcription factor
HUVEC	human umbilical vein endothelial cells
JAG	Jagged
JNK	Jun N-terminal kinase
LNR	Lin-12 Notch-specific repeat
NCR	Notch cytokine region
NICD	Notch intracellular domain
Notch1IC	Notch1 intracellular domain

Notch4IC	Notch4 intracellular domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PECAM	platelet endothelial cell adhesion molecule
PEST	proline, glutamate, serine, threonine-rich sequence
PMSF	phenylmethylsulfonyl fluoride
PTB	phosphotyrosine-binding
RBP-Jκ	Recombination recognition sequence binding protein-Jκ
RT	reverse transcription
SH3	Src homology 3
SKIP	Ski interacting protein
SMA	alpha-smooth muscle actin
SOP	sensory organ precursor
TACE	TNF-α converting enzyme
TAD	transcriptional activator domain
TGF	transforming growth factor
TM-NICD	membrane-tethered Notch intracellular domain
TNF	tumor necrosis factor
VE-cadherin	vascular endothelial-cadherin
VEGF	vascular endothelial growth factor
YFP	yellow fluorescence protein

CONTRIBUTIONS OF OTHERS

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Chapter 1

INTRODUCTION

1.1 NOTCH

1.1.1 Notch structure

The gene encoding the Notch receptor was originally discovered based on the observation that partial loss of function (haploinsufficiency) in *Drosophila* resulted in notches at the wing margin (Artavanis-Tsakonas et al, 1999). Further analysis revealed that complete loss of function mutations in the Notch receptor gene produce a "neurogenic phenotype" in *Drosophila* (i.e. cells destined to become epidermis switch fate and give rise to neural tissue instead) (Artavanis-Tsakonas et al., 1999; Wright, 1970). Notch proteins are single pass (Type I) transmembrane receptors with a modular architecture and are highly conserved throughout evolution (Artavanis-Tsakonas et al., 1999; Baron et al., 2002; Mumm and Kopan, 2000). In mammals, there are four Notch receptors (Notch1, 2, 3 and 4) (Allman et al., 2002; del Amo et al., 1993; Ellisen et al., 1991; Lardelli et al., 1994; Uyttendaele et al., 1996; Weinmaster et al., 1992). Figure 1.1 illustrates the conservation in the domain structure of the Notch receptors between flies, worms and humans.

The extracellular domain of each Notch receptor contains variable numbers of tandem epidermal growth factor (EGF)-like repeats, which are involved in ligand binding (Artavanis-Tsakonas et al., 1999; Baron et al., 2002; Milner and Bigas, 1999). For example, EGF-like repeats 11 and 12 of *Drosophila* Notch are both necessary and sufficient to bind the ligands, Delta and Serrate, *in vitro* (Rebay et al., 1991). Additional extracellular motifs conserved among Notch family members are three membrane-proximal Lin-12 Notch-specific repeats (LNRs) (Fleming, 1998). LNRs function to prevent inappropriate Notch receptor activation prior to ligand binding (Allman et al., 2002; Greenwald, 1994; Kimble et al., 1998). Finally, two conserved cysteine residues, located between the LNRs and the transmembrane domain, are involved in the dimerization of the extracellular and transmembrane-intracellular domain subunits (Lieber et al., 1993; Rand et al., 2000).

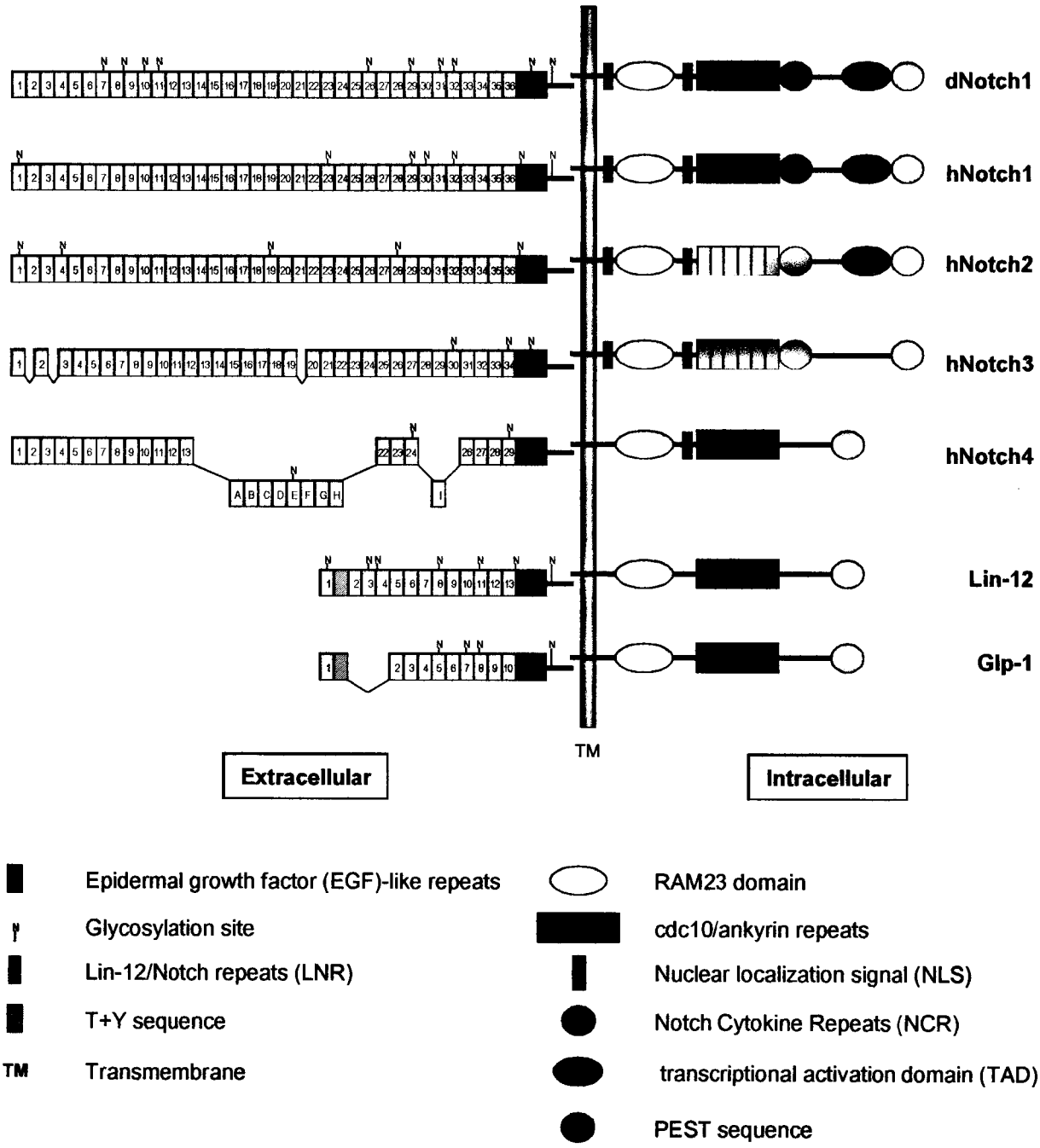


Figure 1.1: Schematic diagram illustrating representative members of the Notch receptor family. The single *Drosophila* (dNotch), 4 human (hNotch), and 2 *C. elegans* (Lin-12 and Glp-1) Notch receptors are depicted. The full-length receptors are expressed at the cell surface as non-covalently linked heterodimers. All Notch receptors contain varying numbers of EGF-like repeats in the extracellular domain that regulate ligand binding (numbering identifies homologous repeats). Glycosylation of specific EGF-like repeats is thought to alter ligand specificity. Other conserved functional domains found in all Notch family members include the LNR (DSL ligand binding and Notch activation), RAM23 (CSL binding), cdc10/ankyrin (CSL binding/protein-protein interactions), and PEST (protein turnover) domains. Conversely, the NCR (cytokine specific effects) and TAD (transactivation) domains are restricted to certain Notch receptors, as indicated. The highest degree of homology amongst the Notch receptors occurs within the cdc10/ankyrin repeats. *Adapted from (Allman et al., 2002) and (Egan et al., 1998).* Four

functionally important regions have been identified in the intracellular domain of Notch. Firstly, the RAM23 domain of Notch binds to the principal mediator of Notch signalling, the CBF1/Suppressor of Hairless/Lag-1 (CSL) transcriptional repressor (described in section.1.2.1) (Kimble and Simpson, 1997; Mizutani et al., 2001; Mumm and Kopan, 2000). The RAM23 domain also binds to Numb, a negative regulator of Notch activity (described in section 1.2.2.4) (Baron et al., 2002). Secondly, the cdc10/ankyrin domain, which is the most conserved region in the Notch gene family, mediates important protein-protein interactions with a number of factors, including CSL, to signal downstream of Notch receptors (Figure 1.4) (Allman et al., 2002; Fleming, 1998; Milner and Bigas, 1999). In all Notch proteins there are 110 amino acids between the transmembrane domain and the cdc10/ankyrin repeat domain. The Notch members are least conserved C-terminal to the cdc10/ankyrin repeats. Only *Drosophila* Notch and mammalian Notch1 and 2 contain a transcriptional activator domain (TAD), the third functionally important Notch intracellular region, which positively regulates Notch dependent transcription events (section 1.2.1.2) (Allman et al., 2002). Finally, a conserved C-terminal proline, glutamate, serine, threonine-rich (PEST) sequence that is thought to be important in protein turnover and the subsequent downregulation of Notch signalling is conserved in all Notch family members (Allman et al., 2002; Fleming, 1998).

Notch receptors are activated by Type I transmembrane ligands of the Delta/Serrate/Lag-2 (DSL) family (Artavanis-Tsakonas et al., 1999; Baron et al., 2002; Fleming, 1998; Lissemore and Starmer, 1999; Mumm and Kopan, 2000). Five mammalian Notch ligands, Delta-like ligand (Dll)1, Dll3, Dll4, Jagged (JAG)1, and JAG2 have been cloned to date (Allman et al., 2002; Bettenhausen et al., 1995; Dunwoodie et al., 1997; Lindsell et al., 1995; Oda et al., 1997a; Shawber et al., 1996a; Shutter et al., 2000). Figure 1.2 illustrates the conservation in the structure of the Notch ligands between flies, worms and humans. All Notch ligands share some structural features, including EGF-like repeats, a DSL domain that is required for Notch receptor binding, and a transmembrane region (Allman et al., 2002; Fleming, 1998; Fleming et al., 1997b; Muskavitch, 1994). Notch ligands are divided into two subclasses, the Delta family and the Serrate family. The two families are distinguished by an extracellular cysteine-rich domain and insertions interrupting some EGF-like repeats of Serrate family members (Allman et al., 2002; Lissemore and Starmer, 1999), as illustrated in Figure 1.2.

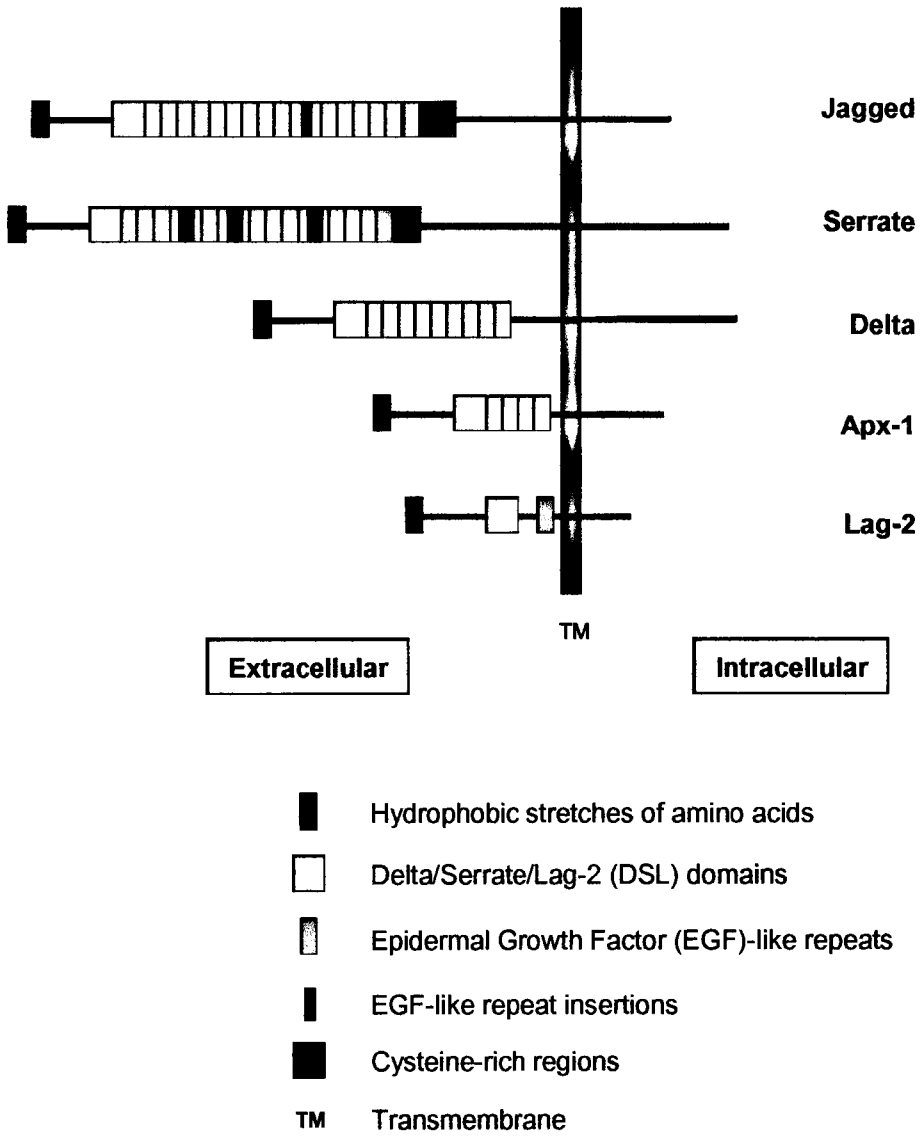


Figure 1.2: Schematic diagram illustrating the structures of the DSL proteins. *Drosophila* (Delta and Serrate), vertebrate (Jagged/Serrate and Delta), and *C. elegans* (APX-1 and LAG-2) Notch ligands are shown. All DSL proteins contain 1 DSL motif (Notch receptor binding), 1-16 EGF-like repeats (Notch receptor binding) and a single transmembrane domain. All Jagged/Serrate ligands contain a cysteine-rich region between the EGF region and the transmembrane domain. Furthermore, *Drosophila* Serrate has insertions in EGF-like repeats 4, 6 and 10 and vertebrate Jagged/Serrate proteins have an insertion in EGF-like repeat 10. The existence of these EGF-like repeat insertions and the cysteine-rich region distinguishes Jagged/Serrate family ligands from Delta family ligands. *Adapted from (Lindsell et al., 1995).*

1.1.2 Notch activation

Detecting physiological levels of active Notch in the nuclei of cells is extremely difficult; however, it has been demonstrated that an active form of Notch translocates to the nucleus in a ligand dependent manner (Artavanis-Tsakonas et al., 1995; Kopan and Goate, 2000; Weinmaster, 1997). Evidence from a number of groups has led to a three-step proteolytic model

for Notch activation, depicted in Figure 1.3A. During transit to the cell membrane, Notch is cleaved in the trans-Golgi by a furin-like convertase; this first cleavage event is referred to as the S1 cleavage (Allman et al., 2002; Blaumueller et al., 1997; Logeat et al., 1998; Mumm and Kopan, 2000). The S1 cleavage generates the Notch extracellular domain and the transmembrane-intracellular domain subunits. The resulting Notch heterodimer is held together by a non-covalent, calcium-dependent interaction (Mumm and Kopan, 2000; Rand et al., 2000). Once at the plasma membrane, this signalling-competent Notch receptor undergoes a second (S2) ligand-dependent cleavage just outside the transmembrane domain (Figure 1.3A & B). The metalloprotease TNF-α converting enzyme (TACE) has been shown to perform the S2 cleavage *in vitro* (Brou et al., 2000); however, the physiological S2 protease remains elusive to date. The majority of the Notch extracellular domain is removed by the ligand-induced S2 cleavage, leaving a membrane-tethered intracellular domain (TM-NICD) (Baron et al., 2002). The TM-NICD receptor fragment then undergoes a constitutive intramembrane cleavage (S3) by the Presenilin-dependent γ-secretase (Figure 1.3A & B) (De Strooper et al., 1999; Mumm et al., 2000; Struhl and Greenwald, 1999; Struhl and Greenwald, 2001; Ye et al., 1999). The protein nicastrin is also required for this S3 cleavage (Yu et al., 2000). The resulting soluble Notch intracellular domain (NICD) translocates to the nucleus where it can interact with the CSL transcriptional repressor family (described in section 1.2.1) and other downstream targets (Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Fortini et al., 1993; Jarriault et al., 1995; Kidd et al., 1998; Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Schweisguth, 1995; Struhl and Adachi, 1998; Struhl et al., 1993; Tamura et al., 1995; Yu et al., 2000).

1.1.3 Notch function

Despite the complexity in establishing a multicellular body plan, organisms rely on repeated use of a small subset of cellular signalling pathways (Pires-daSilva and Sommer, 2003). In other words, each individual cell fate decision does not require a unique signalling mechanism. The variety of outcomes arising from the use of a small number of factors implies that cellular responses depend on the context in which the signals are received. The Notch signalling pathway is one such critical developmental pathway, whereby neighbouring cells expressing Notch receptors and ligands interact to direct cell fate decisions, a process referred to

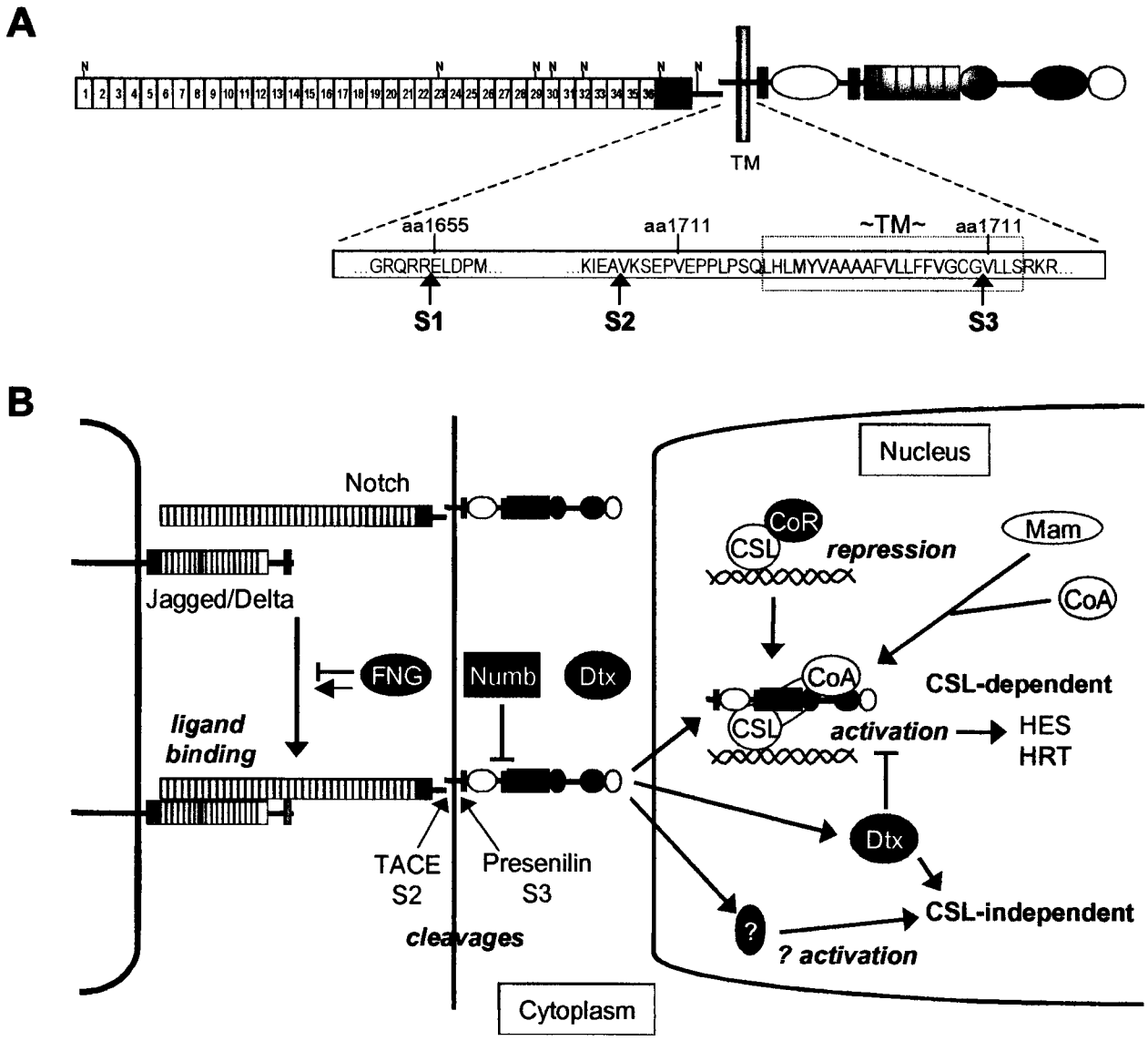


Figure 1.3: Notch signalling requires ligand-induced, proteolytic processing of the full length receptor. (A) The amino acid sequence and the S1, S2, and S3 (site one, etc.) cleavage sites depicted correspond to murine Notch1. The S1 cleavage occurs in the Golgi, producing the extracellular and transmembrane-intracellular subunits of Notch. The resulting heterodimer is expressed at the cell surface and, in response to ligand binding, undergoes the S2 cleavage, which removes the majority of the Notch extracellular domain. Finally, the constitutive S3 generates the NICD that translocates to the nucleus and interacts with target proteins. *Adapted from (Mumm and Kopan, 2000).* **(B)** Schematic of the ligand-induced proteolysis of Notch described in (A). Fringe (FNG) antagonizes the ability of Jagged/Serrate to bind Notch, but agonizes the ability of Delta to bind Notch (described in section 1.2.2.1). In addition, Notch signals are negatively regulated by Numb (section 1.2.2.4) and positively regulated by Deltex (Dtx; section 1.2.2.3). Once inside the nucleus, NICD has been shown to function in both CSL-dependent and -independent pathways. See text for a more detailed description of Notch signalling events (section 1.2). CoA=coactivator complex. CoR=corepressor complex. Mam=mastermind. *Adapted from (Allman et al., 2002).*

as lateral specification (Greenwald, 1998). Neighbouring cells may be equivalent or may be biased towards a particular developmental fate in response to an intrinsic or extrinsic signal (Artavanis-Tsakonas et al., 1999). During Notch lateral specification events, cells taking on the primary differentiation fate express the DSL ligand at high levels. This activates the Notch pathway in juxtaposed cells, which results in these cells remaining undifferentiated or assuming a second differentiation fate (Artavanis-Tsakonas et al., 1999; Frisen and Lendahl, 2001; Lewis, 1998). Notch and DSL interactions now work to stabilize or amplify the differences in the cells originally brought about by stochastic variation and intrinsic or extrinsic factors (Greenwald, 1998). The result is Notch-mediated cell communication based upon differential receptor and ligand expression in neighbouring cells.

There are a number of examples of Notch-mediated cell fate control in development. A classic example of lateral specification is the formation of the anchor cell and the ventral uterine precursor cell of the *Caenorhabditis elegans* (*C. elegans*) hermaphrodite gonad (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999). The two gonadal cells start as developmentally equivalent cells; either cell is capable of differentiating into an anchor cell or a ventral uterine precursor cell. Prior to this differentiation, Notch receptor expression is limited to one cell, and ligand expression to the other (Greenwald, 1998). Notch activation forces one cell to differentiate into the ventral uterine precursor, while loss of Notch function or failed activation in the other cell results in anchor cell differentiation (Artavanis-Tsakonas et al., 1999). The differentiation of the sensory organs of the *Drosophila* peripheral nervous system is an example of nonequivalent precursor cells utilizing Notch to progress to the next developmental stage. The sensory organ precursors (SOPs) divide once to produce two cells, IIa and IIb, both of which divide again to give a hair and a socket cell pair and a neuron and a sheath pair, respectively (Artavanis-Tsakonas et al., 1999; Greenwald, 1998). At each division in the SOP lineage, the protein, Numb (described in section 1.2.2.4), is newly synthesized and asymmetrically segregated into only one of two daughter cells (i.e. IIb, hair and neuron cells) (Greenwald, 1998). Because Numb antagonizes Notch signalling, only those cells that do not receive Numb will adopt a cell fate associated with Notch activation. Thus, although two neighbouring cells both express Notch receptor, only one may be Notch responsive. A final example illustrates the influence of an extrinsic factor on Notch-dependent cell fate acquisition. In the developing eye disc of *Drosophila*, an unknown gradient emanating from the midline of the marginal disc signals to adjacent R3 and R4 photoreceptor precursor cells (Artavanis-Tsakonas et al., 1999).

This signal is transmitted through the Wingless signalling pathway members Frizzled and Disheveled, and leads to the up-regulation of Delta expression. The precursor cell that is closer to the signalling source expresses higher levels of Delta and ultimately acquires the R3 fate. Delta then activates Notch on the adjacent precursor, directing this cell to the R4 fate (Artavanis-Tsakonas et al., 1999; Cooper and Bray, 1999; Fanto and Mlodzik, 1999). The generation of distinct cellular subpopulations by Notch is not restricted to worms and flies. In mammals, Notch is known to play a crucial role in the regulation of cell fate decisions, such as the development of T lymphoid versus B lymphoid cells, pancreatic exocrine versus endocrine cells, and arteries versus veins (Apelqvist et al., 1999; Lawson et al., 2001; Pui et al., 1999).

1.2 NOTCH SIGNALLING

1.2.1 CSL-dependent signalling

1.2.1.1 CSL-mediated transcriptional repression

As indicated in section 1.1.2, Notch activation results in the generation of the soluble NICD, which translocates to the nucleus where it interacts with a number of nuclear factors that regulate transcription, including CSL (Aster et al., 1997; Fortini and Artavanis-Tsakonas, 1994; Hsieh et al., 1996; Matsuno et al., 1997; Tamura et al., 1995). All four mammalian Notch receptors are known to bind and derepress/activate CSL (Mizutani et al., 2001). As indicated earlier, the Notch RAM23 domain is important for NICD/CSL interaction, since deletion of this region leads to weak CSL binding (Kato et al., 1997) or no binding at all (Zhou et al., 2000b). CSL homologs are sequence-specific (CGTGGGAA, (Sakai et al., 1998)) DNA-binding transcription factors, which function as the major mediators of Notch signalling. In the absence of Notch activation, CSL acts as a transcriptional repressor by binding to transcriptional corepressor complexes (Figure 1.3B) (Bray and Furriols, 2001; Dou et al., 1994; Hsieh and Hayward, 1995; Mumm and Kopan, 2000; Piccoli and Spinner, 2001). CSL can bind to at least two corepressor complexes: 1) the SMRT/NcoR/histone deacetylase (HDAC)1 complex (Kao et al., 1998); and 2) the CIR/HDAC2/SAP30 complex (Hsieh et al., 1999; Zhou et al., 2000b). The NICD initiates transcription by antagonizing the CSL/corepressor interaction (Figure 1.3B) (Hsieh et al., 1999; Mumm and Kopan, 2000; Zhou et al., 2000a; Zhou et al., 2000b).

1.2.1.2 Notch-mediated CSL activation

The molecular mechanism by which NICD antagonizes CSL-mediated repression is only partially understood. It is not clear how undetectable amounts of nuclear NICD can turn the CSL/corepressor complex into an activator complex. One potential clue was revealed with the identification of Ski interacting protein (SKIP), which functions as part of the CSL/corepressor complex by binding to CSL at a site distinct from the CSL/SMRT interaction site (Mumm and Kopan, 2000; Zhou et al., 2000a; Zhou et al., 2000b). Interestingly, SKIP is also able to interact with the cdc10/ankyrin repeat domain of NICD (Mumm and Kopan, 2000). Jarriault et al. (1995) and Kopan et al. (1994) found that point mutations (M1 and M2) in the fourth cdc10/ankyrin repeat of Notch disrupted Notch activity, and it was later shown by Zhou et al. (2000b) that M2 mutants are still capable of binding to CSL, but not SKIP. It is now understood that SMRT and NICD compete for CSL binding, while SKIP works to promote NICD/CSL interaction. SKIP binding to SMRT or Notch is mutually exclusive; SKIP/CSL can form a complex with Notch or SMRT, but not both (Mumm and Kopan, 2000). Furthermore, even with a 4-fold excess of SMRT, Notch remains associated with SKIP/CSL providing a potential mechanism by which low levels of NICD can transactivate CSL repressed genes (Mumm and Kopan, 2000; Zhou et al., 2000b).

In addition to SKIP, Kurooka and Honjo (2000) have shown that Notch is able to bind two conserved histone acetyltransferases (HATs), PCAF and GCN5. This interaction requires the Notch cdc10/ankyrin repeats and the putative TAD in mammalian cells (Mumm and Kopan, 2000). However, a TAD deleted *Drosophila* Notch behaved in an identical manner to complete NICD *in vivo* (Tomlinson and Struhl, 1999). Furthermore, since only mammalian Notch1 and 2 contain TAD domains, the requirement of the TAD domain for the interaction of Notch with HATs remains controversial to date. The recruitment of HATs to the NICD/CSL complex likely results in the acetylation of histones, leading to open chromatin and active transcription (Struhl, 1999). A second possibility could be that HATs acetylate SMRT, catalytically promoting NICD/CSL interaction (Mumm and Kopan, 2000). Complicating matters further, it has also been shown that the HAT/NICD/CSL complex requires another molecule, Lag-3/mastermind, to coactivate transcription (Figure 1.3B) (Mumm and Kopan, 2000). Loss of Lag-3 results in a phenotype identical to the complete loss of Notch activity in *C. elegans* (Petcherski and Kimble, 2000). Petcherski and Kimble (2000) showed that *Drosophila* and vertebrate mastermind provides the same function as Lag-3. Although Lag-3 interacts weakly with NICD, *in vitro*, this

interaction is reported to be greatly enhanced by the addition of CSL (Petcherski and Kimble, 2000). Finally, as is the case with SKIP, it is the fourth cdc10/ankyrin repeat of Notch that mediates the NICD/Lag-3 interaction (Mumm and Kopan, 2000).

1.2.1.3 CSL-dependent Notch targets

Acting through CSL, NICD induces the expression of the hairy and Enhancer of split (HES) gene family (Bailey and Posakony, 1995; Jarriault et al., 1995; Kato et al., 1997). The HES family members are basic helix-loop-helix (bHLH) type transcriptional repressors that bind DNA and act as Notch effectors by recruiting transcriptional corepressors and negatively regulating the expression of target genes (Chen et al., 1997; Ishibashi et al., 1995; Ohsako et al., 1994; Van Doren et al., 1994). For example, HES-1 and HES-5 are induced by NICD and function to prevent the differentiation of neuronal precursor cells from mouse embryos (Ohtsuka et al., 1999). Until recently, the HES family was thought to be the only effector of Notch signalling in mammals; however, it is now known that the tissue distribution pattern of Notch receptors and ligands does not always overlap that of HES (Iso et al., 2003). A novel bHLH family named Hey/Hesr/HRT/CHF/gridlock/HERP, hereafter referred to as hairy-related transcription factor (HRT), has recently been identified as a target of Notch. The amino acid sequence and characteristic domains of HRT indicate that it is most closely related to the HES family among the known bHLH proteins (Iso et al., 2003). HRT is expressed in both HES- and non-HES-expressing tissues. Research has demonstrated that both HES and HRT can function as homodimers, but they can also function as heterodimers (Iso et al., 2001). Interestingly, HES and HRT utilize unique mechanisms to repress the transcription of target genes (Iso et al., 2001). The HES genes have a C-terminal tetrapeptide motif, WRPW, which is required for recruitment of the TLE/Groucho corepressor complex (Fisher et al., 1996; Grbavec and Stifani, 1996; Paroush et al., 1994). Conversely, the bHLH domain of the HRT proteins is both necessary and sufficient for transcriptional repression, which is thought to be mediated by the recruitment of a corepressor complex including N-CoR, mSin3A and HDAC1 (Iso et al., 2001).

To date, seven HES members (Akazawa et al., 1992; Bae et al., 2000; Bessho et al., 2001; Hirata et al., 2000; Ishibashi et al., 1993; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Sasai et al., 1992) and three HRT members (Chin et al., 2000; Iso et al., 2001; Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Zhong et al., 2000) have been identified in mammals. However, evidence suggests that only HES-1, -5, -7 and HRT-1, -2, -3 are

physiological targets of Notch (Iso et al., 2003). These studies were performed by transient transfection of constitutively active NICD with HES-1, -5, -7 and HRT-1, -2, -3 reporter gene constructs (Bessho et al., 2001; Iso et al., 2002; Jarriault et al., 1995; Maier and Gessler, 2000; Nakagawa et al., 2000; Nishimura et al., 1998). The concern surrounding these studies is twofold: 1) NICD overexpression may upregulate the expression of other genes that can, in turn, upregulate the expression of HES and HRT; and 2) overexpressed NICD is well above physiologically relevant levels (Iso et al., 2003). To study the effects of Notch signalling under more physiologically relevant conditions, recent studies have employed a coculture system with Notch-ligand expressing cells (Iso et al., 2001; Jarriault et al., 1998; Kuroda et al., 1999; Shawber et al., 1996b). Furthermore, secondary effects caused by the expression of other genes can be ruled out by utilizing cycloheximide, a de novo protein synthesis inhibitor, in the coculture system (Iso et al., 2002; Iso et al., 2001; Kuroda et al., 1999). These coculture experiments are capable of detecting endogenous Notch receptor activation, and provide strong evidence that HES-1, HRT-1 and HRT-2 are primary targets of Notch in tissue culture models (Iso et al., 2003). However, these same studies have illustrated the complexity of Notch signalling associated with multiple receptors, ligands and tissue expression patterns. For example, ligand induced Notch activation failed to induce HRT-2 expression in C2C12 muscle cells, 10T1/2 fibroblasts, 293T, and P19 teratocarcinoma cells (Iso et al., 2001), but induced HRT-2 expression in the aortic smooth muscle cell line, A10 (Izon et al., 2002). These results suggest that targets of Notch may be under cell-type specific regulation in response to ligand activation. In the example above, all the cells lines tested express at least Notch1, 2, and 3 receptors (Iso et al., 2002; Iso et al., 2001), but only A10 smooth muscle cells expressed HRT-2 mRNA following ligand-induced activation.

Mice mutant for specific pathway members have both added to our understanding of, and raised further questions about, the relationship between various Notch receptors, ligands, and effectors. For example, loss of Notch1 results in decreased HES-5 and HRT-1, -2, and -3 expression in various tissues, but has no effect on HES-1 expression in any tissue (de la Pompa et al., 1997; Iso et al., 2003; Leimeister et al., 2000). Notch2 deficient mice display normal HES-1 and HES-5 expression (Hamada et al., 1999), whereas Dll3 null mice demonstrate reduced HES-1, HES-5, and HRT-1 expression in the presomitic mesoderm (Dunwoodie et al., 2002). Loss of Dll1 causes a decrease in expression of HRT-1,-2, and -3 in mice (Leimeister et al., 2000). Finally, CSL knockout mice show reduced HES-5, but not HES-1, expression (de la

Pompa et al., 1997). The fact that HES and HRT expression (particularly HES-1 expression) was not abolished in these mutant mice suggests that other pathways (i.e. other receptors, ligands, or unidentified CSL-independent pathways) may compensate to maintain gene expression in the absence of various Notch family members (Iso et al., 2003).

1.2.2 Modulation of Notch signalling

Section 1.2, thus far, has focused on the "core", ligand activated Notch signalling pathway. It assumes that a ligand on one cell binds and activates a Notch receptor on an adjacent cell; however, a single cell type can express multiple ligands and receptors, and different cell types (e.g. endothelial and smooth muscle) may show different patterns of expression. With all this complexity, it is difficult to define what determines a functional ligand receptor interaction. In addition, it has been well established that Notch signalling is not restricted to CSL transduced events. For instance, *Drosophila* CSL mutant phenotypes differ from Notch mutant phenotypes in the wing, the eye and the embryo (Arias, 1998; Brennan et al., 1999a; Brennan et al., 1999b; Brennan et al., 1997; Ligoxygakis et al., 1998; Rusconi and Corbin, 1999; Zecchini et al., 1999). Furthermore, in vertebrate models, it is now known that Notch regulates development and muscle cell differentiation via CSL-independent pathways (Ordentlich et al., 1998; Shawber et al., 1996b). Complicating matters further, there are a number of molecules that physically interact with Notch (Figure 1.4) and add another dimension to the complex regulation of Notch signalling. A selection of these molecules and their mechanisms of action are discussed below.

1.2.2.1 Fringe

Fringe is a Golgi-localized protein that acts as an N-acetylglucosaminyltransferase, adding N-acetylglucosamine groups to O-linked fucose of specific EGF-like repeats in the Notch extracellular domain (Figures 1.3 and 1.4) (Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000). "Several conserved O-linked fucose sites lie close to or within EGF-like repeats 24-26, which are critical for Serrate- but not Delta-dependent Notch signalling" (Baron et al., 2002). Fringe is often expressed at developmental boundaries during pattern formation, and has been shown to inhibit Serrate-dependent Notch signalling while potentiating Delta-dependent Notch signalling (Bruckner et al., 2000; Fleming et al., 1997a; Panin et al., 1997). Fringe is thought to interfere with the ability of Serrate to form productive signalling complexes with Notch. In support of this hypothesis, Fleming et al. (1997a) showed that the replacement of the

N-terminal receptor-binding domain of *Drosophila* Serrate with that of Delta makes the hybrid receptor resistant to inhibition by Fringe modification. The *Drosophila* protein Fringe has three vertebrate counterparts: Lunatic Fringe, Radical Fringe and Manic Fringe (Fortini, 2000).

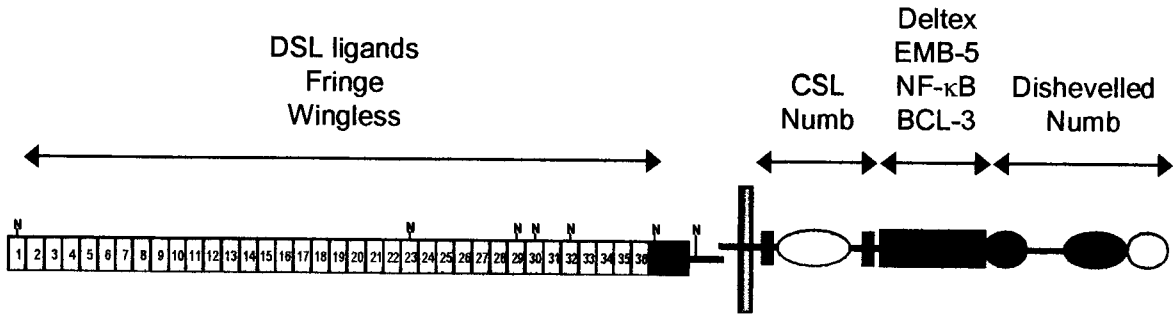


Figure 1.4: Modulators of Notch signalling. The domain structure of Notch is as described in Figure 1.1. Proteins that interact with Notch and the functional domains to which they bind are shown.

1.2.2.2 Wingless

A second extracellular modifier of Notch activity is Wingless, a member of the Wnt family of proteins. Wingless is a secreted protein that binds to its receptor, Frizzled, to stimulate the activation of the cytosolic protein Dishevelled (Baron et al., 2002). Through distinct pathways, activation of Dishevelled culminates in the activation of both β-catenin dependent signalling events and the Jun N-terminal kinase (JNK) pathway (Baron et al., 2002; McEwen and Peifer, 2000). Evidence for a close genetic linkage between Wingless and Notch includes the ability of Notch, in some contexts, to regulate Wingless expression (Baron et al., 2002; Rulifson and Blair, 1995), and the inhibition of Notch signalling through binding to Dishevelled (Axelrod et al., 1996; Rulifson et al., 1996). Wesley et al. (1999) reported that Wingless can bind the Notch extracellular domain (EGF-like repeats 19-36) *in vivo* (Figure 1.4). This same report also showed that the association of Wingless with Notch induces a different transcriptional response from Delta activation of Notch. Delta-dependent Notch signalling leads to the accumulation of NICD, which is not observed upon Notch exposure to Wingless (Wesley, 1999).

1.2.2.3 Deltex

Deltex was initially identified as a positive regulator of Notch signalling in *Drosophila* (Matsuno et al., 1995). On the other hand, enforced expression of Deltex can antagonize Notch

signalling, possibly by competing with NICD for transcriptional coactivators (Izon et al., 2002; Sestan et al., 1999). "The amino acid sequence of Deltex can be divided into three regions separated by glutamine rich sequences" (Baron et al., 2002). The N-terminal domain of Deltex binds to the cdc10/ankyrin repeat region of Notch (Figure 1.4) (Diederich et al., 1994; Matsuno et al., 1995). The N-terminal domain, also contains two WWE domains (Aravind, 2001), which are commonly found in proteins linked to ubiquitination or ADP ribosylation (Baron et al., 2002). The central domain of Deltex contains a proline rich sequence (Matsuno et al., 1998), which may facilitate interactions with Src homology (SH)3-domain containing proteins. The Deltex C-terminal domain contains "a Ring finger domain which may confer E3 ubiquitin ligase activity" (Baron et al., 2002). Wing margin notching and thickened veins are among the phenotypes reported in *Drosophila* containing Deltex mutations (Gorman and Girton, 1992; Xu and Artavanis-Tsakonas, 1990). These Deltex phenotypes resemble those observed in Notch loss of function mutants. In keeping with this observation, Matsuno et al. (1995) reported that these Deltex phenotypes can be rescued by expressing constitutively active NICD. They concluded that Deltex function lies upstream of Notch proteolytic activation (Matsuno et al., 1995). Interestingly, work in mammalian systems has shown that Deltex may function independently of CSL, possibly by down-regulating Ras and JNK-dependent signals (Ordentlich et al., 1998).

1.2.2.4 Numb

A role for Numb in *Drosophila* SOP differentiation was described in section 1.1.3. Numb contains a phosphotyrosine-binding (PTB) domain that facilitates binding to the Notch RAM23 domain and another C-terminal region of Notch (Figure 1.4) (Baron et al., 2002; Lai, 2002). Numb is asymetrically distributed between daughter cells before mitosis, and functions to downregulate Notch signalling in the cell that inherits the Numb protein (Guo et al., 1996; Spana and Doe, 1996). McGill and McGlade (2003) have shown that mammalian Numb mediates the ubiquitination of membrane bound Notch1, which promotes degradation of the Notch1 intracellular domain. In addition to Notch, mammalian Numb can interact with Eps15 (an EH domain-containing endocytic protein), α-adaptin (a clathrin coated pit component) (Jafar-Nejad et al., 2002; Santolini et al., 2000), and "is localized in endocytic vesicles and co-traffics with internalizing cell surface receptors" (Baron et al., 2002). Thus, Numb is also thought to downregulate Notch activity via an endocytic pathway (i.e. by stimulating the endocytosis of Notch) (Baron et al., 2002). In support of this hypothesis, expression of dominant negative Numb results in endocytic perturbations (Baron et al., 2002).

1.3 NOTCH AND EPITHELIAL-TO-MESENCHYMAL TRANSFORMATION (EMT)

1.3.1 Overview of EMT

Epithelial and mesenchymal cells were recognized in the early nineteenth century on the basis of their shape and organization during embryonic development (Thiery, 2002). In 1908, Frank Lillie first described the ability of these cell types to transform between the two states (Lillie, 1908); however, it was not until 1982 that the epithelial-to-mesenchymal transformation (EMT) was recognized as a distinct process (Greenburg and Hay, 1982). Epithelial cells are apical-basal polarized cells that form contiguous sheets on top of the extracellular matrix (ECM) (Hay, 1995; Thiery, 2002). Epithelial cell sheets are held together by a combination of cell-cell adhesion molecules (CAMs) (e.g. E-cadherin) (Prieto and Crossin, 1995) and the formation of junctions (e.g. lateral surface desmosome, zonula adherens, and adherens type junctions) (Farquhar and Palade, 1965; Hay, 1995). The basal actin cortex of the epithelial cells interacts in a stable, non-invasive fashion with the ECM via cellular receptors (Hay, 1995). These cell-cell and cell-ECM contacts "account for both the proper development of epithelial tissues during embryonic life and for the maintenance of homeostasis and architecture of epithelial structures during adult life" (Boyer et al., 2000).

"The goal of EMT is to produce a cell with the capacity to invade the ECM" (Hay, 1995). As such, an epithelial cell undergoes numerous changes during EMT that enable the resulting mesenchymal cell to migrate away from the epithelial origin through the ECM. For example, mesenchymal cells are known to downregulate CAM expression and modify the type of intermediate filaments they express (i.e. from keratin to vimentin intermediate filaments) (Boyer et al., 2000; Hay, 1995; Savagner, 2001; Thiery, 2002). In some cases, the resulting mesenchymal cells synthesize ECM components such as fibronectin and certain types of collagen (Boyer et al., 2000). Mesenchymal cells exhibit front end-back end polarity. Because they are motile cells, mesenchymal cells only form transient contacts with neighbouring cells (Hay, 1995). During migration, the mesenchymal cell is elongated with a leading pseudopodium that extends filipodia, and is thought to synthesize proteolytic enzymes, such as gelatinase, to enhance its invasiveness (Boyer et al., 2000; Hay, 1995; Savagner, 2001).

EMT is fundamental to early development, particularly in vertebrates. It is necessary for the formation of the three-layered embryo during gastrulation (Stern, 1992) and development of the heart, musculoskeletal system, most craniofacial structures and the peripheral nervous system (Boyer et al., 2000; Hay, 1995; Thiery, 2002). It should be emphasized that EMTs do not all exhibit the exact same set of cellular changes; however, both the loss of intercellular junctions and the acquisition of cell motility are always associated with EMT, and are together referred to as "cell scattering" (Boyer et al., 2000). Interestingly, despite the simultaneous or synchronous occurrence of these events during EMT, cell-cell dissociation and cell motility appear to occur independently of one another. For example, Boyer et al. (1989) demonstrated that inhibition of cell motility did not perturb cell-cell dissociation, while Tsukamoto et al. (1999) reported that cell-cell dissociation can be blocked without affecting cell movement.

1.3.2 Mechanism of EMT

In recent years, considerable progress has been made towards elucidating the molecular mechanisms involved in EMT. "In the majority of working models, cell scattering appears as a non-cell-autonomous process, requiring external stimuli to be activated" (Boyer et al., 2000). Some of the stimuli known to be involved in the induction of most *in vivo* EMTs include ECM components, such as collagen and fibronectin, and soluble factors, such as epidermal growth factor, scatter factor/hepatocyte growth factor, fibroblast growth factor and transforming growth factor (TGF)β family members (Hay, 1995; Markwald et al., 1996; Savagner, 2001; Savagner et al., 1994). The Wnt proteins also induce EMT steps *in vivo* during differentiation processes such as segmentation, gastrulation and central nervous system patterning (Polakis, 2000; Savagner et al., 1994). Overall, these exogenous factors alter the transcriptional control of the epithelial gene program and induce the expression of the mesenchymal gene program to produce cells capable of dissociating and migrating away from their neighbours (Hay and Zuk, 1995; Thiery, 2002).

One gene emerging as a "caretaker" of the epithelial phenotype is the prototypic Type I cadherin, E-cadherin (Hay and Zuk, 1995; Thiery, 2002). Type I cadherins mediate calcium-dependent, homophilic interactions between cells, and provide an indirect link to the actin cytoskeleton via the cytoplasmic proteins α- and β-catenin (Takeichi, 1995; Tepass et al., 2000; Thiery, 2002). E-cadherin is required to maintain stable epithelial cell junctions. In 1983, Imhof et al. reported that treatment of epithelial cells with anti-E-cadherin antibodies disrupts cell-cell contacts and induces a mesenchymal phenotype (Imhof et al., 1983). During development, E-

cadherin is lost at sites of EMT in *Drosophila* (Tepass et al., 1996), chick (Edelman et al., 1983), and mouse (Burdsal et al., 1993; Damjanov et al., 1986; Veltmaat et al., 2000). A direct correlation between E-cadherin down regulation and the loss of the epithelial phenotype has also been established *in vitro* (Behrens et al., 1989). N-cadherin, another type I cadherin, is downregulated at other sites of EMT during development, including the neural crest (Hatta et al., 1987) and somites (Birchmeier and Behrens, 1994), highlighting that loss of cell-cell junctions is important in the regulation of EMT.

So how is E-cadherin downregulated during EMT? A number of candidate transcriptional repressors have been identified to date. For example, the zinc-finger transcription factor Snail, originally identified in *Drosophila* as a controlling factor in gastrulation (Leptin, 1991), is known to downregulate transcription of the *Drosophila* E-cadherin orthologue, *shotgun*, to initiate EMT (Hemavathy et al., 2000; Oda et al., 1998). In addition to Snail, a closely related family member, Slug, is observed at sites of EMT in vertebrates (Nieto et al., 1994). Anti-sense and dominant-negative experiments have shown that Slug is necessary for EMT in chick mesoderm (Nieto et al., 1994) and heart (Romano and Runyan, 1999; Romano and Runyan, 2000) formation, and neural crest development in both the chick (Nieto, 2001) and *Xenopus* (Carl et al., 1999; LaBonne and Bronner-Fraser, 2000).

The zinc-finger protein, SIP1, has also been shown to repress E-cadherin expression in epithelial cells (Comijn et al., 2001). Interestingly, Sip1 expression is induced by TGFβ during EMT in the mouse mammary cell line, NMuMG (Thiery, 2002). Furthermore, Sip1 is strongly expressed in cell lines in which the E-cadherin promoter is hypermethylated, suggesting a mechanism for SIP1 repression of E-cadherin (Comijn et al., 2001). Finally, the E2a gene product, E12/E47, is a bHLH transcription factor that blocks E-cadherin expression (Perez-Moreno et al., 2001). E12/E47 has been detected in several cell lines lacking E-cadherin; however, unlike the aforementioned transcriptional repressors, its pattern of expression in the mouse embryo suggests a role in maintaining a mesenchymal phenotype (Thiery, 2002). Thus, any external factor or signalling pathway that is capable of inducing these transcriptional repressors has the potential to induce EMT.

1.3.3 A role for Notch signalling in EMT

1.3.3.1 Notch, EMT and cardiovascular development

Table 1.1 provides examples of EMTs that occur at specific stages during embryonic development. Interestingly, several studies have suggested that the Notch signalling pathway may play a role in EMT during organogenesis, based on the observed expression patterns of Notch pathway members in tissues that undergo EMT. For example, Notch1 is observed in the mouse embryonic whisker follicles, tooth buds and kidney (Weinmaster et al., 1991). However, there is no direct experimental evidence describing a functional role for Notch signalling in EMT to date.

Table 1.1: Examples of EMT during embryonic development

In vivo: development	Stage (mouse)	Transition	
Gastrulation	6.5 d	Epiblast	Three cell layers
Prevalvular mesenchyme (Heart formation)	8 d	Endothelium	Atrial and ventricular septum
Neural crest cells	8 d	Neural plate	Numerous derivatives (bone, muscle, PNS)
Somitogenesis and sclerotome differentiation	9 d	Somite walls	Sclerotome
Palate formation	3.5 d	Oral epithelium	Mesenchymal and epithelial cells, combined with apoptosis
Mullerian tract regression	15 d	Mullerian tract	Mesenchymal cells, combined with apoptosis

Adapted from (Savagner, 2001).

Despite the lack of a functional role in EMT, targeted disruption of Notch pathway members has revealed a critical role for Notch signalling in cardiovascular development. For example, targeted disruption of Notch1 is embryonic lethal and the lack of viability is likely due to defects in angiogenic remodeling (Krebs et al., 2000). Notch4 null animals, on the other hand, are viable and fertile, indicating that Notch4 is dispensable for vascular development (Krebs et al., 2000). Interestingly, however, Notch1/Notch4 double mutants display a more severe phenotype than Notch1 homozygous mutants (Krebs et al., 2000); these double mutants have severe vascular remodeling and angiogenic defects, and die at or before embryonic day E9.5 (Krebs et al., 2000). Interestingly, endothelial cell-specific expression of activated Notch4 also results in embryonic lethality (E9.5-E10.5) as a result of abnormal vessel structure and patterning (Uyttendaele et al., 2001). Although somewhat paradoxical at first glance, the fact that the

phenotype of the activated Notch4 knock-in resembles the Notch1 and Notch1/Notch4 knockouts indicates that proper vascular development requires appropriate levels and regulation of Notch signalling (Gridley, 2001).

Targeted disruption of other Notch pathway members has revealed phenotypes similar to those observed in Notch receptor null mice. For example, JAG1 knockout mice exhibit defective head and yolk sac angiogenesis, and die around E10.5 (Xue et al., 1999), while Dll1 homozygous mutants die from vascular defects and extensive hemorrhaging around E12 (Hrabe de Angelis et al., 1997). Mice lacking various Notch modulators further support the role of Notch signalling in cardiovascular development. For example, mice lacking both Presenilin-1 and -2, which are critical regulators of the Notch receptor S3 cleavage (Section 1.1.2), die before E9.5, probably as a result of defects in cardiac looping (Donoviel et al., 1999). Furthermore, mice with homozygous inactivation of Numb, which downregulates Notch activity via an endocytic pathway (section 1.2.2.4), demonstrate defects in angiogenic remodeling and placental dysfunction resulting in embryonic death at E11.5 (Zilian et al., 2001). Thus, elucidation of the mechanisms by which Notch signalling pathways regulate cardiovascular development is critical to better understand this important process.

1.3.3.2 Notch signalling and Alagille Syndrome (AGS)

The role of Notch in cardiovascular development is further highlighted by the discovery that mutations in the human JAG1 gene cause Alagille Syndrome (AGS) (Li et al., 1997; Oda et al., 1997b). AGS is an autosomal-dominant disorder characterized by variable abnormalities of the heart, liver, eye, vertebrae and kidney. (Alagille et al., 1987; Li et al., 1997; Oda et al., 1997b). McElhinney et al. (2002) recently analyzed 200 patients with a JAG1 mutation or AGS, and determined that 94% of these individuals have cardiovascular abnormalities. Of the cardiovascular anomolies observed, most were right-sided defects with branch pulmonary artery stenosis (a narrowing of the blood vessels that leads to the lungs) (McElhinney et al., 2002).

Two reports by Loomes et al. (1999 and 2002) have characterized the *in situ* expression of JAG1 and the Notch receptors in the developing mammalian heart. Of interest, JAG1 is observed in a distinct layer of endocardium (endothelium) in both the pulmonary outflow tract and the endocardial cushions of the atrioventricular canal at E12.5 (Loomes et al., 1999). A subset of these endocardial cells of the pulmonary outflow tract and atrioventricular canal are

known to undergo EMT during valve leaflet formation (Table 1.1; Figure 1.5A) (Eisenberg and Markwald, 1995). From this point on, EMT of endothelial cells will be referred to as endothelial-to-mesenchymal transformation. Although JAG1 expression is not restricted to endothelial cells, i.e. it is also found in perivascular cells and smooth muscle cells of the developing vessels (Loomes et al., 1999), JAG1 is primarily observed in the right-sided outflow tract, ductus arteriosus and pulmonary arteries of the developing heart, consistent with the right-sided defects in the pulmonary circulation observed in AGS patients (Loomes et al., 1999).

Of the four Notch receptors, the pattern of Notch1 expression in the developing heart is most similar to that seen for JAG1. Like JAG1, Notch1 is observed in the endocardium lining the outflow tract and the atrioventricular canal at E12.5 (Loomes et al., 2002). Notch2 overlaps Notch1 expression in a specific population of endocardial cells of the atrioventricular canal (Loomes et al., 2002). McCright et al. (2002) have also described Notch2 expression in the outflow tract at E11.5 and E14.5. Given the above expression profiles, it is tempting to speculate that JAG1-Notch interactions are involved in endothelial-to-mesenchymal transformation events of the pulmonary outflow tract and atrioventricular canal. Endothelial-to-mesenchymal transformation in these regions is required to form the endocardial cushions, which are the predecessors of the heart valves and septa (Eisenberg and Markwald, 1995). Thus, the cardiac anomalies associated with AGS imply defective cardiac cushion formation (Li et al., 1997; Oda et al., 1997b).

Figure 1.5A and B illustrate the process of endothelial-to-mesenchymal transformation in the pulmonary outflow tract and atrioventricular canal of the developing mouse heart. The primary heart tube consists of an outer myocardium surrounding an inner endocardium, separated by a myocardial derived basement membrane (cardiac jelly) (Eisenberg and Markwald, 1995). The onset of endocardial-to-mesenchymal transformation in the developing heart (E9.5 in the atrioventricular canal, E10.5 in the pulmonary outflow tract (Camenisch et al., 2002a)) is associated with a regionalized swelling of the cardiac jelly (i.e. the formation of the endocardial cushions). Although the molecular mechanisms of endothelial-to-mesenchymal transformation remain poorly elucidated, signals appear to emanate from both the myocardium and the endocardium (Eisenberg and Markwald, 1995; Icardo and Manasek, 1984; Kinsella and Fitzharris, 1982). The accumulation of myocardial derived protein complexes composed of fibronectin and EDTA soluble antigens, collectively referred to as adherons (Eisenberg and Markwald, 1995), in the cardiac jelly is essential for the initiation of endocardial-to-

mesenchymal transformation (Figure 1.5B) (Eisenberg and Markwald, 1995; Markwald et al., 1996). The EDTA soluble proteins are also expressed at other sites of EMT (Markwald et al., 1996), suggesting an overall importance of adherons in mesenchyme induction. In addition to adherons, a number of other molecules have been shown to play a role in endocardial cushion formation. For example, the TGFβ (Figure 1.5B) and homeobox gene superfamilies, bone morphogenic proteins (BMP)-4, -6, and -7, activin receptor-like kinase 2 and a variety of ECM proteins have been suggested to play an important role in endothelial-to-mesenchymal transformation (Camenisch et al., 2002a; Camenisch et al., 2002b; Eisenberg and Markwald, 1995; Kim et al., 2001; Lai et al., 2000; Mjaatvedt et al., 1998; Pierpont et al., 2000). However, the adherons remain the most critical regulators of endocardial cushion formation identified to date (Eisenberg and Markwald, 1995).

How does this relate to a role for Notch in endothelial-to-mesenchymal transformation of the developing heart? Interestingly, it has been shown that even in the restricted regions of endocardial cushion development (i.e. the atrioventricular canal and pulmonary outflow tract), only a subset of the endocardium are competent for activation (Figure 1.5B) (Markwald et al., 1996). The suggestion here is that these competent endothelia have an intrinsic mechanism required for activation. Could there be a requirement for Notch signalling in proper endocardial cushion development? Considering again the cardiac anomalies associated with AGS; it has been proposed that JAG1 haploinsufficiency is one mechanism causing the disease phenotype in humans (Li et al., 1997; Oda et al., 1997b). Although heterozygous deletion of JAG1 in mice does not result in AGS associated cardiac anomolies (Xue et al., 1999), JAG1 null animals die around E10.5, which limits the ability to analyze the role of JAG1 in heart morphogenesis. Interestingly, mice that are doubly heterozygous for a JAG1 null allele and a Notch2 hypomorph display cardiovascular anomalies characteristic of AGS (McCright et al., 2001). These results suggest that other genes in the Notch signalling pathway may contribute to the variety of phenotypes presented by AGS patients. In support of such a hypothesis, three separate groups recently disrupted expression of the Notch target HRT-2 in mice; HRT-2 knockout mice display cardiac defects that resemble those seen in humans with mutations in JAG1, including ventricular septal defects and pulmonary artery stenosis (Donovan et al., 2002; Gessler et al., 2002; Sakata et al., 2002). Overall, experimental evidence demands a look at the functional role

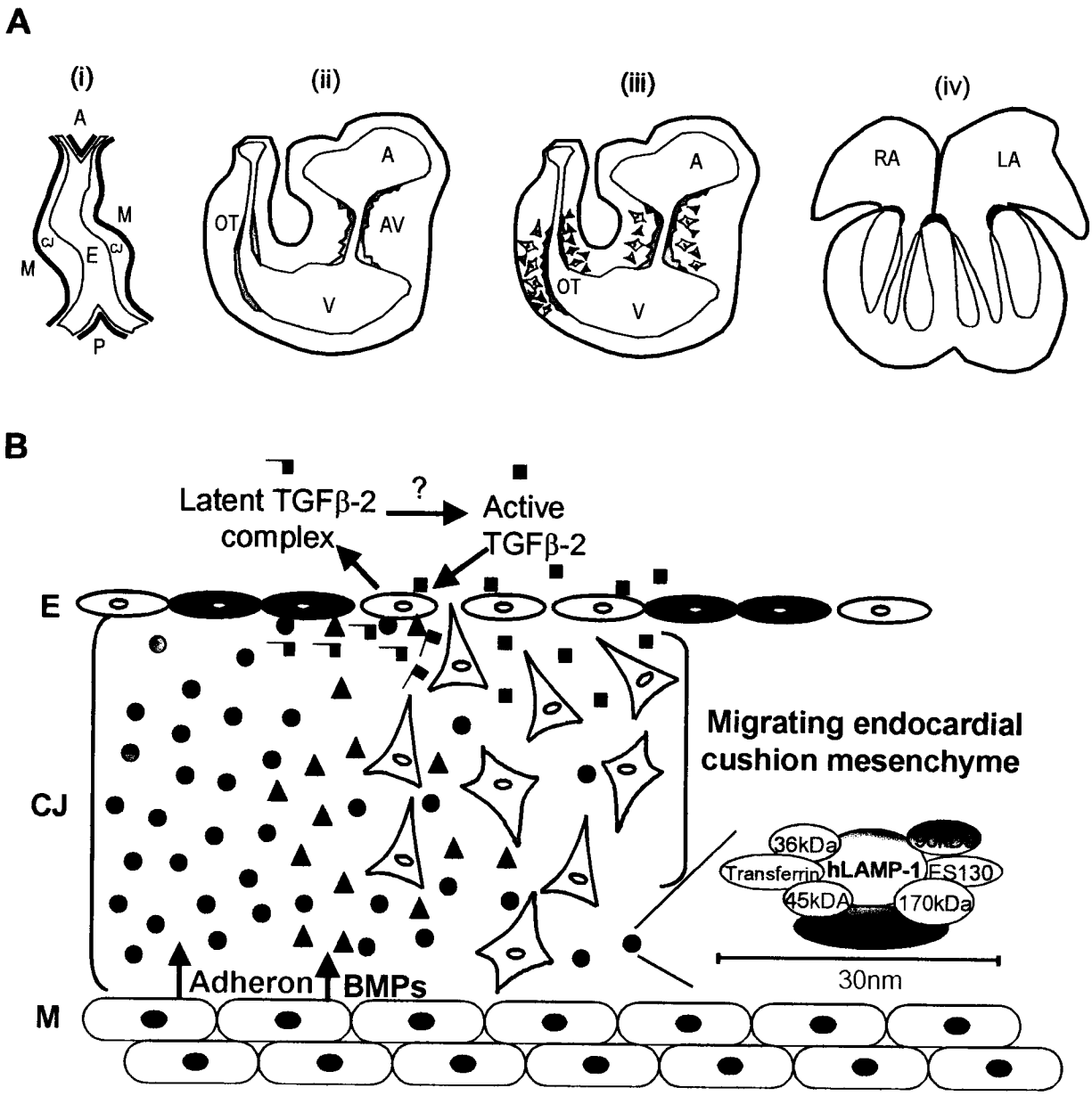


Figure 1.5. Formation of the endocardial cushion tissue in the developing mouse heart. **(A)** (i) The primary heart tube is composed of an outer myocardium (M) and an inner endocardium (E) separated by an acellular matrix called the cardiac jelly (CJ). A=anterior. P=posterior. (ii) After cardiac looping, regionalized thickening of the cardiac jelly in the atrioventricular (AV) canal and pulmonary outflow tract (OT) defines the onset of endocardial cushion tissue formation. (iii) Transformed endocardium (mesenchyme) migrate into the cardiac jelley and form the endocardial cushions, the precursors of the membranous valves and septa. (iv) The blue colouring illustrates the contribution of the endocardial cushion tissue to the valves of the four chambered heart. *Adapted from (Markwald et al., 1996).* **(B)** At the onset of endocardial cushion formation, the cardiac jelly contains myocardial derived adherons, which are protein complexes containing fibronectin and EDTA soluble antigens. Adherons are required for the activation of endocardium and the induction of EMT. The endocardium of the atrioventricular canal and pulmonary ouflow tract contain a subset of endothelium (blue cells) that are competent for activation and transformation. Activated endocardium and migrating mesenchyme secrete TGFβ-2, which may act in an autocrine manner to enhance mesenchyme formation. BMPs have also been shown to enhance mesenchyme formation. *Adapted from (Nakajima et al., 2000) and (Markwald et al., 1996).*

of the Notch signalling pathway in endothelial-to-mesenchymal transformation of the developing heart, and other tissues which require EMT for proper development.

1.4 AIMS OF STUDY

The original aim of this study was to characterize the differences in gene expression in human microvascular endothelial cells (HMEC) overexpressing the activated form of the Notch4 receptor (HMEC-Notch4IC) compared to control cells (HMEC-vector), since previous work in the lab had shown that enforced expression of Notch4IC in HMEC blocked angiogenic remodeling *in vitro*. A cDNA subtraction/hybridization technique (Representational Differential Analysis (RDA)) was employed to uncover Notch4IC induced targets (results will not be presented in this thesis). During the course of these studies, we noticed that the gross morphology of the HMEC-Notch4IC cells was noticeably different from HMEC-vector cells when grown in two dimensions. By light microscopy, HMEC-Notch4IC did not form a proper endothelial monolayer, and seemed to be piling on top of one another. As a starting point, we decided to look at the endothelial-specific adherens junction protein, vascular endothelial (VE)-cadherin. VE-cadherin mediates Ca^{2+}-dependent, homophilic interactions, and is required in the formation of proper endothelial cell-cell junctions. Western blot and immunofluorescence studies showed that HMEC-Notch4IC significantly downregulated VE-cadherin compared to control HMEC. This downregulation was also true for another important endothelial cell junctional molecule, PECAM-1. In light of these findings, we decided to look at the expression of a variety of endothelial cell markers in HMEC-Notch4IC, and showed that all were down to various degrees. HMEC-Notch4IC appeared to be losing the endothelial phenotype, and similar results were observed when Notch1IC was expressed in HMEC.

The morphology of HMEC-Notch4IC and the lack of an endothelial specific cadherin suggested that activated Notch may be inducing an endothelial-to-mesenchymal transformation. ***The aim of this thesis, therefore, was to determine whether overexpression of activated Notch could induce this endothelial-to-mesenchymal transformation in HMEC and primary endothelial cell types***. In addition to cell-cell disruption (i.e. loss of VE-cadherin and PECAM-1), two other hallmarks of this transformation are the expression of the migratory protein alpha-smooth muscle actin (SMA) and the acquisition of cell motility; both were observed in HMEC-Notch4IC and -Notch1IC. To further characterize this transformation, we looked at the role of TGFβ, since the TGFβ family of proteins has been shown to play an important role in EMT

during organogenesis, including heart development. Finally, since mutations in JAG1 are responsible for AGS (in which patients exhibit cardiac defects consistent with improper, EMT-dependent cardiac cushion formation), we attempted to validate our hypothesis that Notch signalling may be involved in endothelial-to-mesenchymal transformation of the heart by looking at expression and effects of the Notch ligand JAG1.

Chapter 2

MATERIALS AND METHODS

2.1 TISSUE CULTURE

2.1.1 Cell lines and reagents

The HMEC-1 microvascular endothelial cell line, hereafter referred to as HMEC was provided by the Centers for Disease Control and Prevention (Atlanta, GA) (Ades et al., 1992). HMEC lines 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 100 U each of penicillin and streptomycin (MCDB-HMEC). Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (Karsan et al., 1997), and maintained in MCDB medium supplemented with 20% heat-inactivated FCS, 20 μg/ml endothelial cell growth supplement (BD Bioscience, Bedford, MA), 16 U/ml heparin and 100 U each of penicillin and streptomycin (MCDB-HUVEC). Human aortic endothelial cells (HAEC) and human microvascular endothelial cells (HMVEC) were purchased from Clonetics (subsidiary of BioWhittaker, Inc., Walkersville, MD) and cultured in Endothelial Growth Media (EGM) (Clonetics) according to manufacturer's specifications. Primary umbilical artery smooth muscle cells (UASMC) were purchased from Clonetics and were cultured in Smooth muscle cell Basal Medium according to manufacturer's specifications. All cells were maintained at 37°C in 5% CO_2.

For conditioned medium experiments, 3-day HMEC-Vector or HMEC-Notch4IC supernatants were 0.45 μm filtered before addition to parental HMEC. Conditioned medium was changed daily. Recombinant TGFβ-1 and TGFβ-2 and mouse monoclonal antibodies (pan-anti-TGFβ neutralizing antibody (clone 1D11) and control IgG1) were purchased from R&D Systems, Inc. (Minneapolis, MN).

2.1.2 Gene transfer

Endothelial cells were transduced using the retroviral vectors MSCV-IRES-YFP (MIY) (gift from Dr. R. K. Humphries) or LNCX, with cDNA constructs encoding the C-terminal HA-tagged Notch4 intracellular domain (Leong et al., 2002), the Notch1 intracellular domain (gift from Dr. S. Artavanis-Tsakonas), or full-length JAG1 (Li et al., 1998). Endothelial cells were

transduced as previously described (Karsan et al., 1996) except that transient transfections of the Ampho Phoenix packaging cell line were performed using Fugene 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). Cells were sorted for YFP expression using a FACS 440 (BD, Franklin Lakes, NJ), or selected with 300 μg/ml neomycin (G418) (LNCX constructs).

2.1.3 Chemotactic assay

The ability of HMEC to migrate towards vascular endothelial growth factor (VEGF; 15 ng/ml, R&D Systems, Inc.) and platelet derived growth factor (PDGF) (PDGF-BB; 20 ng/ml, R&D Systems, Inc.) was measured by a Transwell filter assay (Corning Costar, VWR International) as previously described (Leong et al., 2002). Briefly, polycarbonate filters (8.0 μm pores) of the upper chamber were coated with 50 μl fibrinogen (Sigma; 2.5 mg/ml in phosphate buffered saline (PBS)) and dried overnight. HMEC at 75-100% confluence were trypsinized, washed twice with soybean trypsin inhibitor (10 μg/ml), and resuspended in serum-free MCDB. 3.5 x 10^4 cells were plated in the upper chamber and serum-free MCDB alone (negative control), with VEGF, or with PDGF was plated in the lower chamber. HMEC were incubated with the chemoattractant factors (16 hours, 37°C). The filters were washed with room temperature PBS, fixed (30 minutes, room temperature) in 4% paraformaldehyde, then stained (10 minutes, room temperature) in 0.5% crystal violet/20% methanol. Stained cells were washed with dH_20, and adherent cells were removed from the upper side of the filter with a cotton swab. Cells that had migrated and adhered to the underside of the filter were counted using a Nikon Eclipse TS100 standard inverted microscope (Nikon Corporation, Tokyo Japan).

2.2 PROTEIN ANALYSIS

2.2.1 Total cell lysate preparation

Medium was removed from cell monolayers, and the tissue culture plates were washed once with room temperature PBS. The PBS was removed, and the plates were put on ice. The remaining steps were done at 4°C or on ice as described (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Ice cold lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with the addition of fresh protease inhibitors (10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) and 0.5 mM sodium ortho-vanadate were added at 3.5 μl/cm^2 to the culture plate. The cells were scraped and transferred to a 1.5 ml Eppendorf tube.

The DNA was sheared by passing the lysate through a 21 gauge needle and the lysate was then left on ice for 30-60 minutes. Cellular debris was clarified at 10,000xg for 15 minutes at 4°C and the supernatant (total cell lysate or TCL) was transferred to a new Eppendorf tube.

2.2.2 Immunoblotting

50 μg of total protein/lane (Bio-Rad DC Protein Assay System; Bio-Rad Laboratories, Hercules, CA) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies were used for Western blotting according to manufacturer's recommendations. The mouse antibody against the HA-epitope tag was purchased from BabCo (Richmond, CA), goat anti-VE-cadherin, goat anti-PECAM-1, rabbit anti-Tie1 and rabbit anti-Tie2 were all from Santa Cruz Biotechnology Inc. The mouse anti-eNOS/NOS Type III and anti-fibronectin were purchased from Transduction Laboratories, while the mouse anti-SMA antibody was from Cymbus Biotechnology Ltd. (Hampshire, UK). Mouse anti-alpha-tubulin was purchased from Sigma. Images were acquired using the MulitiImage™ Light Cabinet with FlourChem™ FC Software (Alpha Innotech Corporation, San Leandro, CA)

2.2.3 ELISA

For ELISA analysis, HMEC, HUVEC and HAEC were grown until 75-80% confluent. The cells were washed with PBS, and put in the appropriate medium (Section 2.1.1) for 2 to 3 days. The supernatants were collected at indicated times, and cellular debris was removed by centrifugation (2500 rpm, 5 minutes, 4°C). Human TGFβ-1 and TGFβ-2 ELISAs (R&D Systems, Inc.) were performed according to the manufacturer's instructions.

2.3 RNA ANALYSIS

2.3.1 RNA collection and RT-PCR

RNA was isolated using TRIzol Reagent (Invitrogen, Burlington, ON) and quantitated by spectrophotometry (260 nm). RNA quality was assessed using the 260/280 nm ratio, and by running 1 μl of total RNA on a 1% TAE gel. 1.0-2.5 μg of total RNA for each sample was DNAse treated (Invitrogen) and converted to first strand cDNA. A reverse transcription (RT) reaction was performed for each RNA sample without the addition of the reverse transcriptase as a control for genomic DNA contamination. PCR was performed using 1 μl of cDNA on a PowerBlock II™ EasycyclerTM (Ericomp Inc.). Primer sequence and cycle conditions are

described in Table 2.1. The VE-cadherin and HRT-1 primers are as described (Bach et al., 1998; Henderson et al., 2001). PCR products were resolved on TAE agarose gels and images were acquired using the MulitiImage™ Light Cabinet (Alpha Innotech Corporation).

Table 2.1: Primers and PCR conditions used in this thesis.

TARGET	SENSE	ANTI-SENSE	T_M(°C)	CYCLES	SIZE(bp)
hNotch1	cactgtgggcgggtcc	gttgtattggttcggcaccat	55	35	85
mNotch1	ggccacctcttcactgcttc	ccggaacttcttggtctcca	60	35	529
Notch2	cccaatgggcaagaagtcta	cacaatgtggtggtgggata	53	30	402
Notch3	tgagacgctcgtcagttctt	tggaatgcagtgaagtgagg	53	35	667
hNotch4	tagggctccccagctctc	ggcaggtgcccccatt	60	35	486
mNotch4	caagctcccgtagtcctacttc	ggcaggtgcccccatt	53	35	486
hJAG1	ctatgatgaggggggatgct	cgtccattcaggcactgg	53	35	507
mJAG1	aatggagactccttcacctgt	cgtccattcaggcactgg	53	35	383
Dll4	gcattgtttacattgcatcctg	caagggcgtgcgcgtcaaagta	60	30	456
HES-1	aggcggacattctggaaatg	cggtacttccccagcacactt	55	30	103
HRT-1	ggagaggcgccgctgtagtta	caagggcgtgcgcgtcaaagta	57	28	456
HRT-2	tgagcataggattccgagagtgc	gaaggacagagggaagctgtgtg	57	28	481
HRT-3	cactggtgggacaggattctttg	gtaagcagccgaccctgtaggac	57	28	576
VE-cadherin	cccaccggcgccaaaagagagattgg	ctggttttccttcagctggaagtggt	60	28	1033
VEGFR-2	gactagtaagcgggccaatggaggg	gactagtaacaggaggagagctcagtg	60	35	1742
Tie2	ccatgaagatgcgtcaacaag	gtctgggagacagaacacataagac	50	30	509
SMA	aacactttcctgctcctctct	tcaacctaacaaatggtatcagt	50	35	202
GAPDH	cccatcaccatcttccag	atgaccttgcccacagcc	55	22	445
mGAPDH	gcatggccttccgtgt	gggccgagttgggatagg	53	22	385

2.3.2 RNAse protection assays

For mRNA analysis, HMEC were grown until 75-80% confluent in MCDB-HMEC. The cells were washed with PBS, and put in MCDB + 2% FCS + 100U each of penicillin and streptomycin for 2 to 3 days. RNA was isolated using TRIzol Reagent (Invitrogen). Cytokine mRNA levels were quantitated using a Riboquant™ Multi-Probe RNase Protection Assay (hCK-3 template set; PharMingen, Mississauga, ON) according to the manufacturer's instructions, using [^{33}P]dUTP 9NEG-307H from NEN Life Science Products Inc. (Boston, MA). Quantitation of cytokine mRNA levels, using a phosphorimager (Storm 860, Molecular Dynamics, Sunnyvale, CA), was standardized based on levels of the housekeeping genes GAPDH and L32.

2.4 TRANSIENT TRANSFECTION AND LUCIFERASE ASSAYS

HMEC were grown until 75-80% confluence, washed twice with PBS, trypsinized for 3 minutes at 37°C, then resuspended in MCDB-HMEC. 1.5 x 10^6 cells were pelleted (1000 rpm, 3 minutes, room temperature), washed once with PBS and pelleted as above. The cells were resuspended in 0.4ml 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) with plasmid DNA (2-10 μg total per transfection) and transferred to a 4 mm-gap electroporation cuvette (Bio-Rad; Mississauga, ON). The cell-DNA mixture was left for 10 minutes at room temperature, and then electroporated at a fixed capacitance of 900 μF and 200 V using a Bio-Rad Gene Pulser II instrument (Bio-Rad). The electroporated cells were left for 10 minutes at room temperature, then plated in 12-well culture dishes with pre-warmed (37°C) MCDB-HMEC. The medium was changed after 24 hours. 48 hours post-transfection, dual-luciferase reporter assays were performed according to manufacturer's recommendations (Promega Corporation, Madison, WI) and luminescence was measured on a Tropix™ tube luminometer (BIO/CAN Scientific, Mississauga, ON). The CBF1 reporter, 4xCBF1wt-Luc (pJH23A) (Hsieh et al., 1996), was a gift from Dr. Diane Hayward. The CBF1 reporter contains four CBF1 (CSL) binding motifs cloned in front of the SV40 promoter of the pGL2pro luciferase vector (Promega). The SMA promoter-reporter construct encompassing a 5.4 kb region comprising –2555 to +2813 of the rat SMA gene, pPIact-LUC, was a gift of F. Dandre and G. K. Owens. Transfections were normalized by transfecting cells with 50 ng of the Renilla luciferase plasmid pRL-CMV (Promega).

2.5 MICROSCOPY

Light micrographs were taken with a Nikon COOLPIX 990 on a Nikon Eclipse TS100 standard inverted microscope (Nikon Corporation). For immunofluorescence staining, cells were fixed in 4% paraformaldehyde, and blocked/permeablized in 4% FCS/0.2% TritonX-100/PBS. Primary goat anti-VE-cadherin (Santa Cruz Biotechnology Inc.) and mouse anti-SMA (Cymbus Biotechnology Ltd.) were both used at 1:100 dilutions for 1 hour at room temperature. Alexa 594 secondary antibodies were used according to the manufacturer's recommendations (Molecular Probes, Eugene, OR). Images were acquired using a 1350EX cooled CCD digital camera (QImaging, Burnaby, BC) on a Zeiss Axioplan II Imaging inverted microscope (Carl Zeiss Canada Ltd, Toronto, ON) and analyzed using Northern Eclipse Image Analysis Software (Empix Imaging, Mississauga, ON). For transmission electron microscopy, HMEC-vector and HMEC-Notch4IC were plated in 4-well culture plates coated with Type I collagen (1 mg/ml; Upstate Biotechnology, Inc., Lake Placid, NY). The cells were grown until 75-80% confluent, and then given to our collaborator Dr. Dorovini-Zis (Department of Pathology, Division of Neuropathology, University of British Columbia and General Hospital and Health Sciences Centre) for processing as previously described (Dorovini-Zis et al., 1991).

Chapter 3

NOTCH ACTIVATION INDUCES ENDOTHELIAL-TO-MESENCHYMAL TRANSFORMATION

3.1 INTRODUCTION

As described in section 1.3, recent findings suggest that the Notch family of transmembrane receptors may play a critical role in cardiac cushion development and in the pathogenesis of congenital cardiac malformations. Notwithstanding these suggestions that Notch signalling may be required in cardiac cushion formation, there is no direct evidence that Notch activation is able to induce an endothelial- or even an epithelial-to-mesenchymal transformation. Thus, in this study we examined whether Notch activation can induce an endothelial-to-mesenchymal transformation. Our results show that Notch activation in endothelial cells causes a disruption of the normal cobblestone monolayer associated with areas where cells overlie each other, thus forming multiple layers. Intercellular junctions and expression of the endothelial-specific adherens junction protein, VE-cadherin is lost. Other endothelial markers such as PECAM-1 (CD31), Tie2 and eNOS are also downregulated. Furthermore, Notch-activated endothelial cells begin to express mesenchymal markers including SMA. In endothelial cells, activation of the Notch pathway by JAG1 also activates a SMA promoter-reporter construct and induces SMA protein expression. Our studies suggest that the endothelial-to-mesenchymal transformation involved in cardiac cushion development, at least in part, depends upon activation of the Notch pathway through interaction of Notch receptors with the ligand JAG1.

3.2 RESULTS

3.2.1 Overexpression of activated Notch4 alters HMEC morphology

The Notch signalling pathway plays a critical role in vascular remodeling and angiogenesis. (Krebs et al., 2000; Xue et al., 1999). Our lab is concerned with the biology of Notch4, which is primarily expressed in endothelial and endocardial cells, and has previously been demonstrated to modulate vascular remodeling both *in vitro* and *in vivo* (Leong et al., 2002; Shirayoshi et al., 1997; Uyttendaele et al., 1996). The model for this thesis is the endothelial cell line HMEC, which is an immortalized (SV40 large T-antigen transformed) microvascular cell (Ades et al., 1992). In an effort to determine the suitability of this system, we characterized the expression of the four human Notch receptors and two of the ligands (JAG1, Dll4) by RT-PCR

(Figure 3.1A). We looked at HMEC and primary endothelial cells from different vascular beds (human microvascular endothelial cells (HMVEC), human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC)). Primary umbilical artery smooth muscle cells (UASMC) were used as a negative control, since Notch4 has not been shown to be expressed in smooth muscle (Shirayoshi et al., 1997; Uyttendaele et al., 1996). As seen in Figure 3.1A, HMEC express the receptors and ligands in a pattern similar to primary HMVEC, albeit at much reduced levels for Notch4, JAG1, and Dll4.

The intracellular domain of Notch4 (Notch4IC) acts as a constitutively-active receptor (Gallahan et al., 1996; Jhappan et al., 1992; Soriano et al., 2000; Uyttendaele et al., 1998). Using retrovirally-transduced HMEC, we demonstrated that enforced expression of Notch4IC induces the CSL-dependent targets HES-1 and HRT-1, -2, and -3 (Figure 3.1B) as previously reported, indicating that our construct is functional (Iso et al., 2003). During these studies we noted that HMEC expressing Notch4IC (HMEC-Notch4IC) lost the characteristic cobblestone morphology of confluent endothelial cells as compared to cells expressing the empty vector alone (HMEC-vector) (Figure 3.1C). In regions where the monolayer was disrupted, cells appeared to overlie one another and form multiple layers, suggesting the loss of intercellular endothelial junctions.

3.2.2 Activated Notch4 causes loss of the endothelial phenotype

To confirm the observation that the HMEC-Notch4IC are indeed piling on top of each other, and to further study the morphology and junctional structures, we performed transmission electron microscopy on HMEC-vector and HMEC-Notch4IC. As seen in the photomicrographs shown in Figure 3.2A, HMEC-Notch4IC failed to form contact-inhibited monolayers (i) and proper cell-to-cell junctions (ii), and showed marked overlapping with infrequent, rudimentary cell contacts. In contrast, HMEC-vector endothelial cells retained their capacity to form junctions as shown in Figure 3.2A (iii). These observations suggest that Notch4IC induces a loss of the typical endothelial morphological phenotype.

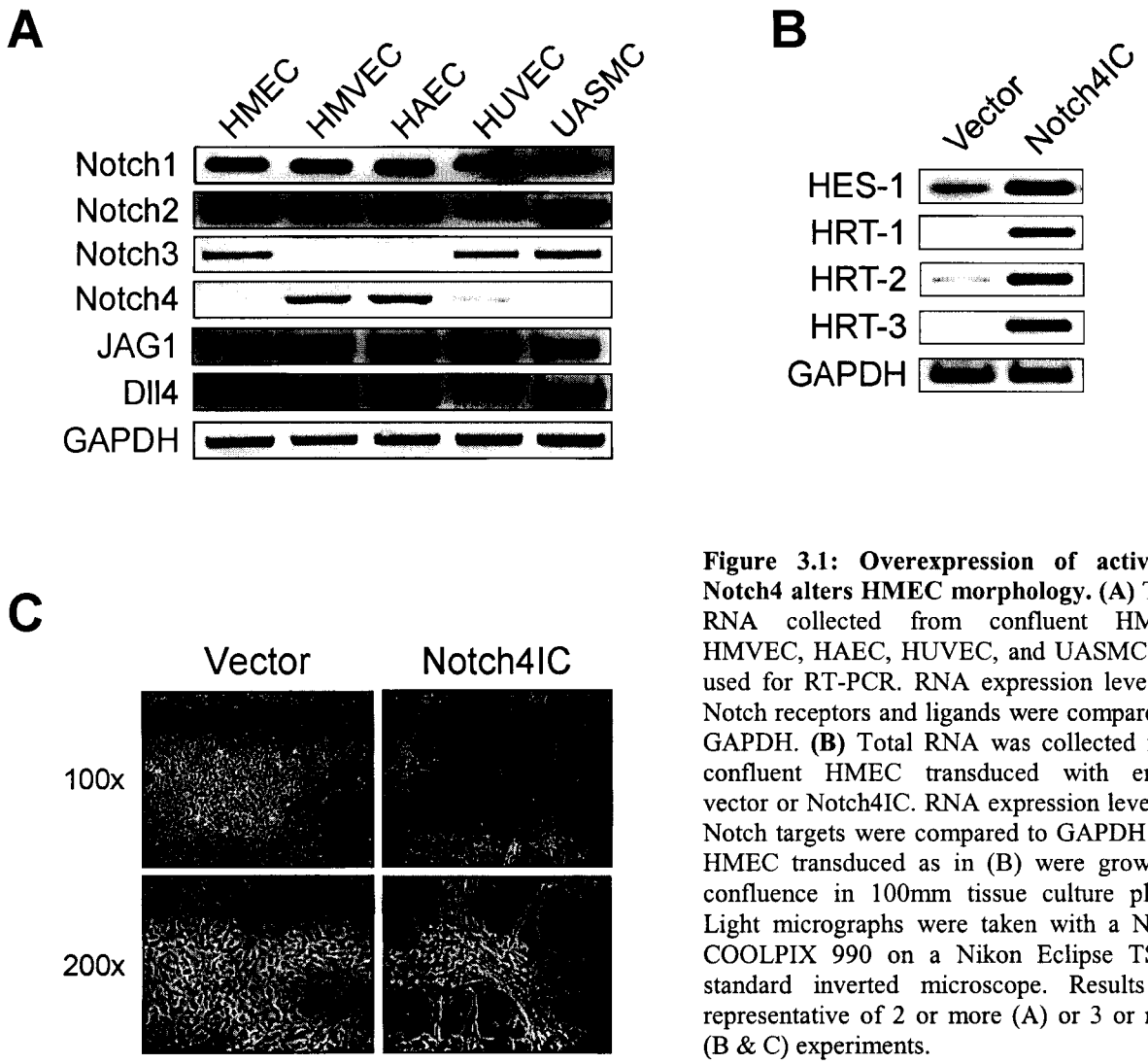


Figure 3.1: Overexpression of activated Notch4 alters HMEC morphology. (A) Total RNA collected from confluent HMEC, HMVEC, HAEC, HUVEC, and UASMC was used for RT-PCR. RNA expression levels of Notch receptors and ligands were compared to GAPDH. **(B)** Total RNA was collected from confluent HMEC transduced with empty vector or Notch4IC. RNA expression levels of Notch targets were compared to GAPDH. **(C)** HMEC transduced as in (B) were grown to confluence in 100mm tissue culture plates. Light micrographs were taken with a Nikon COOLPIX 990 on a Nikon Eclipse TS100 standard inverted microscope. Results are representative of 2 or more (A) or 3 or more (B & C) experiments.

The formation of tight and adherens junctions characterize interactions between endothelial cells. VE-cadherin is a cell adhesion molecule that is localized to interendothelial junctions and is required for the formation of adherens junctions (Breviario et al., 1995; Lampugnani et al., 1992; Salomon et al., 1992). We hypothesized that the lack of proper endothelial junctions and changes in the morphology detected by electron microscopy in HMEC-Notch4IC was a consequence of the loss of VE-cadherin expression. We compared RNA levels for VE-cadherin in HMEC-vector and HMEC-Notch4IC cells and discovered that Notch4IC dramatically reduces VE-cadherin expression (Figure 3.2B). It has been previously reported that activation of Notch4 results in the downregulation of vascular endothelial growth factor receptor

(VEGFR)-2 expression in HUVEC (Taylor et al., 2002). Thus, as a control, we confirmed that VEGFR-2 mRNA levels were reduced in HMEC-Notch4IC (Figure 3.2B). Interestingly, the mRNA of an independent endothelial marker, Tie2, was also downregulated in HMEC-Notch4IC (Figure 3.2B). VE-cadherin protein expression was also reduced as demonstrated by immunoblotting cell lysates from HMEC-vector and HMEC-Notch4IC (Figure 3.2C). To further study changes in the phenotype of HMEC-Notch4IC, we analyzed the protein levels of several endothelial cell markers including PECAM-1 (CD31), Tie1, Tie2 and eNOS. We observed a reduction in expression, to various degrees, of all endothelial markers that were tested (Figure 3.2C).

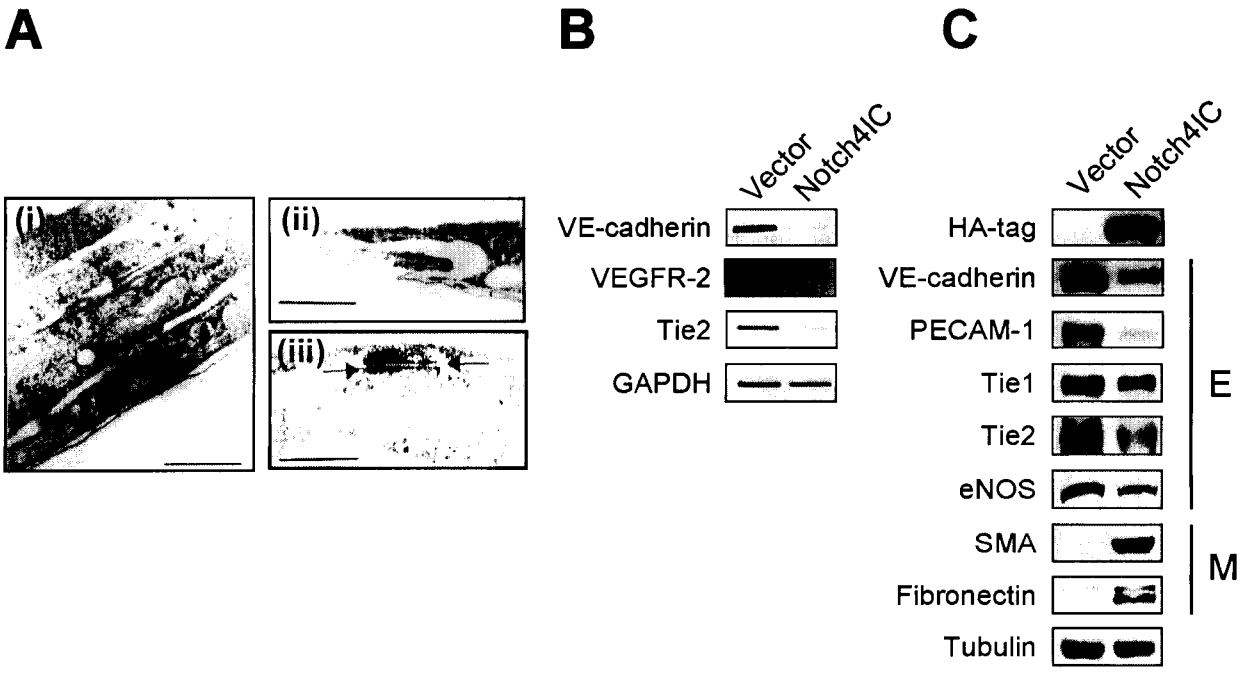


Figure 3.2: Activated Notch4 causes loss of the endothelial phenotype. (A) HMEC transduced with empty vector form confluent monolayers composed of closely associated cells focally formed together by tight junctional complexes with pentilaminar configuration (arrowheads; iii). In contrast, Notch4IC transduced HMEC are arrayed in overlapping cell layers (i) with no evidence of intercellular junctions between adjacent cells (ii). (i) Notch4IC overlapping at 51,000x, scale bar = 1μm; (ii) HMEC-Notch4IC junction at 18,000x, scale bar = 2μm; (iii) HMEC-vector control junction at 61,000x, scale bar = 0.75μm. **(B)** Total RNA collected from confluent HMEC transduced with empty vector or Notch4IC was used for RT-PCR. RNA expression levels were compared to GAPDH. **(C)** Endothelial (E) and mesenchymal (M) markers were analyzed by Western blot. Protein lysates were separated by SDS-PAGE and probed for the indicated markers as described in materials and methods. Membranes were reprobed with anti-tubulin antibodies to show equal loading. Results in (B & C) are representative of 3 or more experiments.

Loss of VEGF receptor expression and reduced cell-to-cell interactions are observed as endocardial (endothelial) cells of the heart transform into mesenchymal cells (Dor et al., 2001). These mesenchymal cells will form the primordia of the heart valves and septa. One of the hallmarks of endothelial-to-mesenchymal transformation is the induction of SMA, which is not normally expressed in endothelial cells, in the activated endocardial cells and migrating mesenchyme (Nakajima et al., 1997). To demonstrate that, in addition to morphologic changes and loss of endothelial phenotype, Notch4-activated cells undergo a mesenchymal transformation, we determined whether SMA was expressed in HMEC-Notch4IC. Immunoblotting shows induction of expression of SMA protein in HMEC-Notch4IC (Figure 3.2C). Cardiac cushion mesenchymal cells also synthesize large amounts of fibronectin during their migration towards the myocardium (Ffrench-Constant and Hynes, 1988; Icardo and Manasek, 1984), and we observed upregulation of fibronectin expression in Notch4-activated cells (Figure 3.2C). Therefore, Notch4 activation in endothelial cells induces several of the phenotypic changes seen in endothelial-to-mesenchymal transformation.

3.2.3 Activated Notch1 causes loss of the endothelial phenotype

In addition to Notch4, the Notch1 gene is expressed in endothelial cells of the embryonic vasculature (Del Amo et al., 1992; Reaume et al., 1992). In the developing mouse heart, Notch1 mRNA has been observed in endocardial cells of the cardiac outflow tract and atrioventricular canal at E12.5 (Loomes et al., 2002). At this stage of development, a subset of these endothelial cells overlying the cardiac cushions will have undergone endothelial-to-mesenchymal transformation (Eisenberg and Markwald, 1995). JAG1 has been shown to activate Notch1, and its expression is similar to that seen for Notch1 in the developing heart (Lindsell et al., 1995; Loomes et al., 1999). RT-PCR data confirmed that expression of an activated Notch1 in HMEC induced all CSL-targets tested (HES-1, HRT-1, -2, and -3) in a similar pattern to that observed with activated Notch4 (Figure 3.3A). We hypothesized that, similar to Notch4, constitutive expression of activated Notch1 (Notch1IC) would induce characteristics associated with a mesenchymal transformation of endothelial cells. By immunofluorescence microscopy we examined the effect of Notch1IC on the expression of the endothelial marker VE-cadherin and the mesenchymal marker SMA, using Notch4IC as the control. As shown in Figure 3.3B, activated Notch4 and Notch1 both caused the loss of VE-cadherin expression and induction of SMA. To compare the kinetics and the efficiency of the transformation induced by activated Notch4 versus Notch1, we retrovirally-transduced HMEC with Notch4IC or Notch1IC, and

analyzed SMA expression by immunofluorescence at 7 and 14 days after the initial infection. The percentage of SMA-positive HMEC transduced with vector, Notch4IC or Notch1IC was 0% ± 0%, 0.52% ± 0.30% and 3.43% ± 0.66% at day 7 and 0% ± 0%, 3.50% ± 1.77% and 15.43% ± 3.65% at day 14, respectively (Figure 3.3C). Thus, activated Notch1 shows a higher propensity to induce a mesenchymal phenotype in HMEC compared to activated Notch4.

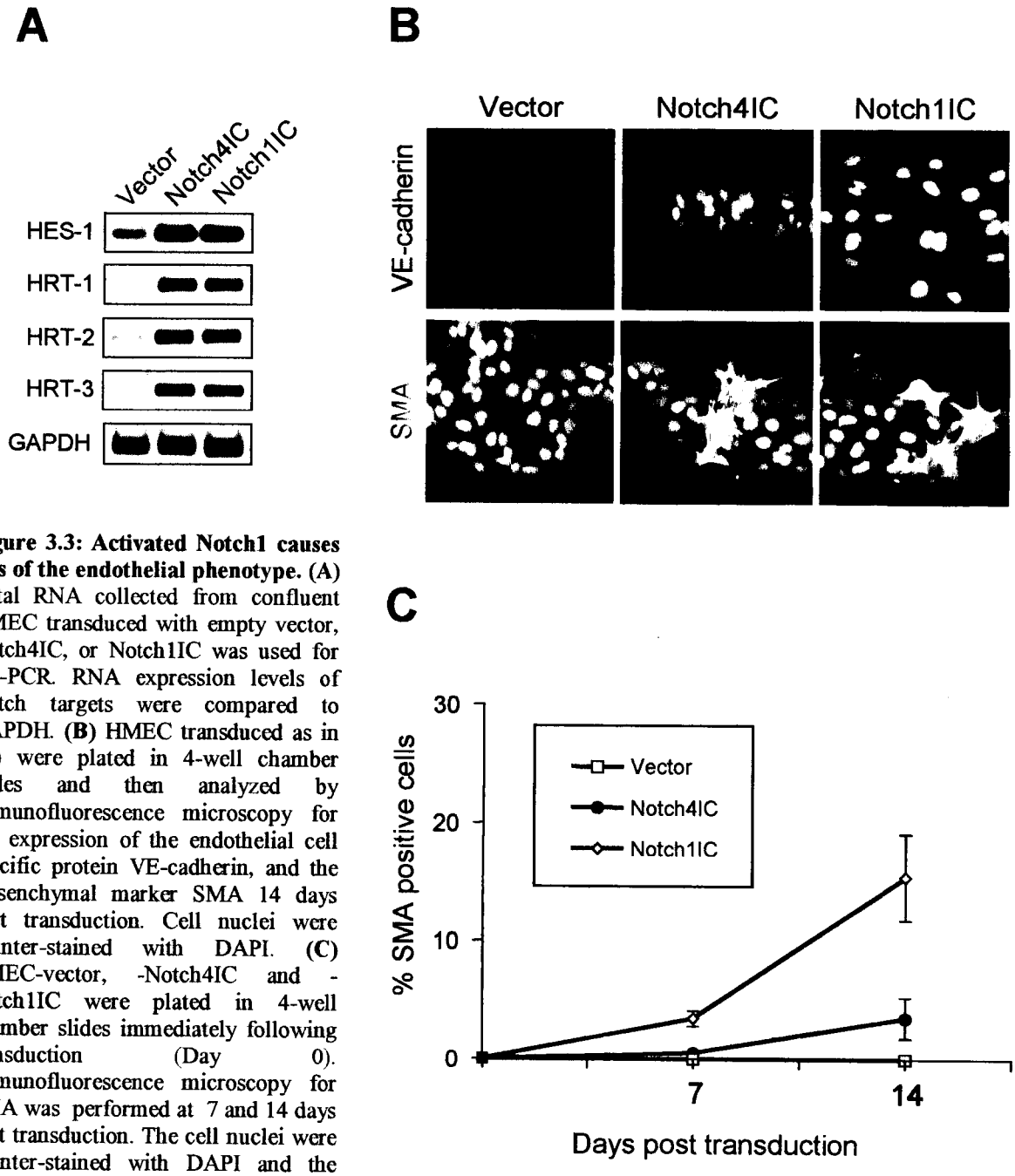


Figure 3.3: Activated Notch1 causes loss of the endothelial phenotype. (A) Total RNA collected from confluent HMEC transduced with empty vector, Notch4IC, or Notch1IC was used for RT-PCR. RNA expression levels of Notch targets were compared to GAPDH. (B) HMEC transduced as in (A) were plated in 4-well chamber slides and then analyzed by immunofluorescence microscopy for the expression of the endothelial cell specific protein VE-cadherin, and the mesenchymal marker SMA 14 days post transduction. Cell nuclei were counter-stained with DAPI. (C) HMEC-vector, -Notch4IC and -Notch1IC were plated in 4-well chamber slides immediately following transduction (Day 0). Immunofluorescence microscopy for SMA was performed at 7 and 14 days post transduction. The cell nuclei were counter-stained with DAPI and the number of SMA positive cells was expressed as a percentage of the total cell number. Results are representative of 2 or more experiments.

3.2.4 Activated Notch1 and Notch4 induce an endothelial-to-mesenchymal transformation

As outlined in section 1.3.1, different cell types undergoing EMT do not always exhibit the same set of cellular changes. However, both the loss of intercellular junctions and the acquisition of cell motility are always associated with EMT (Boyer et al., 2000). We demonstrated the loss of endothelial junctional molecules (VE-cadherin and PECAM-1) and the induction of motility associated proteins (fibronectin and SMA) by activated Notch4 and Notch1 in Figures 3.2C and 3.3B. In order to determine whether Notch directly affects mesenchymal gene expression, we performed a series of promoter assays. Activated Notch receptors have been shown to signal by direct interaction with CSL (CBF1 is the mammalian CSL homologue), and derepression and/or co-activation of transcription at sites containing CSL consensus motifs (Kato et al., 1996). We transiently transfected a CSL-dependent luciferase construct, 4xCBF1wt-Luc, (Section 2.4 and Hsieh et al., 1996) into HMEC transduced with activated Notch4 or empty vector. As seen in Figure 3.4A (left panel), activated Notch4 induced a 3.4 fold increase in CBF1-dependent luciferase activity over control cells. We next transfected HMEC-vector and HMEC-Notch4IC with an SMA promoter-luciferase plasmid, and observed a Notch4-driven increase in SMA promoter activity of 6.6 fold (Figure 3.4A, right panel). RT-PCR confirmed the upregulation of SMA mRNA in both Notch4IC and Notch1IC transduced endothelial cells (Figure 3.4B).

Since expression of Notch may result in secondary changes that upregulate SMA expression, we determined whether activated Notch4 could regulate transcription of SMA, in a transient transfection assay. Parental HMEC were transiently transfected with Notch4IC or empty vector plasmid DNA, and the SMA promoter-luciferase plasmid (Figure 3.4C). To demonstrate that transient transfection of Notch4IC activates a CBF1-dependent promoter, we used the CBF1 promoter-luciferase construct described above (Figure 3.4C). Activated Notch4 induced a 6.1 fold and 7.7 fold increase in CBF1 and SMA promoter luciferase expression, respectively, 48 hours post transfection. These findings suggest that transcription of SMA is induced early after Notch activation.

Platelet derived growth factor (PDGF) is a known chemotactic factor for mesenchymal cells in various contexts, including EMT (Paranya et al., 2001). In particular, PDGF receptor-α is expressed between E9-10.5 in the endocardium and endocardial cushions. This receptor is also expressed at high levels at later stages when endothelial-to-mesenchymal transition is required

for atrial and ventricular valve formation. (Ataliotis and Mercola, 1997). To determine whether the phenotypic changes induced by Notch resulted in a functional change in the endothelial cells, we examined the chemotactic response of HMEC-vector, HMEC-Notch4IC, or HMEC-Notch1IC transduced endothelial cells to PDGF-BB. As seen in Figure 3.4D, Notch-activated endothelial cells were able to migrate towards PDGF-BB in a modified Boyden chamber chemotaxis assay, whereas vector-transduced cells did not. In contrast, vector-transduced cells migrated towards VEGF, while Notch-activated cells did not. Hence, Notch-induced endothelial transformation modifies the functional response of these cells compatible with that of a mesenchymal phenotype.

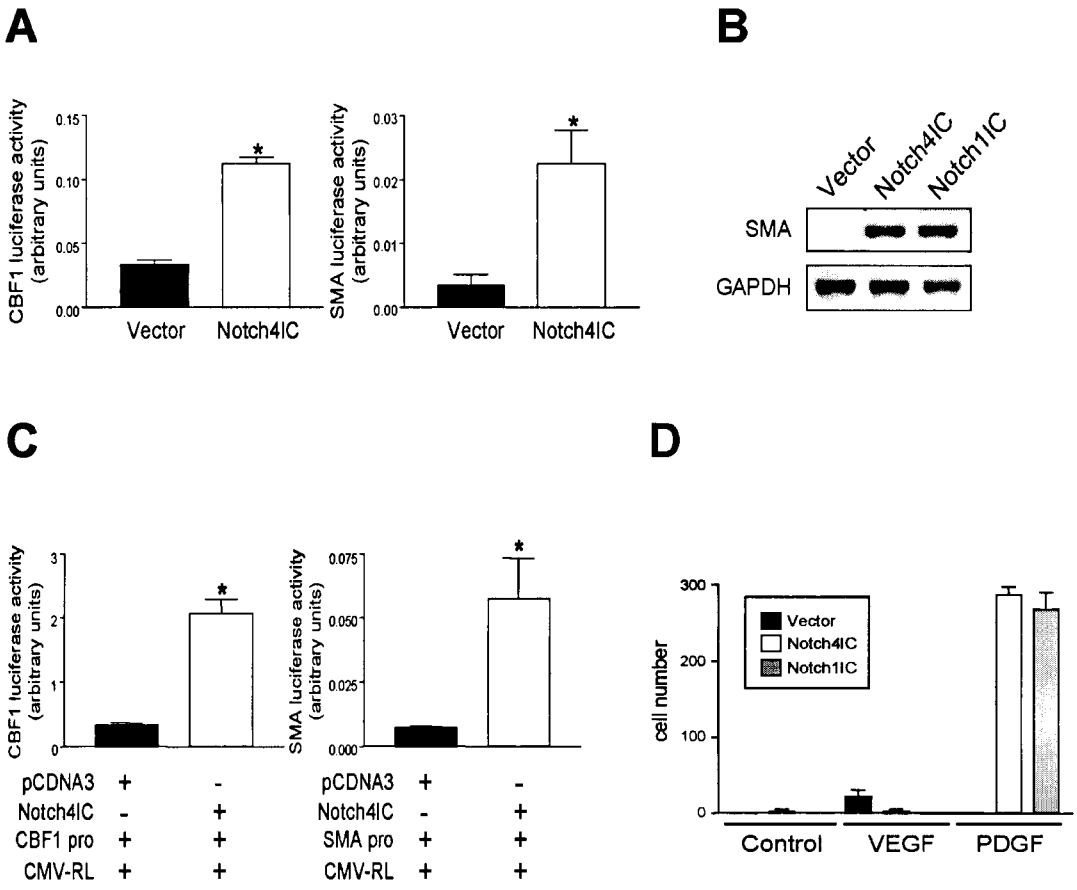


Figure 3.4: Activated Notch1 and Notch4 induce an endothelial-to-mesenchymal transformation. (A) HMEC transduced with empty vector or Notch4IC were transiently transfected with a CBF1-dependent luciferase construct (left panel), or an SMA promoter luciferase construct (right panel). **(B)** Total RNA collected from confluent HMEC transduced with empty vector, Notch4IC, or Notch1IC was used for RT-PCR. RNA expression levels of SMA were compared to GAPDH. Results are representative of 3 experiments. **(C)** Parental HMEC were transiently transfected with a CBF1-dependent luciferase construct (left panel) or an SMA promoter luciferase construct (right panel), and either Notch4IC or control expression plasmid. Luciferase activity in (B & C) is the relative luminescence units normalized to Renilla luciferase activity. $p<0.05$. **(D)** Migration of HMEC transduced as in (A) towards media (control), 15ng/ml VEGF, or 20ng/ml PDGF-BB. Each graph illustrates results obtained from one experiment performed in triplicate, and is representative of 2 (D) or 3 or more (B & C) individual experiments.

3.2.5 Activated Notch induces TGFβ production in HMEC, but not primary endothelial cells

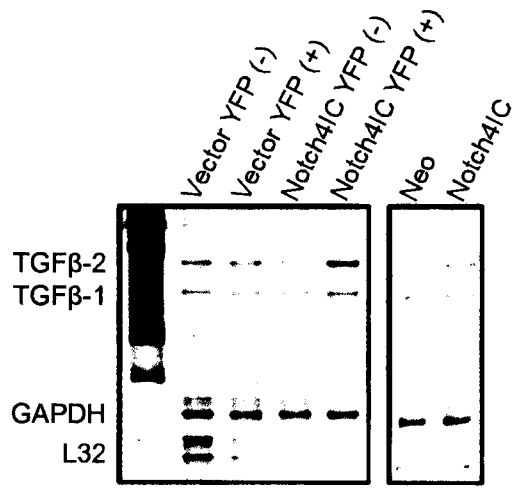
It is possible that activated Notch induces a mesenchymal transformation in conjunction with activation by other molecules. In this regard, TGFβ has been suggested to play an important role in endothelial-to-mesenchymal transformation in the endocardial cushion (Boyer et al., 1999; Brown et al., 1996; Brown et al., 1999; Ramsdell and Markwald, 1997). We thus assayed for TGFβ mRNA and protein expression in HMEC-vector and HMEC-Notch4IC. RNAse protection assays on cell lysates (Figure 3.5A & B) and ELISAs on cell supernatants (Figure 3.5C) showed an upregulation of TGFβ-2, but not TGFβ-1, mRNA and protein respectively, in HMEC-Notch4IC cells. However, this upregulation of TGFβ-2 was not observed in primary endothelial cells expressing Notch4IC or Notch1IC (Figure 3.5C, right panel) even though enforced expression of Notch1 and Notch4 induced an endothelial-to-mesenchymal transformation in these cells (data not shown; experiments performed by Dr. Michela Noseda).

To determine whether TGFβ stimulation was sufficient to induce an endothelial-to-mesenchymal transformation, we treated HMEC with recombinant TGFβ-1 or TGFβ-2 (2.5 ng/ml or 5 ng/ml) for up to 28 days. These concentrations are at the high end of those seen by ELISA, and are at or above concentrations used to induce endothelial-to-mesenchymal transformation events in TGFβ responsive endothelium (Brown et al., 1999; Paranya et al., 2001) Daily replacement of exogenous TGFβ-1 or TGFβ-2 did not induce morphological changes or the expression of SMA (data not shown), indicating that neither TGFβ-1 nor TGFβ-2 alone is sufficient to transform the endothelial cells tested.

3.2.6 The Notch induced mesenchymal transformation is cell autonomous

To determine whether the Notch-induced mesenchymal transformation is cell autonomous, we examined SMA expression using flow cytometry. The retroviral vector used in these studies contains yellow fluorescence protein (YFP) that is linked to the transgene through an internal ribosomal entry site. Thus, cells that express YFP also express Notch4IC. Results obtained by flow cytometry demonstrated that only the YFP-positive (Notch4IC-expressing) cells exhibited SMA expression (Figure 3.6A). This finding was confirmed by performing high-purity cell sorting of YFP-positive and YFP-negative populations of both HMEC-vector and

A



B

MIY System	raw data	GAPDH corrected	L32 corrected	raw data	GAPDH corrected	L32 corrected
Vector (V) (-)	103601	284285	400901	105961	290762	410035
Vector (V) (+)	80780	264175	417199	87248	285326	450603
Notch4IC (N4) (-)	79604	325871	427475	69334	283828	372323
Notch4IC (N4) (+)	134133	394341	722694	365353	1074110	1968481
	N4(+)/V(-)	1.4	1.8	N4(+)/V(-)	3.7	4.8
	N4(+)/N4(-	1.2	1.7	N4(+)/N4(-	3.8	5.3
LNCX System	raw data	GAPDH corrected	L32 corrected	raw data	GAPDH corrected	L32 corrected
Neo (N)	57004	151722	340597	20014	53271	119587
Notch4IC (N4)	99246	242051	670455	106306	259271	718150

C

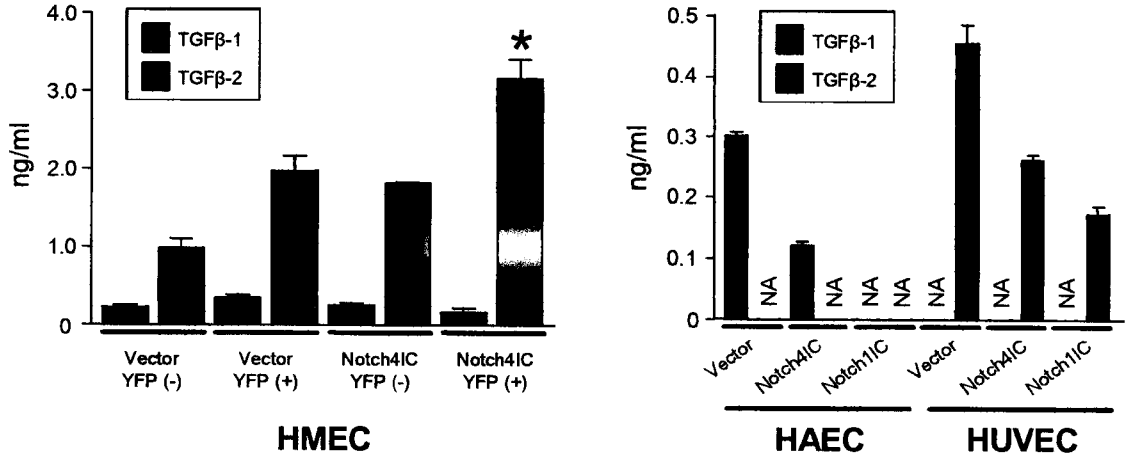


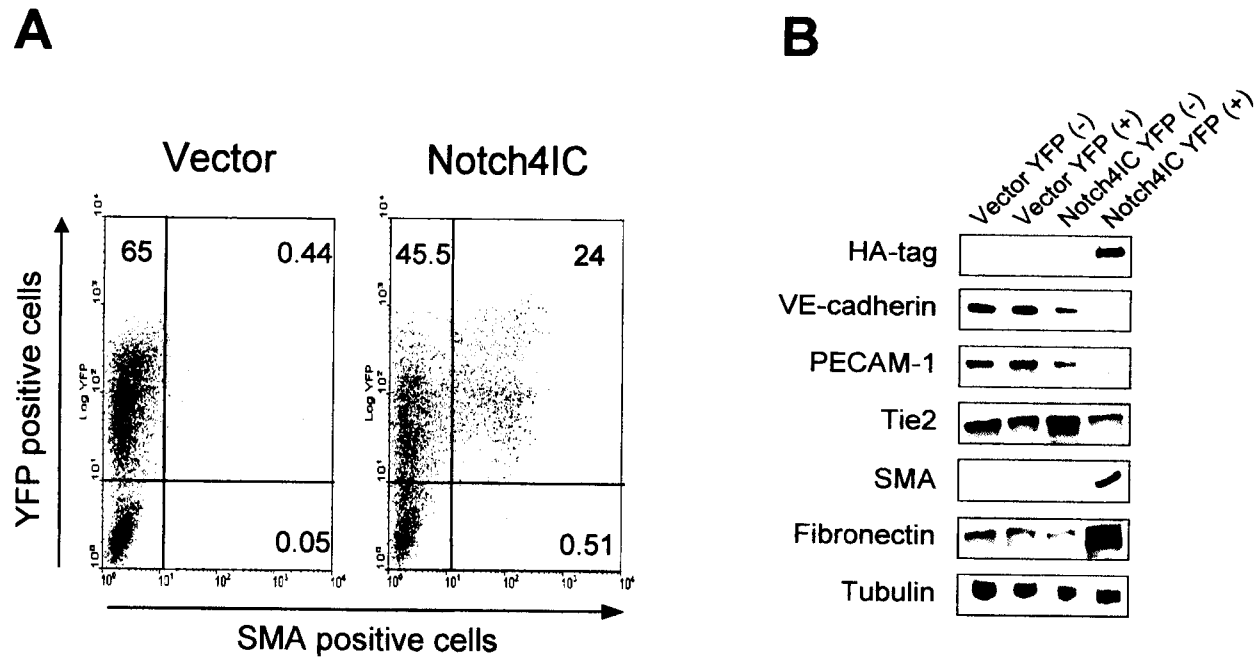
Figure 3.5: Activated Notch induces TGFβ production in HMEC, but not primary endothelial cells. (A) Total RNA collected from confluent HMEC transduced with empty vector or Notch4IC was used for RNA Protection Assays (R&D Systems, Inc.). The left panel shows HMEC transduced with the retroviral vector MIY (MSCVpac-IRES-Yellow Fluorescent Protein (YFP)). Retrovirally infected cells were high purity sorted into YFP positive and negative populations. Sorted cells were used between passages 1-5. The right panel illustrates HMEC tranduced with the retroviral vector LNCX. Cells were retrovirally infected and then selected for resistance to neomycin. Polyclonal populations were used between passages 1-5. Bands were resolved using a Phosphorimager. **(B)** Table showing the

relative total RNA levels for TGFβ-1 and TGFβ-2 from (A). RNA levels were corrected to the housekeeping genes GAPDH and L32. The fold increase in RNA for the activated Notch4 expressing cells versus the control cell lines are highlighted. **(C)** HMEC (left panel) were transduced with empty vector or activated Notch4, and sorted for YFP as in (A; left panel). Following 48 hours in culture, TGFβ-1 and -2 levels in the supernatants were determined by ELISA (R&D Systems, Inc.). Primary HAEC and HUVEC (right panel) were transduced with empty vector, Notch1IC or Notch4IC and sorted for YFP. Supernatants from the YFP positive populations were collected and measured as for HMEC. $p<0.05$. Results are representative of 2 or more experiments.

HMEC-Notch4IC transduced endothelial cells. Immunoblotting of the sorted populations showed downregulation of endothelial-specific proteins and upregulation of mesenchymal markers only in YFP positive HMEC-Notch4IC (Figure 3.6B). Thus, only endothelial cells that express activated Notch undergo mesenchymal transformation.

While TGFβ alone may not be sufficient to induce a mesenchymal transformation, it may be necessary, in conjunction with Notch activation, to induce endothelial-to-mesenchymal transformation. To confirm that the Notch4IC- and Notch1IC-induced mesenchymal transformation was entirely independent of TGFβ, we added a pan-anti-TGFβ-neutralizing antibody to HMEC transduced with empty vector, Notch4IC, or Notch1IC beginning at the time of retroviral transduction, followed by daily replacement with neutralizing antibody. The pan-anti-TGFβ-neutralizing mouse monoclonal antibody, used at concentrations that completely blocked TGFβ-dependent signalling (Figure 3.6C, top panel) (Dasch et al., 1989; Zavadil, 2003), did not inhibit or reduce the Notch4IC- or Notch1IC-induced morphological changes (data not shown) or SMA expression (Figure 3.6C, bottom panel).

The above findings imply that the Notch-induced mesenchymal transformation is not TGFβ–dependent. To determine whether Notch-transduced endothelial cells secrete other soluble factors that are capable of causing phenotypic changes, we added three-day conditioned medium from HMEC-vector and HMEC-Notch4IC to parental HMEC. We did not observe morphologic changes or SMA expression in HMEC treated daily with conditioned medium from Notch4IC-transduced HMEC over a 28-day period (data not shown). Taken together, the above findings strongly suggest that the Notch-induced mesenchymal transformation is cell autonomous, and does not require the presence of an additional secreted factor.



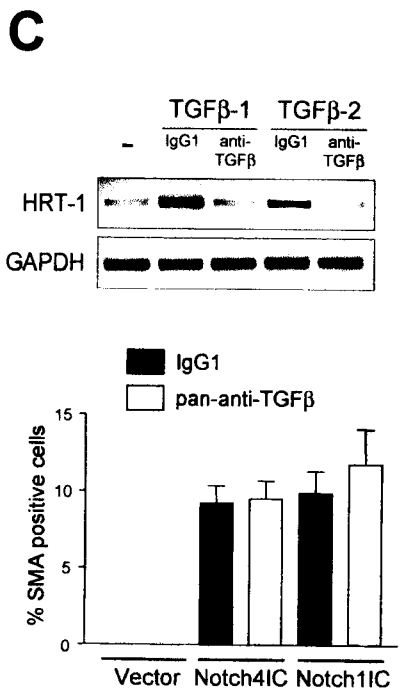


Figure 3.6: The Notch induced mesenchymal transformation is cell autonomous. **(A)** HMEC were transduced with empty vector or Notch4IC and analyzed by flow cytometry for YFP and SMA expression after 21 days. **(B)** HMEC were transduced with empty vector or Notch4IC and high purity sorted into YFP positive and negative populations. The sorted cells were then analyzed by immunoblotting for the expression of endothelial and mesenchymal markers. **(C)** HMEC at 90-100% confluence were starved for 16 hours in MCDB + 2% FCS. Cells were then stimulated ± TGFβ-1 (10 ng/ml) or TFGβ-2 (10 ng/ml) (both from R&D Systems, Inc) in the presence of control antibody (IgG1; 8 μg/ml) or pan-anti-TGFβ antibody (8 μg/ml). Total RNA was collected and used for RT-PCR. RNA levels were compared to GAPDH (top panel). HMEC were pre-treated with either control IgG1 (8 μg/ml) or a pan-anti-TGF-β neutralizing antibody (8 μg/ml), and then transduced with empty vector, Notch4IC or Notch1IC. The medium was changed daily with the addition of fresh IgG1 (8 μg/ml) or pan-anti-TGFβ (8 μg/ml). HMEC were analyzed by immunofluorescence for the expression of SMA 14 days post transduction. The cell nuclei were counter-stained with DAPI, and the number of SMA positive cells was expressed as a percentage of the total cell number (bottom panel). Results in (A & C) are representative of 3 or more experiments. Results in (B) are representative of 2 experiments.

3.2.7 Activated JAG1 induces characteristics of endothelial-to-mesenchymal transformation, and is observed in regions of the heart which undergo this transformation

JAG1 is a mammalian ligand that activates Notch1 (Lindsell et al., 1995). In the effort to determine whether JAG1-Notch interactions induce the characteristics of endothelial-to-mesenchymal transformation that we observed with constitutive expression of activated Notch4 or Notch1, we retrovirally transduced HMEC with empty vector or wild-type JAG1 (HMEC-JAG1). FACS analysis on transduced HMEC showed YFP expression levels of approximately 50% (data not shown); thus, we considered these unsorted HMEC-vector and HMEC-JAG1 as mixed populations. Western blots for the endothelial junctional molecules VE-cadherin and PECAM-1 show that these proteins are downregulated by JAG1, similar to the results obtained with activated Notch (Figure 3.7A, and compare to Figure 3.2C). We could not detect the expression of the mesenchymal marker SMA by Western blot, but did observe a slight induction of SMA by the more sensitive technique of immunofluorescence (Figure 3.7B).

In another experiment, we transiently transfected the HMEC-vector and HMEC-JAG1 mixed (i.e. unsorted) cultures with the CBF1-dependent promoter-luciferase construct. We observed 3.8 fold higher CBF1-luciferase activity in HMEC-JAG1-expressing mixed endothelial cultures compared to HMEC-vector mixed cultures, demonstrating that enforced expression of JAG1 activates CBF1-dependent Notch signalling (Figure 3.7C, left panel). In simultaneous experiments, mixed cultures with HMEC-JAG1 also induced a 2.1 fold increase in SMA promoter luciferase activity over HMEC-vector cultures, suggesting that JAG1-Notch interactions are capable of upregulating SMA expression (Figure 3.7C, right panel). However, these experiments do not rule out the possibility that JAG1 is inducing a paracrine factor that is responsible for the induction of the CBF1-dependent and SMA promoter luciferase activities.

At E12.5 of murine development, there is overlapping expression of JAG1 and Notch1 in endothelial cells of the cardiac cushion (Loomes et al., 2002; Loomes et al., 1999). However, formation of the cardiac cushion tissue, via endothelial-to-mesenchymal transformation, is described as beginning at E9.5 in the atrioventricular canal, and a day later, E10.5, in the ventricular outflow tract (Camenisch et al., 2002a). RT-PCR of various regions of the mouse heart shows expression of JAG1 and Notch1 in the outflow tract at E9.5, a time that better coincides with the onset of endothelial-to-mesenchymal transformation in this region. Furthermore, the expression of JAG1 at this time is consistent with the predominantly right-sided

cardiac anomalies seen in AGS (Li et al., 1997; McElhinney et al., 2002; Oda et al., 1997b). Taken together, our results are consistent with JAG1-Notch interactions contributing to endothelial transdifferentiation to a mesenchymal phenotype.

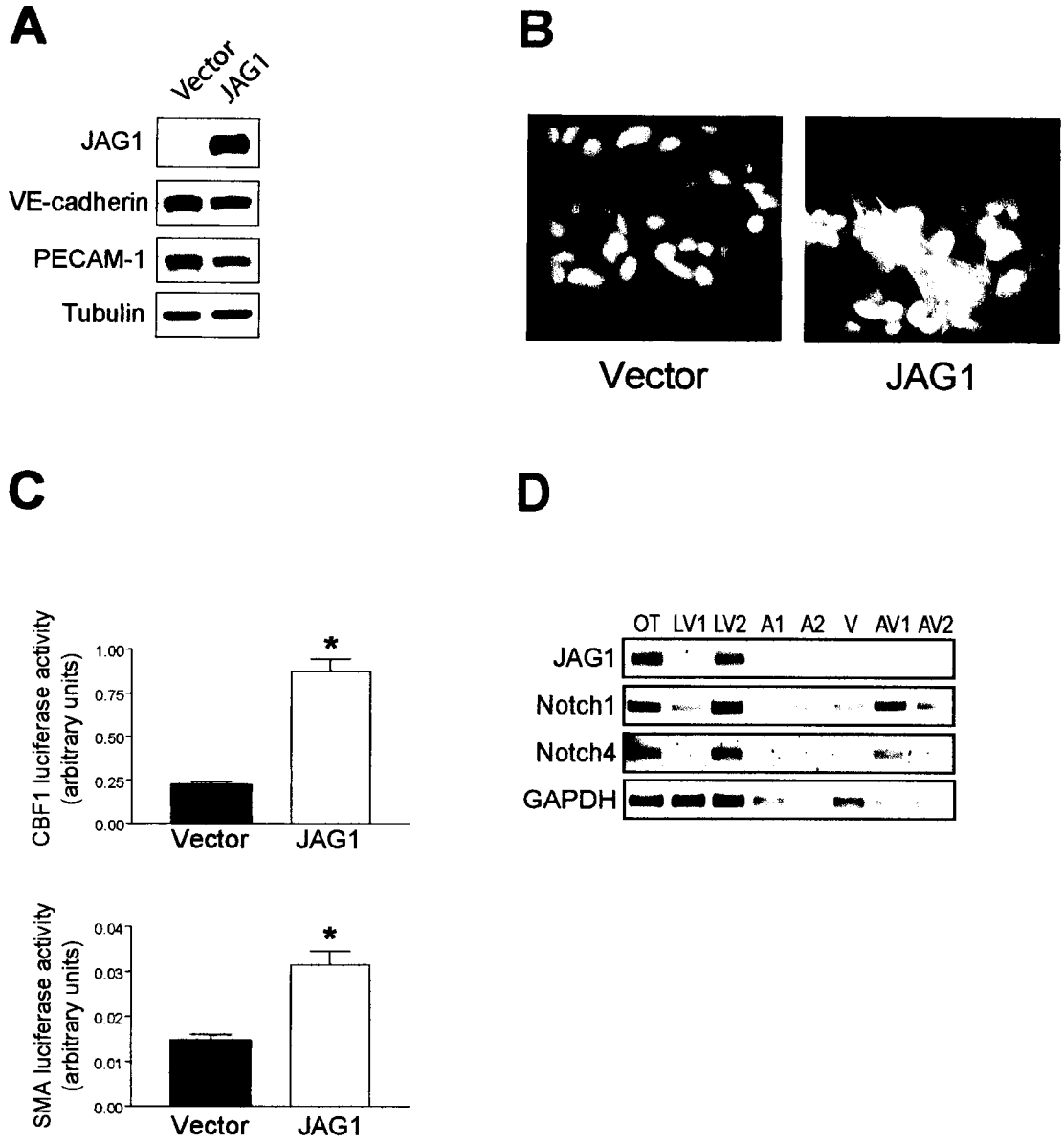


Figure 3.7: Activated JAG1 induces characteristics of endothelial-to-mesenchymal transformation, and is observed in regions of the heart which undergo this transformation. **(A)** HMEC transduced with empty vector or JAG1 were grown as polyclonal populations for up to five passages. The endothelial junctional markers VE-cadherin and PECAM-1 were analyzed by Western blot. Protein lysates were separated by SDS-PAGE and probed as described in materials and methods. Membranes were reprobed with anti-tubulin antibodies to show equal loading. **(B)** HMEC-vector and HMEC-JAG1 were analyzed for expression of SMA by immunofluorescence 14 days post transduction. Results in (A & B) are representative of 2 experiments. **(C)** HMEC transduced as in (A) were transiently transfected with a CBF1-dependent luciferase construct (left panel), or an SMA promoter luciferase construct (right panel). Each graph illustrates results obtained from one experiment performed in triplicate, and is representative of 3 or more individual experiments. Luciferase activity is the relative luminescence units normalized to Renilla luciferase activity. $p<0.05$. **(D)** PCR for JAG1, Notch1 and Notch4 was performed on cDNA from murine hearts. OT (ouflow tract), LV1, 2 (left ventricle sample 1, 2), A1, 2 (atria sample 1, 2), are from E9.5 hearts, and V (ventricles), AV1, 2 (atrioventricular canal sample 1, 2) are from E11.5 hearts. This experiment was only performed once.

3.3 DISCUSSION

We have shown that enforced expression of the activated form of the Notch4 or Notch1 receptor in endothelial cells causes transdifferentiation to a mesenchymal phenotype. In addition to morphologic changes consistent with a mesenchymal phenotype, activation of Notch mediates induction of the two hallmarks of EMT: 1) the loss of cell-cell junctions (i.e. loss of the endothelial junctional molecules VE-cadherin and PECAM-1); and 2) cell motility (Boyer et al., 2000), with the associated induction of SMA (Nakajima et al., 1999) and fibronectin (Eisenberg and Markwald, 1995). Since we and others (Loomes et al., 1999; Loomes et al., 2002) have shown that Notch receptors and the ligand, JAG1, are expressed in regions of the heart where endothelial-to-mesenchymal transformation is required for proper development, the results described herein are consistent with a role for JAG1-Notch interactions in promoting mesenchymal transformation in cardiac cushion development. Although specific defects of cardiac cushion development have not been reported in Notch1 or Notch1/Notch4 null mutants, these animals die at a very early stage of development (E9.5), at a point prior to significant cushion development (Kaufman, 1992; Krebs et al., 2000). Transgenic mice expressing constitutively-active Notch4 under the endothelial-specific VEGFR-2 promoter are reported to have enlarged hearts, but specific cardiac defects have not yet been reported (Uyttendaele et al., 2001).

We also demonstrate the ability of JAG1 to activate endothelial-to-mesenchymal transformation with the downregulation of VE-cadherin and PECAM-1, and the induction of SMA expression. JAG1 has been identified as the gene causing AGS, an autosomal dominant disorder characterized by multiple developmental abnormalities (Li et al., 1997; Oda et al., 1997b). In particular, patients with AGS demonstrate a number of cardiovascular defects that are consistent with improper cardiac cushion development, including atrial and ventricular septal defects, pulmonary atresia/stenosis, and a combination of these referred to as Tetralogy of Fallot (Alagille et al., 1987; Eisenberg and Markwald, 1995; Pierpont et al., 2000). However, most commonly these patients suffer from pulmonary stenosis/hypoplasia, which is consistent with our demonstration of expression of JAG1 in the ventricular outflow tract at a time during development when endothelial-to-mesenchymal transformation is proceeding (Camenisch et al., 2002a; McElhinney et al., 2002). Interestingly, more recent studies have shown that mutations of JAG1 are associated with defects of cardiac cushion development independent of the other developmental anomalies reported in AGS (Krantz et al., 1999).

Furthermore, given that both Notch receptors and the ligand JAG1 are expressed in regions of the heart where endothelial-to-mesenchymal transformation is required for proper development, the results described herein are consistent with a role for JAG1-Notch interactions in promoting mesenchymal transformation in cardiac cushion formation (Loomes et al., 2002; Loomes et al., 1999). This is particularly true in the ventricular outflow tract where we were able to detect expression of JAG1 and Notch1 by RT-PCR at embryonic day 9.5, and is consistent with the propensity of AGS patients to develop pulmonary stenosis/hypoplasia. In addition to the early requirement for endothelial-to-mesenchymal transformation during endocardial cushion formation, endothelial transformation is also required at later stages of cardiac development when the cushions merge to form the membranous septa. Since some AGS patients develop atrial and ventricular septal defects, it is tempting to speculate that JAG1 is required for endothelial-to-mesenchymal transformation during fusion of the cardiac cushions in the atrioventricular canal. The question then arises as to whether Notch activation is required during endocardial cushion formation in the atrioventricular canal. To speculate again, we would envision a role for other Notch ligands in endocardial cushion formation in the atrioventricular canal. A likely candidate for this role would be the endothelial-restricted ligand, Dll4, which has been reported to be expressed in the endocardium beginning at E8.5 and functions as a ligand for Notch1 and Notch4 (Mailhos et al., 2001; Rao et al., 2000; Shutter et al., 2000). However, further experiments would be required to determine whether Dll4 could also induce an endothelial-to-mesenchymal transformation.

TGFβ has been shown to play an important role in the endothelial-to-mesenchymal transformation seen during cardiac cushion formation. However, it is clear that there are TGFβ-dependent and -independent mechanisms involved in endocardial cell differentiation (Boyer et al., 1999). Our studies demonstrate that Notch-mediated mesenchymal transformation is independent of TGFβ secretion. This finding is consistent with the evidence that inhibition of TGFβ signalling does not block induction of SMA in the avian model system, confirming that while TGFβ may play an important role, it cannot be entirely responsible for endothelial-to-mesenchymal transformation (Boyer et al., 1999; Ramsdell and Markwald, 1997). Our results are also in accordance with previous studies showing that TGFβ does not induce SMA in microvascular endothelial cells (Paranya et al., 2001). While TGFβ–1 has been shown to induce SMA in bovine aortic endothelial cells (Arciniegas et al., 1992), we found that bovine aortic

endothelial cells spontaneously expressed SMA following passaging of cells in culture, as recently described ((Frid et al., 2002) and data not shown).

Further evidence that Notch signalling is involved in cardiac cushion development was revealed by recently reported targeted mutations of the HRT-2 gene. HRT-2 has been shown to be a downstream effector of Notch signalling (Iso et al., 2002; Iso et al., 2001; Nakagawa et al., 2000). Mice with null mutations of HRT-2 display a number of cardiac malformations. These abnormalities include atrioventricular septal defects, Tetralogy of Fallot, and tricuspid atresia; defects that resemble those associated with mutations of JAG1 seen in humans, and which are known to be secondary to anomalous cardiac cushion differentiation (Donovan et al., 2002; Gessler et al., 2002; Sakata et al., 2002).

Overall, our results indicate a crucial role for JAG1-Notch interactions in endothelial-to-mesenchymal transformation, which would explain the cardiac cushion anomalies seen in patients with JAG1 mutations. However, how Notch interacts with other factors (e.g. members of the TGFβ and homeobox gene superfamilies) implicated in endothelial-to-mesenchymal transformation during cardiac cushion development remains to be established.

Chapter 4

SUMMARY AND PERSPECTIVES

In summary, we have provided evidence that implicates JAG1-Notch signalling in endothelial-to-mesenchymal transformation. Endothelial-to-mesenchymal transformation is critical in the development of the endocardial cushion tissue of the heart, which contributes to the formation of the membranous valves and septa (Eisenberg and Markwald, 1995). The importance of our findings are highlighted by the fact that cardiac malformations (such as ventricular septal defects, pulmonary stenosis and Tetralogy of Fallot) are the most frequent type of congenital birth defects, present in ~1% of live births (Icardo, 1992; Olson and Srivastava, 1996; Srivastava, 2000). Furthermore, studies have demonstrated that mutations in the Notch ligand, JAG1, are responsible for the autosomal dominant disorder AGS (Li et al., 1997; Oda et al., 1997b). The majority of AGS patients present cardiovascular anomolies including atrioventricular septal defects, pulmonary stenosis and Tetralogy of Fallot which are defects associated with defective endocardial cushion formation (Eldadah et al., 2001; Le Caignec et al., 2002; Li et al., 1997; McElhinney et al., 2002; Oda et al., 1997b).

Given that JAG1 mutations cause AGS, and the high incidence of cardiovascular defects in these patients, Loomes et al. (1999; 2002) characterized JAG1 and Notch receptor expression in the developing mouse heart. They showed that JAG1 and Notch1 are expressed in a subset of endocardial cells in the atrioventricular canal and pulmonary outflow tract at E12.5. These endocardium are the cells that will undergo endothelial-to-mesenchymal transformation in response to intrinsic and extrinsic stimuli to form the endocardial cushions (Eisenberg and Markwald, 1995; Markwald et al., 1996). However, the onset of endothelial-to-mesenchymal transformation in the formation of the cushion tissue begins at E9.5 in the atrioventricular canal and E10.5 in the pulmonary outflow tract (Camenisch et al., 2002a; Markwald et al., 1977; Markwald et al., 1975). We add to their data by showing expression of JAG1 and Notch1 in the pulmonary outflow tract (E9.5) and atrioventricular canal (E11.5) by RT-PCR. In collaboration with Dr. Pamela Hoodless, we also detected expression of JAG1, Notch1 and Notch4 by *in situ* hybridization in the endocardium of the pulmonary outflow tract at E10.5-11.5. Taken together, these expression data are consistent with a potential role for JAG1-Notch interactions in the onset of cardiac cushion formation. Furthermore, we provided functional evidence that Notch

signalling induces the characteristics of cells undergoing EMT, namely loss of cell-cell junctions and the acquisition of cell motility (Boyer et al., 2000).

We have shown that the Notch-induced endothelial-to-mesenchymal transformation is independent of the TGFβ gene family, which is known to play a key role in EMT events (Boyer et al., 1999; Brown et al., 1996; Brown et al., 1999; Ramsdell and Markwald, 1997; Thiery, 2002). Interestingly, preliminary experiments in our lab suggest this transformation is also independent of the "core" Notch-CSL signalling pathway. We found that enforced expression of either HRT-1 or HRT-2 in HMEC did not induce the morphological changes or SMA expression seen in activated Notch transduced HMEC. However, these results are very preliminary, and have not considered enforced expression of other CSL targets (e.g. HES-1), or the combined expression of two or more targets in the same cell. Such experiments will be necessary given that activated Notch induced all CSL targets tested (Figures 3.1 & 3.2).

Our laboratory is expanding these studies to examine which domains of Notch are important to mediate this endothelial-to-mesenchymal transformation. Preliminary results indicate that the cdc10/ankyrin repeat domain is necessary, but not sufficient, to promote the Notch induced endothelial-to-mesenchymal transformation (Farrell MacKenzie, *personal communication*). Since the cdc10/ankyrin repeat domain is known to mediate important protein-protein interactions, future work will require a look at Notch modulators that interact with this domain. One such protein is Deltex (section 1.2.2.3), which may function independently of CSL (Ordentlich et al., 1998). A Deltex-Notch interaction mediating an endothelial-to-mesenchymal transformation independent of CSL would fit with our HRT overexpression results described above. However, a number of different scenarios are possible given the sheer number of Notch pathway receptors, ligands, modulators and targets. We are in the process of expressing wild-type Deltex and the endothelial cell-restricted ligand Dll4 in HMEC, to determine if either molecule is involved in endothelial-to-mesenchymal transformation.

So, what are the benefits of elucidating the mechanisms by which Notch activation induces endothelial-to-mesenchymal transformation? Every year in the United States, valvular disease leads to 60,000 valve replacement surgeries, and existing technologies have yet to produce a "model" replacement valve (Paranya et al., 2001). Understanding how endogenous molecules direct the development of valvular tissue will lead to the ability to engineer more suitable replacement valves. Furthermore, although not discussed in this thesis, EMT is known to

play a role in the transformation of carcinomas to invasive, metastatic carcinomas (Thiery, 2002). Thus, further studies on the role of Notch signalling in the regulation of EMT may also help us understand and control this pathological transformation.

In conclusion, these studies have revealed some new and important insights into the role of Notch receptors and ligands in the regulation of endothelial-to-mesenchymal transformation. Furthermore, they suggest that the continued exploration of these intriguing molecules and the signalling pathways that they activate will prove invaluable for a comprehensive understanding of endocardial cushion formation in the developing heart.

REFERENCES

Ades, E.W., Candal, F.J., Swerlick, R.A., George, V.G., Summers, S., Bosse, D.C. and Lawley, T.J. (1992) HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol*, **99**, 683-90.

Akazawa, C., Sasai, Y., Nakanishi, S. and Kageyama, R. (1992) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J Biol Chem*, **267**, 21879-85.

Alagille, D., Estrada, A., Hadchouel, M., Gautier, M., Odievre, M. and Dommergues, J.P. (1987) Syndromic paucity of interlobular bile ducts (Alagille syndrome or arteriohepatic dysplasia): review of 80 cases. *J Pediatr*, **110**, 195-200.

Allman, D., Punt, J.A., Izon, D.J., Aster, J.C. and Pear, W.S. (2002) An invitation to T and more: notch signaling in lymphopoiesis. *Cell*, **109 Suppl**, S1-11.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D.J., Honjo, T., de Angelis, M.H., Lendahl, U. and Edlund, H. (1999) Notch signalling controls pancreatic cell differentiation. *Nature.*, **400**, 877-881.

Aravind, L. (2001) The WWE domain: a common interaction module in protein ubiquitination and ADP ribosylation. *Trends Biochem Sci*, **26**, 273-5.

Arciniegas, E., Sutton, A.B., Allen, T.D. and Schor, A.M. (1992) Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J Cell Sci*, **103**, 521-9.

Arias, A.M. (1998) Interactions Between Wingless and Notch During the Assignation of Cell Fates in Drosophila. *International Journal of Developmental Biology*, **42**, 325-333.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E. (1995) Notch signaling. *Science*, **268**, 225-32.

Artavanis-Tsakonas, S., Rand, M.D. and Lake, R.J. (1999) Notch signaling: Cell fate control and signal integration in development. *Science.*, **284**, 770-776.

Aster, J.C., Robertson, E.S., Hasserjian, R.P., Turner, J.R., Kieff, E. and Sklar, J. (1997) Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-Jkappa or nuclear localization sequences retain the ability to associate with RBP-Jkappa and activate transcription. *J Biol Chem*, **272**, 11336-43.

Ataliotis, P. and Mercola, M. (1997) Distribution and functions of platelet-derived growth factors and their receptors during embryogenesis. *Int Rev Cytol*, **172**, 95-127.

Axelrod, J.D., Matsuno, K., Artavanistsakonas, S. and Perrimon, N. (1996) Interaction Between Wingless and Notch Signaling Pathways Mediated By Dishevelled. *Science*, **271**, 1826-1832.

Bach, T.L., Barsigian, C., Chalupowicz, D.G., Busler, D., Yaen, C.H., Grant, D.S. and Martinez, J. (1998) VE-Cadherin mediates endothelial cell capillary tube formation in fibrin and collagen gels. *Exp Cell Res*, **238**, 324-34.

Bae, S., Bessho, Y., Hojo, M. and Kageyama, R. (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development*, **127**, 2933-43.

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

Baron, M., Aslam, H., Flasza, M., Fostier, M., Higgs, J.E., Mazaleyrat, S.L. and Wilkin, M.B. (2002) Multiple levels of Notch signal regulation. *Mol Membr Biol*, **19**, 27-38.

Behrens, J., Mareel, M.M., Van Roy, F.M. and Birchmeier, W. (1989) Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol*, **108**, 2435-47.

Bessho, Y., Miyoshi, G., Sakata, R. and Kageyama, R. (2001) Hes7: a bHLH-type repressor gene regulated by Notch and expressed in the presomitic mesoderm. *Genes Cells*, **6**, 175-85.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J.L. and Gossler, A. (1995) Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. *Development*, **121**, 2407-18.

Birchmeier, W. and Behrens, J. (1994) Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta*, **1198**, 11-26.

Blaumueller, C.M., Qi, H., Zagouras, P. and Artavanis-Tsakonas, S. (1997) Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell*, **90**, 281-91.

Boyer, A.S., Erickson, C.P. and Runyan, R.B. (1999) Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGFbeta signal transduction mechanisms. *Dev Dyn*, **214**, 81-91.

Boyer, B., Tucker, G.C., Valles, A.M., Franke, W.W. and Thiery, J.P. (1989) Rearrangements of desmosomal and cytoskeletal proteins during the transition from epithelial to fibroblastoid organization in cultured rat bladder carcinoma cells. *J Cell Biol*, **109**, 1495-509.

Boyer, B., Valles, A.M. and Edme, N. (2000) Induction and regulation of epithelial-mesenchymal transitions. *Biochem Pharmacol*, **60**, 1091-9.

Bray, S. and Furriols, M. (2001) Notch pathway: making sense of suppressor of hairless. *Curr Biol*, **11**, R217-21.

Brennan, K., Baylies, M. and Arias, A.M. (1999a) Repression by Notch is required before Wingless signalling during muscle progenitor cell development in Drosophila. *Curr Biol*, **9**, 707-10.

Brennan, K., Klein, T., Wilder, E. and Arias, A.M. (1999b) Wingless modulates the effects of dominant negative notch molecules in the developing wing of Drosophila. *Developmental Biology*, **216**, 210-229.

Brennan, K., Tateson, R., Lewis, K. and Arias, A.M. (1997) A functional analysis of Notch mutations in Drosophila. *Genetics*, **147**, 177-88.

Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M.G. and Dejana, E. (1995) Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. *Arterioscler Thromb Vasc Biol*, **15**, 1229-39.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R.A. and Israel, A. (2000) A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell*, **5**, 207-16.

Brown, C.B., Boyer, A.S., Runyan, R.B. and Barnett, J.V. (1996) Antibodies to the Type II TGFbeta receptor block cell activation and migration during atrioventricular cushion transformation in the heart. *Dev Biol*, **174**, 248-57.

Brown, C.B., Boyer, A.S., Runyan, R.B. and Barnett, J.V. (1999) Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science*, **283**, 2080-2.

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

Burdsal, C.A., Damsky, C.H. and Pedersen, R.A. (1993) The role of E-cadherin and integrins in mesoderm differentiation and migration at the mammalian primitive streak. *Development*, **118**, 829-44.

Camenisch, T.D., Molin, D.G., Person, A., Runyan, R.B., Gittenberger-de Groot, A.C., McDonald, J.A. and Klewer, S.E. (2002a) Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev Biol*, **248**, 170-81.

Camenisch, T.D., Schroeder, J.A., Bradley, J., Klewer, S.E. and McDonald, J.A. (2002b) Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. *Nat Med*, **8**, 850-5.

Carl, T.F., Dufton, C., Hanken, J. and Klymkowsky, M.W. (1999) Inhibition of neural crest migration in Xenopus using antisense slug RNA. *Dev Biol*, **213**, 101-15.

Chen, H., Thiagalingam, A., Chopra, H., Borges, M.W., Feder, J.N., Nelkin, B.D., Baylin, S.B. and Ball, D.W. (1997) Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. *Proc Natl Acad Sci U S A*, **94**, 5355-60.

Chin, M.T., Maemura, K., Fukumoto, S., Jain, M.K., Layne, M.D., Watanabe, M., Hsieh, C.M. and Lee, M.E. (2000) Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system. *J Biol Chem*, **275**, 6381-7.

Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huylebroeck, D. and van Roy, F. (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell*, **7**, 1267-78.

Cooper, M.T. and Bray, S.J. (1999) Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. *Nature*, **397**, 526-30.

Damjanov, I., Damjanov, A. and Damsky, C.H. (1986) Developmentally regulated expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in peri-implantation mouse embryos and extraembryonic membranes. *Dev Biol*, **116**, 194-202.

Dasch, J.R., Pace, D.R., Waegell, W., Inenaga, D. and Ellingsworth, L. (1989) Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. *J Immunol*, **142**, 1536-41.

de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakano, T., Honjo, T., Mak, T.W., Rossant, J. and Conlon, R.A. (1997) Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development*, **124**, 1139-48.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A. and Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature*, **398**, 518-22.

del Amo, F.F., Gendron-Maguire, M., Swiatek, P.J., Jenkins, N.A., Copeland, N.G. and Gridley, T. (1993) Cloning, analysis, and chromosomal localization of Notch-1, a mouse homolog of Drosophila Notch. *Genomics*, **15**, 259-64.

Del Amo, F.F., Smith, D.E., Swiatek, P.J., Gendron-Maguire, M., Greenspan, R.J., McMahon, A.P. and Gridley, T. (1992) Expression pattern of Motch, a mouse homolog of Drosophila Notch, suggests an important role in early postimplantation mouse development. *Development*, **115**, 737-44.

Diederich, R.J., Matsuno, K., Hing, H. and Artavanis-Tsakonas, S. (1994) Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development*, **120**, 473-81.

Donovan, J., Kordylewska, A., Jan, Y.N. and Utset, M.F. (2002) Tetralogy of fallot and other congenital heart defects in Hey2 mutant mice. *Curr Biol*, **12**, 1605-10.

Donoviel, D.B., Hadjantonakis, A.K., Ikeda, M., Zheng, H., Hyslop, P.S. and Bernstein, A. (1999) Mice lacking both presenilin genes exhibit early embryonic patterning defects. *Genes & Development.*, **13**, 2801-2810.

Dor, Y., Camenisch, T.D., Itin, A., Fishman, G.I., McDonald, J.A., Carmeliet, P. and Keshet, E. (2001) A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects. *Development*, **128**, 1531-8.

Dorovini-Zis, K., Prameya, R. and Bowman, P.D. (1991) Culture and characterization of microvascular endothelial cells derived from human brain. *Lab Invest*, **64**, 425-36.

Dou, S., Zeng, X., Cortes, P., Erdjument-Bromage, H., Tempst, P., Honjo, T. and Vales, L.D. (1994) The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. *Mol Cell Biol*, **14**, 3310-9.

Dunwoodie, S.L., Clements, M., Sparrow, D.B., Sa, X., Conlon, R.A. and Beddington, R.S. (2002) Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm. *Development*, **129**, 1795-806.

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

Ear, T., Giguere, P., Fleury, A., Stankova, J., Payet, M.D. and Dupuis, G. (2001) High efficiency transient transfection of genes in human umbilical vein endothelial cells by electroporation. *J Immunol Methods*, **257**, 41-9.

Edelman, G.M., Gallin, W.J., Delouvee, A., Cunningham, B.A. and Thiery, J.P. (1983) Early epochal maps of two different cell adhesion molecules. *Proc Natl Acad Sci U S A*, **80**, 4384-8.

Egan, S.E., St-Pierre, B. and Leow, C.C. (1998) Notch receptors, partners and regulators: from conserved domains to powerful functions. *Curr Top Microbiol Immunol*, **228**, 273-324.

Eisenberg, L.M. and Markwald, R.R. (1995) Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ Res*, **77**, 1-6.

Eldadah, Z.A., Hamosh, A., Biery, N.J., Montgomery, R.A., Duke, M., Elkins, R. and Dietz, H.C. (2001) Familial Tetralogy of Fallot caused by mutation in the jagged1 gene. *Hum Mol Genet*, **10**, 163-9.

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D. and Sklar, J. (1991) TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell*, **66**, 649-61.

Fanto, M. and Mlodzik, M. (1999) Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the Drosophila eye. *Nature*, **397**, 523-6.

Farquhar, M.G. and Palade, G.E. (1965) Cell junctions in amphibian skin. *J Cell Biol*, **26**, 263-91.

Ffrench-Constant, C. and Hynes, R.O. (1988) Patterns of fibronectin gene expression and splicing during cell migration in chicken embryos. *Development*, **104**, 369-82.

Fisher, A.L., Ohsako, S. and Caudy, M. (1996) The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol Cell Biol*, **16**, 2670-7.

Fleming, R.J. (1998) Structural conservation of Notch receptors and ligands. *Seminars in Cell & Developmental Biology*, **9**, 599-607.

Fleming, R.J., Gu, Y. and Hukriede, N.A. (1997a) Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. *Development*, **124**, 2973-81.

Fleming, R.J., Purcell, K. and Artavanistsakonas, S. (1997b) The Notch Receptor and Its Ligands. *Trends in Cell Biology*, **7**, 437-441.

Fortini, M.E. (2000) Fringe benefits to carbohydrates. *Nature*, **406**, 357-8.

Fortini, M.E. and Artavanis-Tsakonas, S. (1994) The suppressor of hairless protein participates in notch receptor signaling. *Cell*, **79**, 273-82.

Fortini, M.E., Rebay, I., Caron, L.A. and Artavanis-Tsakonas, S. (1993) An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. *Nature*, **365**, 555-7.

Frid, M.G., Kale, V.A. and Stenmark, K.R. (2002) Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ Res*, **90**, 1189-96.

Frisen, J. and Lendahl, U. (2001) Oh no, Notch again! *Bioessays*, **23**, 3-7.

Gallahan, D., Jhappan, C., Robinson, G., Hennighausen, L., Sharp, R., Kordon, E., Callahan, R., Merlino, G. and Smith, G.H. (1996) Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. *Cancer Res*, **56**, 1775-85.

Gessler, M., Knobeloch, K.P., Helisch, A., Amann, K., Schumacher, N., Rohde, E., Fischer, A. and Leimeister, C. (2002) Mouse gridlock: no aortic coarctation or deficiency, but fatal cardiac defects in Hey2 -/- mice. *Curr Biol*, **12**, 1601-4.

Gorman, M.J. and Girton, J.R. (1992) A genetic analysis of deltex and its interaction with the Notch locus in Drosophila melanogaster. *Genetics*, **131**, 99-112.

Grbavec, D. and Stifani, S. (1996) Molecular Interaction Between Tle1 and the Carboxyl-Terminal Domain of Hes-1 Containing the Wrpw Motif. *Biochemical & Biophysical Research Communications*, **223**, 701-705.

Greenburg, G. and Hay, E.D. (1982) Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *J Cell Biol*, **95**, 333-9.

Greenwald, I. (1994) Structure/function studies of lin-12/Notch proteins. *Curr Opin Genet Dev*, **4**, 556-62.

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

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

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

Hamada, Y., Kadokawa, Y., Okabe, M., Ikawa, M., Coleman, J.R. and Tsujimoto, Y. (1999) Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development*, **126**, 3415-24.

Hatta, K., Takagi, S., Fujisawa, H. and Takeichi, M. (1987) Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev Biol*, **120**, 215-27.

Hay, E.D. (1995) An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)*, **154**, 8-20.

Hay, E.D. and Zuk, A. (1995) Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. *Am J Kidney Dis*, **26**, 678-90.

Hemavathy, K., Ashraf, S.I. and Ip, Y.T. (2000) Snail/slug family of repressors: slowly going into the fast lane of development and cancer. *Gene*, **257**, 1-12.

Henderson, A.M., Wang, S.J., Taylor, A.C., Aitkenhead, M. and Hughes, C.C. (2001) The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation. *J Biol Chem*, **276**, 6169-76.

Hirata, H., Ohtsuka, T., Bessho, Y. and Kageyama, R. (2000) Generation of structurally and functionally distinct factors from the basic helix-loop-helix gene Hes3 by alternative first exons. *J Biol Chem*, **275**, 19083-9.

Hrabe de Angelis, M., McIntyre, J., 2nd and Gossler, A. (1997) Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature*, **386**, 717-21.

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

Hsieh, J.J., Henkel, T., Salmon, P., Robey, E., Peterson, M.G. and Hayward, S.D. (1996) Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol Cell Biol*, **16**, 952-9.

Hsieh, J.J., Zhou, S., Chen, L., Young, D.B. and Hayward, S.D. (1999) CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. *Proc Natl Acad Sci U S A*, **96**, 23-8.

Icardo, J.M. and Manasek, F.J. (1984) An indirect immunofluorescence study of the distribution of fibronectin during the formation of the cushion tissue mesenchyme in the embryonic heart. *Dev Biol*, **101**, 336-45.

Icardo, J.M.and Manasek, F.J. (1992) *Cardiogenesis: Development, mechanisms and embryology.* Raven Press, Ltd., New York.

Imhof, B.A., Vollmers, H.P., Goodman, S.L. and Birchmeier, W. (1983) Cell-cell interaction and polarity of epithelial cells: specific perturbation using a monoclonal antibody. *Cell*, **35**, 667-75.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R. and Guillemot, F. (1995) Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev*, **9**, 3136-48.

Ishibashi, M., Sasai, Y., Nakanishi, S. and Kageyama, R. (1993) Molecular characterization of HES-2, a mammalian helix-loop-helix factor structurally related to Drosophila hairy and Enhancer of split. *Eur J Biochem*, **215**, 645-52.

Iso, T., Chung, G., Hamamori, Y. and Kedes, L. (2002) HERP1 is a cell type-specific primary target of Notch. *J Biol Chem*, **277**, 6598-607.

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

Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H.Y., Chung, G., Kedes, L. and Hamamori, Y. (2001) HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol Cell Biol*, **21**, 6080-9.

Izon, D.J., Aster, J.C., He, Y., Weng, A., Karnell, F.G., Patriub, V., Xu, L., Bakkour, S., Rodriguez, C., Allman, D. and Pear, W.S. (2002) Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity*, **16**, 231-43.

Jafar-Nejad, H., Norga, K. and Bellen, H. (2002) Numb: "Adapting" notch for endocytosis. *Dev Cell*, **3**, 155-6.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R. and Israel, A. (1995) Signalling downstream of activated mammalian Notch. *Nature*, **377**, 355-8.

Jarriault, S., Le Bail, O., Hirsinger, E., Pourquie, O., Logeat, F., Strong, C.F., Brou, C., Seidah, N.G. and Isra l, A. (1998) Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol*, **18**, 7423-31.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G.H., Merlino, G. and Callahan, R. (1992) Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev*, **6**, 345-55.

Kao, H.Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C.R., Evans, R.M. and Kadesch, T. (1998) A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev*, **12**, 2269-77.

Karsan, A., Yee, E. and Harlan, J.M. (1996) Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. *J Biol Chem*, **271**, 27201-4.

Karsan, A., Yee, E., Poirier, G.G., Zhou, P., Craig, R. and Harlan, J.M. (1997) Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. *Am J Pathol*, **151**, 1775-84.

Kato, H., Sakai, T., Tamura, K., Minoguchi, S., Shirayoshi, Y., Hamada, Y., Tsujimoto, Y. and Honjo, T. (1996) Functional conservation of mouse Notch receptor family members. *FEBS Lett*, **395**, 221-4.

Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura-Okazaki, S., Tamura, K. and Honjo, T. (1997) Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development*, **124**, 4133-41.

Kaufman, M.H. (1992) *The Atlas of Mouse Development*. Academic Press, London, United Kingdom.

Kidd, S., Lieber, T. and Young, M.W. (1998) Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. *Genes Dev*, **12**, 3728-40.

Kim, R.Y., Robertson, E.J. and Solloway, M.J. (2001) Bmp6 and Bmp7 are required for cushion formation and septation in the developing mouse heart. *Dev Biol*, **235**, 449-66.

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

Kimble, J. and Simpson, P. (1997) The LIN-12/NOTCH signaling pathway and its regulation. *Annual Review of Cell & Developmental Biology*, **13**, 333-361.

Kinsella, M.G. and Fitzharris, T.P. (1982) Control of cell migration in atrioventricular pads during chick early heart development: analysis of cushion tissue migration in vitro. *Dev Biol*, **91**, 1-10.

Kokubo, H., Lun, Y. and Johnson, R.L. (1999) Identification and expression of a novel family of bHLH cDNAs related to Drosophila hairy and enhancer of split. *Biochem Biophys Res Commun*, **260**, 459-65.

Kopan, R. and Goate, A. (2000) A common enzyme connects notch signaling and Alzheimer's disease. *Genes Dev*, **14**, 2799-806.

Kopan, R., Nye, J.S. and Weintraub, H. (1994) The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development*, **120**, 2385-96.

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

Koyano-Nakagawa, N., Kim, J., Anderson, D. and Kintner, C. (2000) Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. *Development*, **127**, 4203-16.

Krantz, I.D., Smith, R., Colliton, R.P., Tinkel, H., Zackai, E.H., Piccoli, D.A., Goldmuntz, E. and Spinner, N.B. (1999) Jagged1 mutations in patients ascertained with isolated congenital heart defects. *Am J Med Genet*, **84**, 56-60.

Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G.H., Stark, K.L. and Gridley, T. (2000) Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev*, **14**, 1343-52.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H. and Honjo, T. (1999) Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J Biol Chem*, **274**, 7238-44.

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

LaBonne, C. and Bronner-Fraser, M. (2000) Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev Biol*, **221**, 195-205.

Lai, E.C. (2002) Protein degradation: four E3s for the notch pathway. *Curr Biol*, **12**, R74-8.

Lai, Y.T., Beason, K.B., Brames, G.P., Desgrosellier, J.S., Cleggett, M.C., Shaw, M.V., Brown, C.B. and Barnett, J.V. (2000) Activin receptor-like kinase 2 can mediate atrioventricular cushion transformation. *Dev Biol*, **222**, 1-11.

Lampugnani, M.G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L.P. and Dejana, E. (1992) A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J Cell Biol*, **118**, 1511-22.

Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994) The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech Dev*, **46**, 123-36.

Lawson, N.D., Scheer, N., Pham, V.N., Kim, C.H., Chitnis, A.B., Campos-Ortega, J.A. and Weinstein, B.M. (2001) Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development*, **128**, 3675-83.

Le Caignec, C., Lefevre, M., Schott, J.J., Chaventre, A., Gayet, M., Calais, C. and Moisan, J.P. (2002) Familial deafness, congenital heart defects, and posterior embryotoxon caused by cysteine substitution in the first epidermal-growth-factor-like domain of jagged 1. *Am J Hum Genet*, **71**, 180-6.

Lecourtois, M. and Schweisguth, F. (1998) Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. *Curr Biol*, **8**, 771-4.

Leimeister, C., Dale, K., Fischer, A., Klamt, B., Hrabe de Angelis, M., Radtke, F., McGrew, M.J., Pourquie, O. and Gessler, M. (2000) Oscillating expression of c-Hey2 in the presomitic mesoderm suggests that the segmentation clock may use combinatorial signaling through multiple interacting bHLH factors. *Dev Biol*, **227**, 91-103.

Leimeister, C., Externbrink, A., Klamt, B. and Gessler, M. (1999) Hey genes: a novel subfamily of hairy- and Enhancer of split related genes specifically expressed during mouse embryogenesis. *Mech Dev*, **85**, 173-7.

Leong, K.G., Hu, X., Li, L., Noseda, M., Larrivee, B., Hull, C., Hood, L., Wong, F. and Karsan, A. (2002) Activated Notch4 inhibits angiogenesis: role of beta 1-integrin activation. *Mol Cell Biol*, **22**, 2830-41.

Leptin, M. (1991) twist and snail as positive and negative regulators during Drosophila mesoderm development. *Genes Dev*, **5**, 1568-76.

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

Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trask, B.J., Kuo, W.L., Cochran, J., Costa, T., Pierpont, M.E., Rand, E.B., Piccoli, D.A., Hood, L. and Spinner, N.B. (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet*, **16**, 243-51.

Li, L., Milner, L.A., Deng, Y., Iwata, M., Banta, A., Graf, L., Marcovina, S., Friedman, C., Trask, B.J., Hood, L. and Torok-Storb, B. (1998) The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity*, **8**, 43-55.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M.W. (1993) Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev*, **7**, 1949-65.

Ligoxygakis, P., Yu, S.Y., Delidakis, C. and Baker, N.E. (1998) A subset of notch functions during Drosophila eye development require Su(H) and the E(spl) gene complex. *Development*, **125**, 2893-900.

Lillie, F. (1908) *The Development of the Chick*. Henry Holt and Co., New York, NY.

Lindsell, C.E., Shawber, C.J., Boulter, J. and Weinmaster, G. (1995) Jagged: a mammalian ligand that activates Notch1. *Cell*, **80**, 909-17.

Lissemore, J.L. and Starmer, W.T. (1999) Phylogenetic analysis of vertebrate and invertebrate Delta/Serrate/LAG-2 (DSL) proteins. *Molecular Phylogenetics & Evolution.*, **11**, 308-319.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N.G. and Israel, A. (1998) The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A*, **95**, 8108-12.

Loomes, K.M., Taichman, D.B., Glover, C.L., Williams, P.T., Markowitz, J.E., Piccoli, D.A., Baldwin, H.S. and Oakey, R.J. (2002) Characterization of Notch receptor expression in the developing mammalian heart and liver. *Am J Med Genet*, **112**, 181-9.

Loomes, K.M., Underkoffler, L.A., Morabito, J., Gottlieb, S., Piccoli, D.A., Spinner, N.B., Baldwin, H.S. and Oakey, R.J. (1999) The expression of Jagged1 in the developing mammalian heart correlates with cardiovascular disease in Alagille syndrome. *Human Molecular Genetics*, **8**, 2443-2449.

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

Mailhos, C., Modlich, U., Lewis, J., Harris, A., Bicknell, R. and Ish-Horowicz, D. (2001) Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation*, **69**, 135-44.

Markwald, R., Eisenberg, C., Eisenberg, L., Trusk, T. and Sugi, Y. (1996) Epithelial-mesenchymal transformations in early avian heart development. *Acta Anat (Basel)*, **156**, 173-86.

Markwald, R.R., Fitzharris, T.P. and Manasek, F.J. (1977) Structural development of endocardial cushions. *Am J Anat*, **148**, 85-119.

Markwald, R.R., Fitzharris, T.P. and Smith, W.N. (1975) Sturctural analysis of endocardial cytodifferentiation. *Dev Biol*, **42**, 160-80.

Matsuno, K., Diederich, R.J., Go, M.J., Blaumueller, C.M. and Artavanis-Tsakonas, S. (1995) Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development*, **121**, 2633-44.

Matsuno, K., Eastman, D., Mitsiades, T., Quinn, A.M., Carcanciu, M.L., Ordentlich, P., Kadesch, T. and Artavanis-Tsakonas, S. (1998) Human deltex is a conserved regulator of Notch signalling. *Nat Genet*, **19**, 74-8.

Matsuno, K., Go, M.J., Sun, X., Eastman, D.S. and Artavanis-Tsakonas, S. (1997) Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements. *Development*, **124**, 4265-73.

McCright, B., Gao, X., Shen, L., Lozier, J., Lan, Y., Maguire, M., Herzlinger, D., Weinmaster, G., Jiang, R. and Gridley, T. (2001) Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. *Development*, **128**, 491-502.

McCright, B., Lozier, J. and Gridley, T. (2002) A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development*, **129**, 1075-82.

McElhinney, D.B., Krantz, I.D., Bason, L., Piccoli, D.A., Emerick, K.M., Spinner, N.B. and Goldmuntz, E. (2002) Analysis of cardiovascular phenotype and genotype-phenotype correlation in individuals with a JAG1 mutation and/or Alagille syndrome. *Circulation*, **106**, 2567-74.

McEwen, D.G. and Peifer, M. (2000) Wnt signaling: Moving in a new direction. *Curr Biol*, **10**, R562-4.

McGill, M.A. and McGlade, C.J. (2003) Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J Biol Chem*, **278**, 23196-203

Milner, L.A. and Bigas, A. (1999) Notch as a mediator of cell fate determination in hematopoiesis: Evidence and speculation. *Blood.*, **93**, 2431-48.

Mizutani, T., Taniguchi, Y., Aoki, T., Hashimoto, N. and Honjo, T. (2001) Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members. *Proc Natl Acad Sci U S A*, **98**, 9026-31.

Mjaatvedt, C.H., Yamamura, H., Capehart, A.A., Turner, D. and Markwald, R.R. (1998) The Cspg2 gene, disrupted in the hdf mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol*, **202**, 56-66.

Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K.D., Haltiwanger, R.S. and Vogt, T.F. (2000 Jul 27) Fringe is a glycosyltransferase that modifies Notch. *Nature*, **406**, 369-75.

Mumm, J.S. and Kopan, R. (2000) Notch signaling: from the outside in. *Dev Biol*, **228**, 151-65.

Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J. and Kopan, R. (2000) A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Mol Cell*, **5**, 197-206.

Munro, S. and Freeman, M. (2000) The notch signalling regulator fringe acts in the Golgi apparatus and requires the glycosyltransferase signature motif DXD. *Curr Biol*, **10**, 813-20.

Muskavitch, M.A. (1994) Delta-notch signaling and Drosophila cell fate choice. *Dev Biol*, **166**, 415-30.

Nakagawa, O., McFadden, D.G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D. and Olson, E.N. (2000) Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. *Proc Natl Acad Sci U S A*, **97**, 13655-60.

Nakagawa, O., Nakagawa, M., Richardson, J.A., Olson, E.N. and Srivastava, D. (1999) HRT1, HRT2, and HRT3: A New Subclass of bHLH Transcription Factors Marking Specific Cardiac, Somitic, and Pharyngeal Arch Segments. *Dev Biol*, **216**, 72-84.

Nakajima, Y., Mironov, V., Yamagishi, T., Nakamura, H. and Markwald, R.R. (1997) Expression of smooth muscle alpha-actin in mesenchymal cells during formation of avian endocardial cushion tissue: a role for transforming growth factor beta3. *Dev Dyn*, **209**, 296-309.

Nakajima, Y., Yamagishi, T., Hokari, S. and Nakamura, H. (2000) Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). *Anat Rec*, **258**, 119-27.

Nakajima, Y., Yamagishi, T., Yoshimura, K., Nomura, M. and Nakamura, H. (1999) Antisense oligodeoxynucleotide complementary to smooth muscle alpha-actin inhibits endothelial-mesenchymal transformation during chick cardiogenesis. *Dev Dyn*, **216**, 489-98.

Nieto, M.A. (2001) The early steps of neural crest development. *Mech Dev*, **105**, 27-35.

Nieto, M.A., Sargent, M.G., Wilkinson, D.G. and Cooke, J. (1994) Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science*, **264**, 835-9.

Nishimura, M., Isaka, F., Ishibashi, M., Tomita, K., Tsuda, H., Nakanishi, S. and Kageyama, R. (1998) Structure, chromosomal locus, and promoter of mouse Hes2 gene, a homologue of Drosophila hairy and Enhancer of split. *Genomics*, **49**, 69-75.

Oda, H., Tsukita, S. and Takeichi, M. (1998) Dynamic behavior of the cadherin-based cell-cell adhesion system during Drosophila gastrulation. *Dev Biol*, **203**, 435-50.

Oda, T., Elkahloun, A.G., Meltzer, P.S. and Chandrasekharappa, S.C. (1997a) Identification and cloning of the human homolog (JAG1) of the rat Jagged1 gene from the Alagille syndrome critical region at 20p12. *Genomics*, **43**, 376-9.

Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S. and Chandrasekharappa, S.C. (1997b) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet*, **16**, 235-42.

Ohsako, S., Hyer, J., Panganiban, G., Oliver, I. and Caudy, M. (1994) Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. *Genes Dev*, **8**, 2743-55.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999) Hes1 and Hes5 as Notch effectors in mammalian neuronal differentiation. *EMBO Journal.*, **18**, 2196-2207.

Olson, E.N. and Srivastava, D. (1996) Molecular pathways controlling heart development. *Science*, **272**, 671-6.

Ordentlich, P., Lin, A., Shen, C.P., Blaumueller, C., Matsuno, K., Artavanis-Tsakonas, S. and Kadesch, T. (1998) Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol*, **18**, 2230-9.

Panin, V.M., Papayannopoulos, V., Wilson, R. and Irvine, K.D. (1997) Fringe modulates Notch-ligand interactions. *Nature*, **387**, 908-12.

Paranya, G., Vineberg, S., Dvorin, E., Kaushal, S., Roth, S.J., Rabkin, E., Schoen, F.J. and Bischoff, J. (2001) Aortic valve endothelial cells undergo transforming growth factor-beta-mediated and non-transforming growth factor-beta-mediated transdifferentiation in vitro. *Am J Pathol*, **159**, 1335-43.

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

Perez-Moreno, M.A., Locascio, A., Rodrigo, I., Dhondt, G., Portillo, F., Nieto, M.A. and Cano, A. (2001) A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. *J Biol Chem*, **276**, 27424-31.

Petcherski, A.G. and Kimble, J. (2000) LAG-3 is a putative transcriptional activator in the C. elegans Notch pathway. *Nature*, **405**, 364-8.

Piccoli, D.A. and Spinner, N.B. (2001) Alagille syndrome and the Jagged1 gene. *Semin Liver Dis*, **21**, 525-34.

Pierpont, M.E., Markwald, R.R. and Lin, A.E. (2000) Genetic aspects of atrioventricular septal defects. *Am J Med Genet*, **97**, 289-96.

Pires-daSilva, A. and Sommer, R.J. (2003) The evolution of signalling pathways in animal development. *Nat Rev Genet*, **4**, 39-49.

Pissarra, L., Henrique, D. and Duarte, A. (2000) Expression of hes6, a new member of the Hairy/Enhancer-of-split family, in mouse development. *Mech Dev*, **95**, 275-8.

Polakis, P. (2000) Wnt signaling and cancer. *Genes Dev*, **14**, 1837-51.

Prieto, A.L. and Crossin, K.L. (1995) Cell-cell adhesion molecules in epithelial-mesenchymal transformations. *Acta Anat (Basel)*, **154**, 21-33.

Pui, J.C., Allman, D., Xu, L., DeRocco, S., Karnell, F.G., Bakkour, S., Lee, J.Y., Kadesch, T., Hardy, R.R., Aster, J.C. and Pear, W.S. (1999) Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity*, **11**, 299-308.

Ramsdell, A.F. and Markwald, R.R. (1997) Induction of endocardial cushion tissue in the avian heart is regulated, in part, by TGFbeta-3-mediated autocrine signaling. *Dev Biol*, **188**, 64-74.

Rand, M.D., Grimm, L.M., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S.C., Sklar, J. and Aster, J.C. (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. *Mol Cell Biol*, **20**, 1825-35.

Rao, P.K., Dorsch, M., Chickering, T., Zheng, G., Jiang, C., Goodearl, A., Kadesch, T., and McCarthy, S. (2000) Isolation and characterization of the notch ligand delta4. *Exp Cell Res*, **260**, 379-386.

Reaume, A.G., Conlon, R.A., Zirngibl, R., Yamaguchi, T.P. and Rossant, J. (1992) Expression analysis of a Notch homologue in the mouse embryo. *Dev Biol*, **154**, 377-87.

Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991) Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell*, **67**, 687-99.

Romano, L.A. and Runyan, R.B. (1999) Slug is a mediator of epithelial-mesenchymal cell transformation in the developing chicken heart. *Dev Biol*, **212**, 243-54.

Romano, L.A. and Runyan, R.B. (2000) Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. *Dev Biol*, **223**, 91-102.

Rulifson, E.J. and Blair, S.S. (1995) Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. *Development*, **121**, 2813-24.

Rulifson, E.J., Micchelli, C.A., Axelrod, J.D., Perrimon, N. and Blair, S.S. (1996) wingless refines its own expression domain on the Drosophila wing margin. *Nature*, **384**, 72-4.

Rusconi, J.C. and Corbin, V. (1999) A widespread and early requirement for a novel Notch function during Drosophila embryogenesis. *Developmental Biology.*, **215**, 388-398.

Sakai, T., Taniguchi, Y., Tamura, K., Minoguchi, S., Fukuhara, T., Strobl, L.J., Zimber-Strobl, U., Bornkamm, G.W. and Honjo, T. (1998) Functional replacement of the intracellular region of the Notch1 receptor by Epstein-Barr virus nuclear antigen 2. *J Virol*, **72**, 6034-9.

Sakata, Y., Kamei, C.N., Nakagami, H., Bronson, R., Liao, J.K. and Chin, M.T. (2002) Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. *Proc Natl Acad Sci U S A*, **99**, 16197-202.

Salomon, D., Ayalon, O., Patel-King, R., Hynes, R.O. and Geiger, B. (1992) Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *J Cell Sci*, **102**, 7-17.

Santolini, E., Puri, C., Salcini, A.E., Gagliani, M.C., Pelicci, P.G., Tacchetti, C. and Di Fiore, P.P. (2000) Numb is an endocytic protein. *J Cell Biol*, **151**, 1345-52.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev*, **6**, 2620-34.

Savagner, P. (2001) Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays*, **23**, 912-23.

Savagner, P., Boyer, B., Valles, A.M., Jouanneau, J. and Thiery, J.P. (1994) Modulations of the epithelial phenotype during embryogenesis and cancer progression. *Cancer Treat Res*, **71**, 229-49.

Schroeter, E.H., Kisslinger, J.A. and Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature*, **393**, 382-6.

Schweisguth, F. (1995) Suppressor of Hairless is required for signal reception during lateral inhibition in the Drosophila pupal notum. *Development*, **121**, 1875-84.

Sestan, N., Artavanis-Tsakonas, S. and Rakic, P. (1999) Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science*, **286**, 741-6.

Shawber, C., Boulter, J., Lindsell, C.E. and Weinmaster, G. (1996a) Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev Biol*, **180**, 370-6.

Shawber, C., Nofziger, D., Hsieh, J.J., Lindsell, C., Bogler, O., Hayward, D. and Weinmaster, G. (1996b) Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development*, **122**, 3765-73.

Shirayoshi, Y., Yuasa, Y., Suzuki, T., Sugaya, K., Kawase, E., Ikemura, T. and Nakatsuji, N. (1997) Proto-oncogene of int-3, a mouse Notch homologue, is expressed in endothelial cells during early embryogenesis. *Genes Cells*, **2**, 213-24.

Shutter, J.R., Scully, S., Fan, W., Richards, W.G., Kitajewski, J., Deblandre, G.A., Kintner, C.R. and Stark, K.L. (2000) Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev*, **14**, 1313-8.

Soriano, J.V., Uyttendaele, H., Kitajewski, J. and Montesano, R. (2000) Expression of an activated Notch4(int-3) oncoprotein disrupts morphogenesis and induces an invasive phenotype in mammary epithelial cells in vitro. *Int J Cancer*, **86**, 652-9.

Spana, E.P. and Doe, C.Q. (1996) Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron*, **17**, 21-6.

Srivastava, D. (2000) Congenital heart defects: trapping the genetic culprits. *Circ Res*, **86**, 917-8.

Stern, C.a.I., P. (1992) *Gastrulation*. the company of biologists ltd., Cambridge UK.

Struhl, G. and Adachi, A. (1998) Nuclear access and action of notch in vivo. *Cell*, **93**, 649-60.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993) Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell*, **74**, 331-45.

Struhl, G. and Greenwald, I. (1999) Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature*, **398**, 522-5.

Struhl, G. and Greenwald, I. (2001) Presenilin-mediated transmembrane cleavage is required for Notch signal transduction in Drosophila. *Proc Natl Acad Sci U S A*, **98**, 229-34.

Struhl, K. (1999) Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. *Cell*, **98**, 1-4.

Takeichi, M. (1995) Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol*, **7**, 619-27.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T. and Honjo, T. (1995) Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). *Curr Biol*, **5**, 1416-23.

Taylor, K.L., Henderson, A.M. and Hughes, C.C. (2002) Notch Activation during Endothelial Cell Network Formation in Vitro Targets the Basic HLH Transcription Factor HESR-1 and Downregulates VEGFR-2/KDR Expression. *Microvasc Res*, **64**, 372-83.

Tepass, U., Gruszynski-DeFeo, E., Haag, T.A., Omatyar, L., Torok, T. and Hartenstein, V. (1996) shotgun encodes Drosophila E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Dev*, **10**, 672-85.

Tepass, U., Truong, K., Godt, D., Ikura, M. and Peifer, M. (2000) Cadherins in embryonic and neural morphogenesis. *Nat Rev Mol Cell Biol*, **1**, 91-100.

Thiery, J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*, **2**, 442-54.

Tomlinson, A. and Struhl, G. (1999) Decoding vectorial information from a gradient: sequential roles of the receptors Frizzled and Notch in establishing planar polarity in the Drosophila eye. *Development*, **126**, 5725-38.

Tsukamoto, T. and Nigam, S.K. (1999) Cell-cell dissociation upon epithelial cell scattering requires a step mediated by the proteasome. *J Biol Chem*, **274**, 24579-84.

Uyttendaele, H., Ho, J., Rossant, J. and Kitajewski, J. (2001) Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proc Natl Acad Sci U S A*, **98**, 5643-8.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D. and Kitajewski, J. (1996) Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development*, **122**, 2251-9.

Uyttendaele, H., Soriano, J.V., Montesano, R. and Kitajewski, J. (1998) Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. *Dev Biol*, **196**, 204-17.

Van Doren, M., Bailey, A.M., Esnayra, J., Ede, K. and Posakony, J.W. (1994) Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. *Genes Dev*, **8**, 2729-42.

Veltmaat, J.M., Orelio, C.C., Ward-Van Oostwaard, D., Van Rooijen, M.A., Mummery, C.L. and Defize, L.H. (2000) Snail is an immediate early target gene of parathyroid hormone related peptide signaling in parietal endoderm formation. *Int J Dev Biol*, **44**, 297-307.

Weinmaster, G. (1997) The ins and outs of notch signaling. *Mol Cell Neurosci*, **9**, 91-102.

Weinmaster, G., Roberts, V.J. and Lemke, G. (1991) A homolog of Drosophila Notch expressed during mammalian development. *Development*, **113**, 199-205.

Weinmaster, G., Roberts, V.J. and Lemke, G. (1992) Notch2: a second mammalian Notch gene. *Development*, **116**, 931-41.

Wesley, C.S. (1999) Notch and wingless regulate expression of cuticle patterning genes. *Mol Cell Biol*, **19**, 5743-58.

Wright, T.R. (1970) The genetics of embryogenesis in Drosophila. *Adv Genet*, **15**, 261-395.

Xu, T. and Artavanis-Tsakonas, S. (1990) deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. *Genetics*, **126**, 665-77.

Xue, Y., Gao, X., Lindsell, C.E., Norton, C.R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E.B., Weinmaster, G. and Gridley, T. (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet*, **8**, 723-30.

Ye, Y., Lukinova, N. and Fortini, M.E. (1999) Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. *Nature*, **398**, 525-9.

Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D.S., Holmes, E., Milman, P., Liang, Y., Zhang, D.M., Xu, D.H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L.S., Sorbi, S., Bruni, A., Fraser, P. and St George-Hyslop, P. (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature*, **407**, 48-54.

Zavadil, J., Cermak, L, Bottinger, E. (2003) TGF-beta Activation of the Notch Function is Required for Epithelial-to-Mesenchymal Transition. *American Association for Cancer Research*, Toronto, ON.

Zecchini, V., Brennan, K. and Martinez-Arias, A. (1999) An activity of Notch regulates JNK signalling and affects dorsal closure in Drosophila. *Curr Biol*, **9**, 460-9.

Zhong, T.P., Rosenberg, M., Mohideen, M.A., Weinstein, B. and Fishman, M.C. (2000) gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science*, **287**, 1820-4.

Zhou, S., Fujimuro, M., Hsieh, J.J., Chen, L. and Hayward, S.D. (2000a) A role for SKIP in EBNA2 activation of CBF1-repressed promoters. *J Virol*, **74**, 1939-47.

Zhou, S., Fujimuro, M., Hsieh, J.J., Chen, L., Miyamoto, A., Weinmaster, G. and Hayward, S.D. (2000b) SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function. *Mol Cell Biol*, **20**, 2400-10.

Zilian, O., Saner, C., Hagedorn, L., Lee, H.Y., Sauberli, E., Suter, U., Sommer, L. and Aguet, M. (2001) Multiple roles of mouse Numb in tuning developmental cell fates. *Curr Biol*, **11**, 494-501.