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Notch Signaling in Development and Cancer

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Notch is an evolutionarily conserved local cell signaling mechanism that participates in a variety of cellular processes: cell fate specification, differentiation, proliferation, apoptosis, adhesion, epithelial-mesenchymal transition, migration, and angiogenesis. These processes can be subverted in Notch-mediated pathological situations. In the first part of this review, we will discuss the role of Notch in vertebrate central nervous system development, somitogenesis, cardiovascular and endocrine development, with attention to the mechanisms by which Notch regulates cell fate specification and patterning in these tissues. In the second part, we will review the molecular aspects of Notch-mediated neoplasias, where Notch can act as an oncogene or as a tumor suppressor. From all these studies, it becomes evident that the outcome of Notch signaling is strictly context-dependent and differences in the strength, timing, cell type, and context of the signal may affect the final outcome. It is essential to understand how Notch integrates inputs from other signaling pathways and how specificity is achieved, because this knowledge may be relevant for future therapeutic applications. (*Endocrine Reviews* 28: 339–363, 2007)

I. Introduction: Elements of the Notch Signaling Pathway

NOTCH IS ONE of the fundamental signaling pathways that regulate metazoan development and adult tissue homeostasis. The Notch mutant was initially described in *Drosophila*, based on its dominant wing-notching phenotype (1). The study of the embryonic lethal phenotype caused by complete lack of Notch function (2) and its complex allelic series and genetic interactions (3) brought Notch to the forefront, so that in the mid-1980s the *Drosophila* Notch gene product was identified (4, 5).

Notch is a local signaling mechanism that is evolutionarily conserved throughout the animal kingdom. Mammals have four Notch proteins (Notch 1–4; Refs. 6–10 and Fig. 1A) that are membrane-bound type I receptors (with a single-pass transmembrane domain), harboring a large extracellular domain involved in ligand binding, and a cytoplasmatic domain involved in signal transduction. The extracellular domain contains a variable number of epidermal growth factor (EGF)-like repeats that are critical for binding interactions (11, 12). The EGF-like repeats are followed by three cysteine-rich LIN12/Notch repeats (LNR) that prevent signaling in the absence of the ligand. The Notch intracellular domain (NICD) contains a RAM23 domain (13), six ankyrin/cdc10 repeats involved in protein-protein interactions (14), two nuclear localization signals (N1 and N2), a transcriptional activation domain (TAD) that differs among the four receptors, and a PEST sequence [rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] that negatively regulates protein stability (15).

The Notch receptors are synthesized as single precursor proteins that are cleaved by a furin-convertase activity (16) at site 1 or S1 (Fig. 1B) during transport to the cell surface, where they are expressed as heterodimers (17). The mammalian Notch ligands Delta1 (18), Delta3 (19), Delta4 (20), Jagged1 (21), and Jagged2 (22) are named after the *Drosophila* homologs Delta and Serrate, respectively, and are also membrane-bound. They have an amino-terminal domain termed DSL (for Delta, Serrate and LAG-2 domain), followed by a variable number of EGF-like repeats. In addition, Jagged1 and Jagged2 harbor a cysteine-rich domain (CR; Fig. 1A).

Notch signaling is regulated by posttranslational modification events, such as glycosylation, and by other modifications involving the extracellular domains of both receptors and ligands, such as the extension of sugar residues by the

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Abbreviations: A-P, Anterior-posterior; bHLH, basic helix-loop-helix; CNS, central nervous system; CoR, corepressor; CR, cysteine-rich domain; DSL, Delta, Serrate and LAG-2 domain; E, embryonic day; EGF, epidermal growth factor; EMT, epithelial-mesenchyme transition; FGF10, fibroblast growth factor 10; HNSCC, head and neck SCC; HSC, hematopoietic stem cell; Lfng, lunatic fringe; LNR, LIN12/Notch repeats; MAN, Mastermind-Like 1; MMTV, mouse mammary tumor virus; NICD, Notch intracellular domain; PAE, porcine aortic endothelial; PEST, sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T); PKA, protein kinase A; PPR, PTH/PTHrP receptor; PSM, presomitic mesoderm; SCC, squamous cell carcinoma; Shh, sonic hedgehog; TACE, TNF-α-converting-enzyme; TAD, transcriptional activation domain; T-ALL, T cell acute lymphoblastic leukemia; WAP, whey acidic protein.

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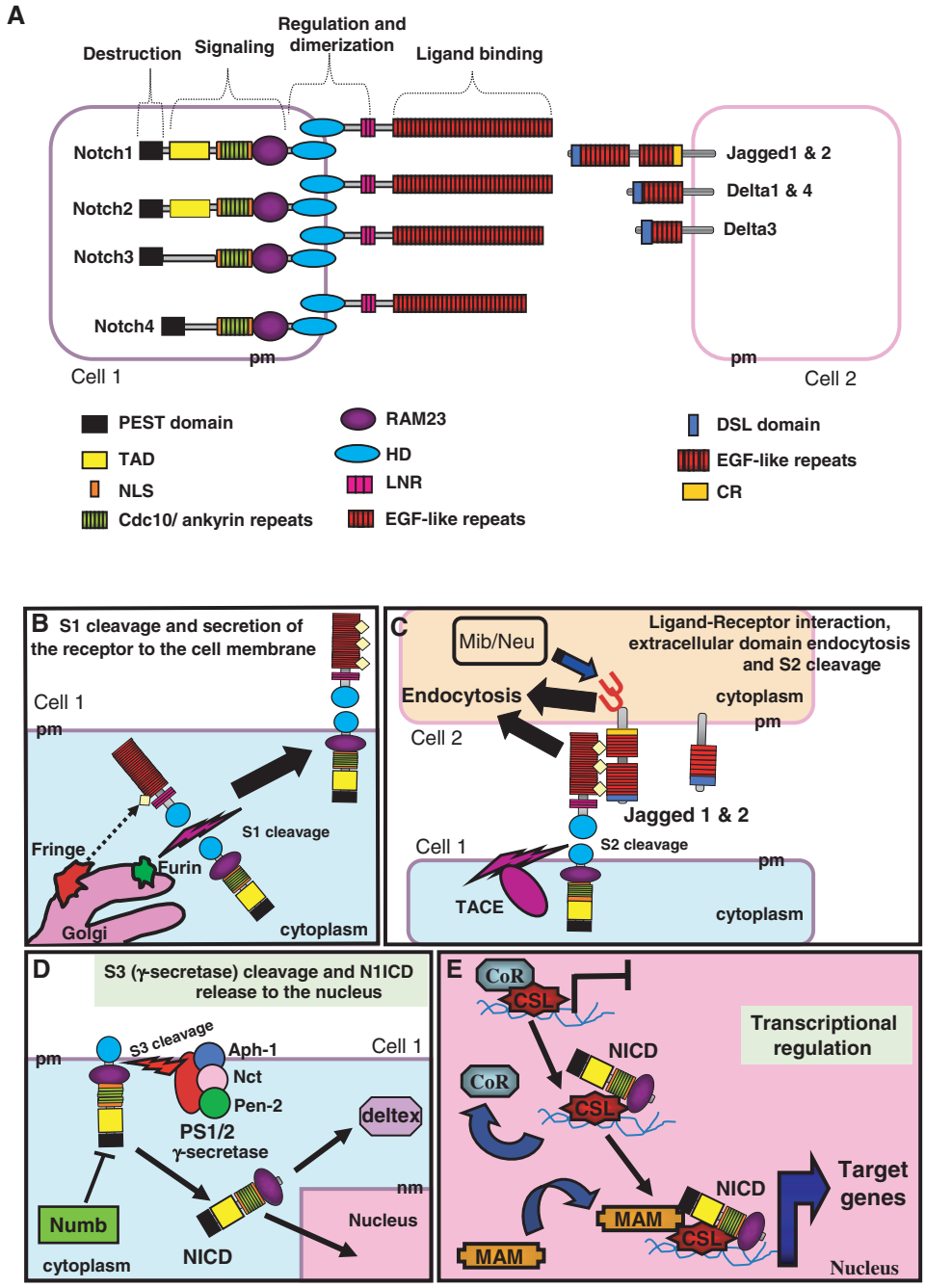

FIG. 1. Notch receptors and ligands and schematic representation of Notch signaling. A, Vertebrates have four Notch receptors (Notch1–4) expressed in the signal-receiving cell (Cell 1). The extracellular domain of Notch has between 29 and 36 EGF-like repeats (36 in Notch1 and Notch2, 34 in Notch3, 29 in Notch4) involved in ligand binding, followed by three cysteine-rich LNRs. The LNR domain prevents ligand-independent activation of the receptor and is followed by the heterodimerization domain (HD). The cytoplasmic part of the receptor contains the RAM23 domain, six cdc10/ankyrin repeats, two nuclear localization signals (NLS), a transcriptional transactivation domain (TAD), and a PEST sequence. Vertebrate receptors can be activated by at least five ligands (Jagged1 and 2 and Delta1, 3, and 4), expressed in the signaling cell (Cell 2). The ligands share an N-terminal DSL structure. Both Delta and Jagged ligands have EGF-like repeats in the extracellular domain, but only Jagged1 and Jagged2 harbor an additional cysteine-rich (CR) sequence downstream of the EGF-like repeats. B, The Notch receptor is secreted to the cell membrane in a furin convertase-dependent step (site 1 or S1 cleavage) that takes place in the Golgi. In this cell compartment, the glycosyltransferase fringe elongates previously attached fucose residues. Notch is expressed in the cell membrane as a heterodimer. C, Binding to Delta or Jagged ligands initiates two consecutive proteolytic cleavage events; the first is mediated by the ADAM protease TACE and occurs on the extracellular side of Notch, near the transmembrane domain (site 2 cleavage). D, The second cleavage (S3) occurs within the transmembrane domain and is mediated by γ-secretase activity, a complex composed of four different integral membrane proteins: presenilin, nicastrin (Nct), Aph-1, and Pen-2 (286). NICD is released and translocates to the nucleus. In the cytoplasm, the Numb protein negatively regulates Notch signaling, possibly by promoting receptor turnover. Deltex proteins may transduce Notch signals independently of CSL. E, In the nucleus NICD binds to the CSL transcription factor, converting it from a transcriptional repressor into a transcriptional activator by displacing a CoR complex and recruiting coactivators such as MAML1 (MAM). This leads to transcriptional activation of downstream target genes. pm, Plasma membrane; nm, nuclear membrane.

glycosyl transferase fringe (23, 24) (Fig. 1B). This prevents Notch activation by Jagged but not by Delta ligands (Refs. 25 and 26; reviewed in Ref. 27). Notch signaling initiates through ligand-receptor interactions between neighboring cells, leading to two consecutive proteolytic cleavages of the receptor, which ultimately liberate NICD (Fig. 1, C and D). Thus, after ligand binding, the ubiquitin ligases mind bomb (28, 29) or neuralized (30–32) interact with the ligand intracellular domain to promote its ubiquitination and internalization (28) (Fig. 1C). Ligand endocytosis leads to a conformational change in Notch that allows ADAM (metalloprotease and disintegrin) protease TACE (TNF-α-converting-enzyme), to cleave the receptor at a second site (S2) on the extracellular side, near the transmembrane domain (33) (Fig. 1C); the released extracellular portion of the receptor is then transendocytosed to the ligand-expressing cell (34) (Fig. 1C). The third cleavage (S3) occurs within the transmembrane domain and is mediated by a γ-secretase activity whose key components are presenilin and nicastrin (35) (Fig. 1D). This final cleavage liberates NICD, which subsequently translocates to the nucleus where it binds via its RAM23 domain to the transcription factor CSL (*C*BF1 in humans, *S*uppressor of Hairless in *Drosophila*, *L*AG in *Caenorhabditis elegans*), also called RBPJK in mice. In the absence of Notch activity, CSL proteins bind to promoters of its target genes and recruit histone deacetylases (36) and corepressors (CoR; Fig. 1E) that inhibit transcription. The corepressor molecules include SMRT/NcoR (37) and SHARP/MINT/SPEN (38). The NICD/CSL interaction converts CSL from a transcriptional repressor into a transcriptional activator by displacing the corepressor complex and recruiting coactivators (Fig. 1E) such as Mastermind-Like 1 (MAM; Fig. 1E) (39) and histone acetyltransferase (40). A number of additional proteins modulate Notch signaling, including the RING-domain E3 ubiquitin ligase deltex (41, 42) and the phosphotyrosine-binding domain (PTB)-containing proteins numb and numb-like (43), which act as context-dependent negative or positive Notch regulators (Fig. 1D).

To date, only a few target genes have been identified; some are Notch-dependent in various tissues, whereas others are tissue-specific. The best-known Notch target genes are members of the basic helix-loop-helix (bHLH) hairy/enhancer of split (*Hes*) family, the related HRT/*Herp* (Hes-related repressor protein) transcription factor family (44), the cell cycle regulator p21 (45), the Notch pathway element Notch-regulated ankyrin repeat protein (*Nrarp*) (46), *deltex1*, and the *pre-T cell receptor-α* gene (47). For an in-depth study of the molecular intricacies of Notch signaling elements see Refs. 48–51.

Here we will examine the role of Notch in specific developmental processes such as central nervous system (CNS) development, somitogenesis, cardiovascular development, and endocrine development, with attention to the distinct mechanisms by which Notch regulates cell specification and patterning in these tissues. We will then analyze the role of Notch in cancer, both in leukemia and in solid tumors, and describe studies that suggest possible means of therapeutic intervention. As a rule, when we refer to humans, the elements of the pathway are named in capital letters (*i.e.*, NOTCH1 receptor or *NOTCH1* gene), and when we refer to experimental models, the elements of the pathway are named in lowercase (Notch1 protein or *Notch1* gene).

II. Notch in Vertebrate CNS Development

During vertebrate CNS development, the primitive neuroepithelium gives rise to two main lineages, neurons and glia. Neurons are generated in embryonic life from multipotent progenitors close to the ventricle and, after their final mitotic division, migrate away from their birthplace to their ultimate destinations, where they terminally differentiate and integrate into the brain circuitry. Glial cells, in contrast, are generated in the proliferating subventricular zone at late embryonic and early postnatal stages.

During the past 10 yr, the role of Notch in the differentiation, morphogenesis, and function of the CNS has become increasingly valued. The phenotypic analyses of Notch-targeted mutants in mice and functional manipulation in other vertebrates have greatly benefited from knowledge generated by research in *Drosophila* neurogenesis (52). Poulson (2) was the first to associate lack of Notch function with an embryonic lethal phenotype in *Drosophila*; it is caused by failure of the early neurogenic ectoderm to segregate neural and epidermal cell lineages. In homozygous Notch mutant embryos, all cells become neuroblasts, which leads to hypertrophy of the neural tissue at the expense of the epidermis, giving rise to the so-called neurogenic phenotype (2). In vertebrates, Notch is required when the epidermal and neural lineages have already segregated; its inactivation results in a "neurogenic phenotype" represented by premature differentiation of neuronal progenitors, leading to the interpretation that Notch maintains a progenitor state and inhibits differentiation.

A. Notch promotes progenitor diversification and inhibits neuronal differentiation

Notch1 was the first Notch pathway gene to be disrupted by homologous recombination (53, 54). Mutant embryos die at midgestation [embryonic day 11 (E11)] with defective somitogenesis and placentation, although little attention was given to a possible neural phenotype. Subsequently, a detailed analysis of neural development in *Notch1* targeted mutants was reported (55). This study examined the expression of pathway components such as *Hes1*, *Hes5*, and *Delta1* and of early differentiation markers such as *NeuroD* (or *Neurod2*), *Math4A* (or *Neurog2*), and *NSCL-1* (or *Nhlh1*). Consistent with the view that Notch activity is needed for progenitor maintenance, expression of these markers was increased in mutants. In addition, *Hes5* expression was reduced in *Notch1* mutants, although *Hes1* expression appeared to be unaffected. This result was unexpected in light of the *Hes1* mutant phenotype (56) and the extensive literature supporting the idea that *Hes1* is a primary Notch/CSL target (reviewed in Ref. 57). Similar results in *RBPJk* targeted mutants (55) suggested that whereas *Hes1* may well be a true Notch target, it is also likely to be activated by other signaling pathways. After the original *Notch1* deletion studies, targeted alleles of *Notch2* (58, 59), *Notch3* (60), and *Notch4* (61) were also generated, as well as conditional (floxed) alleles of

Notch1 (62). Although *Notch3* and *Notch4* do not have detectable phenotypes when deleted, *Notch2* mutants, similarly to *Notch1* mutants, die around E11 (58). In contrast to *Notch1* mutants, however, *Notch2* mutants do not show alterations in *Hes5* expression in the CNS. *Notch2* mutants undergo widespread cell death in the CNS starting around E9 (58), but it is not clear whether this phenotype reflects a role for *Notch2* in the developing CNS or whether it occurs as a consequence of other embryonic perturbations.

To circumvent the early lethality of *Notch1* deletion, several studies have addressed the effect of deleting this receptor in specific brain structures. In one case, conditional Cre-loxP-mediated recombination was used to delete *Notch1* from the medial cerebellar primordium (63). Consistent with the traditional model of Notch function in the nervous system, the authors found that *Notch1* deletion resulted in upregulation of the proneural genes *Mash1* and *Math1* and precocious neuronal differentiation. More recently, conditional deletion of *Notch1* in the neural progenitor pool using a nestin-Cre promoter also resulted in precocious neuronal differentiation (64). Deletion of *Notch1* in the telencephalon, using the foxg1-Cre line, led to reduced neuron numbers later in development, most likely resulting from precocious neuron differentiation and earlier progenitor pool depletion (65). In support of this finding, the telencephalic deletion of *Notch1* led to a reduction in progenitor frequency (assayed as neurospheres) *in vitro* (66). This result is consistent with the reduced neurosphere frequencies observed after standard deletion of *Notch1*, *RBPJk*, *PS1* and *PS2* (67), or *Hes1* and *Hes5* (68). In summary, the conditional deletions of *Notch1* support the view that Notch signaling inhibits neuron differentiation and maintains the neural progenitor pool.

In addition to the receptor mutations, many Notch ligand mutations have been examined in mice. A few studies have analyzed the effect of deleting *Delta1* on neural development (66, 69). One of these studies found that *Delta1* mutants had decreased *Hes5* expression, consistent with the predicted reduction in Notch activation (66). This finding was previously documented in a comparative study of the role of Notch in somitogenesis (70). In addition, the study by Yun *et al.* (66) found that *Delta1* targeted mutants showed a decrease in radial progenitor markers and an increase in neuronal markers. These findings support the view that Notch signaling inhibits neuron differentiation in the developing CNS. Based on comparisons of the *Delta1*, *Mash1*, and other mutants, however, the authors suggest that Notch signaling might also regulate the diversification of the progenitor pool into distinct progenitor subtypes. This function would precede the role of Notch in inhibiting the differentiation of mature cell types (neurons and oligodendrocytes) and could convert a homogeneous proliferative pool into a heterogeneous mixture of stem cells, neuroblasts, and glioblasts. Further evidence that Notch signaling may generate progenitor diversity was obtained by *in vitro* analysis of *Delta1* targeted mutants (69). This report suggested that Notch signaling first specifies glial progenitors and then functions in those cells to promote astrocyte *vs.* oligodendrocyte fate. Both this study and the work described above indicate that in mice, Notch influences multiple choice points in the neural progenitor lineage.

As discussed previously, the Notch signaling cascade is transduced primarily through the transcriptional regulator CSL (RBPJK in mice), when nuclear translocation of NICD converts CSL from a repressor to an activator. Consistent with the *Notch1* mutant phenotype, *RBPJk* targeted mutants show altered gene expression, suggestive of widespread precocious neuron differentiation (such as decreased *Hes5* and increased *Delta1* and *NeuroD*) (55). As in the case of the other Notch pathway genes, conditional deletion of *RBPJk* in the CNS is likely to be highly informative.

The most widely accepted Notch/CSL targets are the *Hes* (71) and the *HRT* gene families (72). Although there are seven *Hes* genes, not all are clear Notch targets, and studies in the mammalian CNS have focused on *Hes1* and *Hes5*. *Hes1* mutant embryos show severe defects in neural development, including lack of cranial neural tube closure and anencephaly (56). Because these animals die perinatally, it is possible to examine alterations in gene expression at late developmental stages. Consistent with the canonical model, precocious neurogenesis in *Hes1* mutants was suggested by early expression of *Mash1*, *NSCL1*, and neurofilament markers. Studies with double mutant combinations reveal that *Hes1* and *Hes5* have redundant functions in the CNS, regulating the differentiation of the neural progenitor pool. *Hes1; Hes5* double mutants thus have a more severe phenotype than single *Hes1* and *Hes5* mutant phenotypes combined (68). *Hes1* mutants also show increased *Hes5* expression, suggesting the existence of a compensatory mechanism between these Notch targets (56). Lastly, although *Hes5* mutants showed some precocious neuronal differentiation, these animals were largely normal, suggesting that *Hes1* is able to compensate for lack of *Hes5* almost completely.

Inactivation of recently identified Notch signaling elements also leads to altered neurogenesis. Targeted mutagenesis of *mind bomb1* causes a phenotype relatively similar to that of *Notch1* or *RBPJk* mutants, which result in embryonic lethality at E10.5. There is also a strong neurogenic phenotype in the CNS, with premature neurons undergoing apoptosis soon after differentiation. Aberrant neurogenesis is a direct consequence of lowered *Hes1* and *Hes5* expression resulting from the inability to generate N1ICD (73).

B. Notch in gliogenesis

In contrast to its "permissive" role in neuronal differentiation, Notch appears to have an instructive role in gliogenesis, directly promoting the differentiation of many glial subtypes. The results of *in vivo* studies involving mouse (74, 75), zebrafish (76), chick (77, 78), and *Xenopus* (79) are consistent with an instructive role for Notch signals in gliogenesis, through their conventional bHLH targets. Activation of Notch signaling favors the generation of Müller glia cells at the expense of neurons, whereas reduced Notch signaling induces production of ganglion cells, causing a reduction in the number of Müller glia.

In vertebrates (55, 80) as in *Drosophila* (81) early neurogenesis, Notch signaling operates among cells belonging to an equivalence group—because they express the same set of molecules and are functionally equivalent—and controls their commitment to differentiate via a mechanism termed

lateral inhibition (82). How a given cell within an equivalence group adopts the neuronal fate or not depends on the Notch ligand expression level. If a progenitor cell expresses more Delta ligand than its neighbors, it will become a neuron and will transmit an inhibitory signal to the Notch-expressing progenitors in contact with it, preventing these progenitors from differentiating prematurely into neurons and from expressing Delta. As a consequence, the cell that produces more ligand forces its neighbors to produce less; ultimately, individual neighboring cells are driven into different developmental pathways (83). Molecular genetic studies show that in this situation, ligand production is regulated by a negative feedback loop (84). This interpretation of the gene expression patterns has been documented extensively by experiments in *Xenopus* (79, 80), chick (78, 85), zebrafish (86–88), and mouse (55). Another example of lateral inhibition is the formation of sensory hair cells in the vertebrate inner ear (89), where a single cell within an equivalence domain expresses high Delta levels.

More detailed analyses of Notch function in the CNS has revealed that Notch is likely to regulate progenitor pool diversification and neuronal maturation (90). Emerging data also suggest that Notch signaling has a role in neuronal function in the adult brain (91). It will be of great interest to determine which components of the Notch signaling cascade function in each of these processes. Figure 2 summarizes the roles of Notch in embryonic and adult CNS.

III. Notch in Somitogenesis

The somites are blocks of paraxial mesoderm cells lying at either side of the neural tube. They give rise to the vertebrae of the axial skeleton and their associated muscles and tendons, which retain a segmental or metameric pattern. In all vertebrates, somites are generated sequentially from the presomitic mesoderm (PSM), the unsegmented paraxial mesoderm at the caudal end of the embryo (92). At the rostral end of the PSM, clefts appear, and successive blocks of somite tissue split off; meanwhile, the embryo grows caudally, with a relatively constant amount of PSM tissue. Once formed, somitic cells differentiate progressively to give rise to five major cell types: the bone, cartilage and tendons of the trunk, skeletal muscles of the body, and the dermis of the back (93, 94). Each somite is also subdivided into anterior and posterior compartments (A-P polarity), a subdivision that is already established in the anterior PSM (95). In addition, the different morphological specification of somites is established early in the PSM and relies mostly on the activity of *Hox* genes (96). The connection between somitogenesis and the nested expression domains of *Hox* genes in the paraxial mesoderm has been established (97), although the coordination of these two patterning processes is poorly understood. Notch involvement in somitogenesis was first suggested by the defects in somite morphology observed in mice with targeted mutations in the *Notch1* (54) and *RBPJk* (98) genes. In *Notch1* mutants, the PSM generates irregular somites in which the positioning of segmental boundaries is abnormal (54). Similarly, *Delta1* targeted mutants show abnormal somitogenesis with loss of A-P polarity (99). This phenotype is more severe in *RBPJk* mutants, which have fewer somites and a largely unsegmented paraxial mesoderm (70, 98) (Fig. 3, A–D). Studies using dominant-negative or constitutively activated Notch showed that perturbing Notch signaling in *Xenopus* produces similar phenotypes; somitic cells differentiated normally into myocytes, but the segmental organization of these cells is lost (100). The similarity of these phenotypes suggested that Notch is critical in the patterning process leading to somite boundary formation (92) and the establishment of the A-P polarity of somites (99, 101).

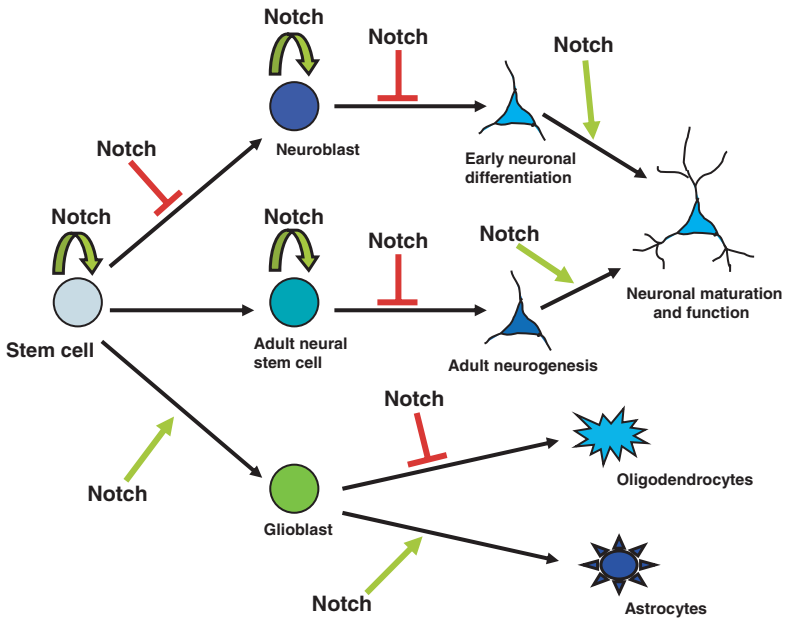

FIG. 2. Notch signaling in the developing and adult CNS. Processes that require Notch activity are labeled with *green arrows*, and those that require Notch inhibition are labeled with *red truncated arrows*. The *green semicircular arrows* indicate the requirement of Notch for progenitor pool maintenance. See text for details. [Adapted from K. Yoon and N. Gaiano: *Nature Neuroscience* 8:709–715, 2005 (287) with permission from Macmillan Publishers Ltd.]

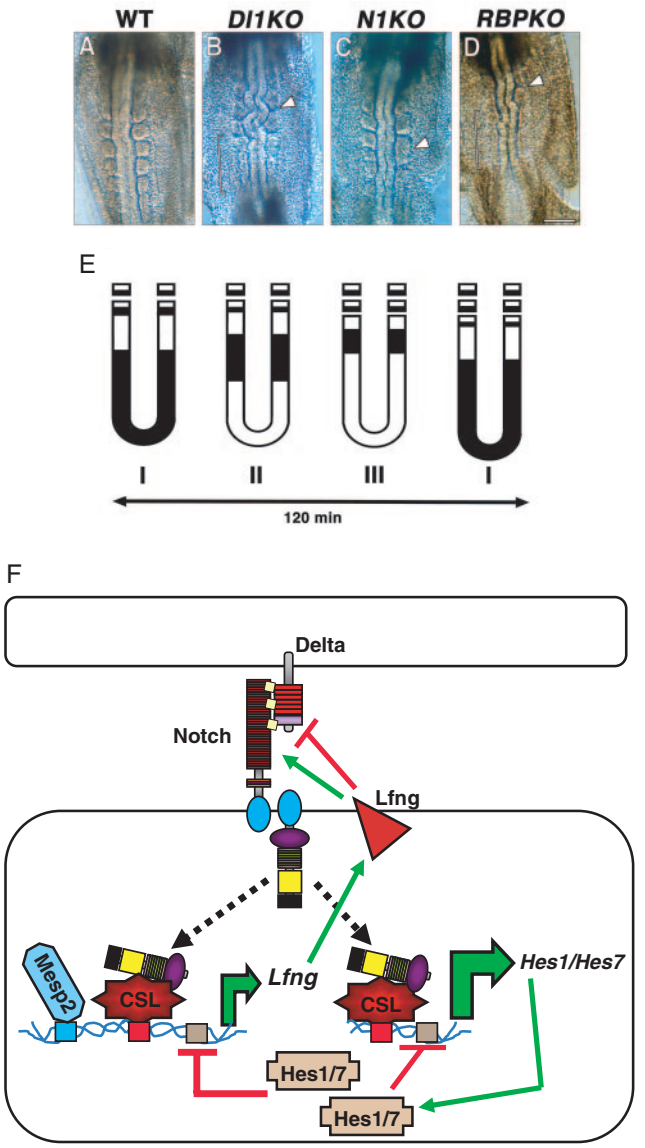

FIG. 3. Notch signaling is required for somite boundary formation. A–D, Dorsal view of the somitic region of E8.5 embryos. A, Wild-type embryo. B, *Delta1* targeted embryo. Note irregular somites (*arrowhead*) and unsegmented paraxial mesoderm. C, *Notch1* mutant embryo. Note irregular and fused somites (*arrowhead*). D, *RBPJk* mutant embryo. Note a few irregular somites (*arrowhead*) and a large stretch of unsegmented paraxial mesoderm. *Scale bar*, 50 μm. E, Oscillatory expression in the PSM (*black*). *chairy1* mRNA sweeps repeatedly across the PSM in a posterior-to-anterior direction, and each cycle is synchronous with the formation of a new somite. Blackened area represents the moving front of *chairy1* expression. F, Notch and the segmentation clock. In the posterior PSM, Delta-Notch signaling activates *Lfng*, *Hes1*, and *Hes7*. Lfng potentiates Delta-Notch interaction (*green arrow*), whereas Hes1 and Hes7 binding to E boxes (*brown*) repress *Lfng* and their own transcription, via a negative feedback loop. After Hes1/Hes7 proteins disappear, their transcription is initiated *de novo* by activators such as Notch. Thus, *Lfng* expression oscillates in the same phase as *Hes1/7* expression. In the anterior PSM, Mesp2 binds to N boxes (*blue*) in the *Lfng* promoter and activates its expression. Lfng in this situation inhibits Delta-Notch1 interaction (*red truncated arrow*). As a result, both waves of Notch1 activity and Lfng are arrested, and a boundary between Notch1 activity domain and the Mesp2 expression domain is generated, which leads to a new segmental boundary. [Modified from Refs. 105, 108, 109, and 113.]

A great advance in the molecular analysis of segmentation was the discovery of *cHairy1*, a chick gene whose expression oscillates within the PSM, with a periodicity corresponding to a segmental cycle (102) (Fig. 3E). The *cHairy1* expression pattern provided the first molecular insight into a potential segmental clock. In addition, *cHairy1* belongs to a large family of bHLH transcriptional repressors, including proteins encoded by genes that are known to be direct Notch targets in other species, such as *Hes1* (103) in the mouse and the *Enhancer of Split* [E(spl)] genes in *Drosophila* (104). These observations, together with the defective somitogenesis of Notch targeted mutants described above, raised the possibility that the dynamic changes in *cHairy1* expression within the PSM could be driven by changes in Notch signaling, as part of a segmentation clock.

Oscillatory expression of other bHLH repressor genes in the PSM was later observed in other vertebrate embryos, although the gene with dynamic expression is not always the same. *Hes7* (105) and *Her1/7* (106, 107) are thus rhythmically expressed in the mouse and zebrafish embryo, respectively. In *Xenopus*, homologs of the oscillating zebrafish *Her1* gene are expressed in the PSM, but their expression does not oscillate (101). The emerging picture is that Notch signaling undergoes a dramatic spatial and temporal change within the PSM during each segmental cycle. Segmentation appears to rely on two major components; an oscillator, the segmentation clock, sets the periodicity of somite formation, and a dynamic wave-front defines the level at which PSM cells respond to the clock, providing a mechanism that spaces the segment boundaries (108).

How does the segmentation clock work? A negative feedback loop has been proposed as the major mechanism. A study by Hirata *et al.* (109) showed that the segmentation clock can be mimicked in cultured cells. After serum stimulation, expression of both *Hes1* mRNA and protein oscillates with a 2-h cycle. This coincides with the cycle of the segmentation clock in mouse (Fig. 3E), suggesting that the oscillators in cultured cells and the PSM share the same mechanism. Hes1 protein oscillation is delayed by approximately 15 min relative to *Hes1* mRNA oscillation. Both *Hes1* mRNA and Hes1 protein have very short half-lives, which enable 2-h cycle oscillatory gene expression. Hes1 protein is degraded by the ubiquitin-proteasome pathway (109). When stabilized by proteasome inhibitors, Hes1 protein constitutively represses its own transcription by binding directly to the *Hes1* promoter. Conversely, in the absence of functional Hes1 protein, *Hes1* transcription is constitutively up-regulated. The negative feedback loop in which Hes1 protein periodically represses its own transcription is thus the central mechanism for Hes1 oscillation (Fig. 3F). It is likely that Hes1 protein represses *Hes1* mRNA synthesis, but is rapidly degraded, allowing the next cycle of mRNA synthesis (109). Because *Hes1* transcription is constitutively up-regulated in the absence of functional Hes1 protein, there appears to be a steady level of transcriptional activators in cultured cells after serum stimulation.

Because a simple negative feedback loop would be insufficient to maintain stable oscillation, another cycling factor might be involved. Such a cycling factor is not yet known for cultured cells, but for the segmentation clock, lunatic fringe

(Lfng) in mouse (Fig. 3F) and chick and deltaC in zebrafish might be implicated in maintaining stable oscillation in the PSM. Dale *et al.* (110) found that Lfng also establishes a negative feedback loop in chick, and that, in addition to *Lfng* mRNA, Lfng protein exhibits oscillatory expression in the PSM. Activation of Notch signaling induces *Lfng* transcription, but Lfng protein inhibits Notch signaling and thereby represses its own transcription. Lfng thus also periodically represses its own expression, like Hes1 and Hes7. Analysis of the regulatory region of *Lfng* in mouse confirmed that it includes a site that mediates direct stimulation by activated Notch (111) but showed that it contains sites that mediate inhibition by bHLH proteins such as the Hes family members (112). This led to a model in which the inhibitory action of *Hes* genes plays a key role, and Lfng is assumed to potentiate Delta-Notch signaling (Fig. 3F), as in *Drosophila* (25). In contrast to this proposition, Morimoto *et al.* (113) have shown that Notch1 activity in mice oscillates in the posterior PSM. Somite boundaries would form at the interface between Notch1-activated and Notch1-repressed domains. This interface would be generated by suppression of Notch activity by the bHLH transcription factor Mesp2 through induction of *Lfng* that, in contrast with its role in the wing margin (25), would inhibit Notch activation. It thus becomes clear that the function of Lfng could vary, depending on cell context and biological system. Another important finding is that Wnt signaling is also involved in the segmentation clock (114). Lfng oscillation is lost in vestigial tail mice (hypomorphic *Wnt3a* mutants), indicating that the Wnt pathway interacts with the Notch pathway, although the precise mechanism remains to be determined. Although great advances have been made in the past decade in understanding aspects of the somitogenesis process, many aspects, such as the molecular machinery that underlies the segmentation clock, remain to be explained. For a more detailed review on the molecular aspects of somitogenesis see Refs. 108, 115, and 116.

IV. Notch in Cardiovascular Development and Homeostasis

The cardiovascular organ system is the first to form during vertebrate embryogenesis, when the heart develops from the cardiogenic mesoderm to form the double-walled primary heart tube. This tube consists of two cell types: an inner layer of endocardial endothelial cells, and an outer myocardial layer which is separated from the inner layer by an extracellular matrix (the cardiac jelly). Endocardial endothelium and cardiomyocytes together constitute the primitive heart tube. The first rhythmic cardiac contractions are initiated at this double-walled stage of heart development (117). Interaction between endocardial endothelium and myocardium leads to formation of ventricular trabeculae, highly organized sheets of cardiomyocytes forming muscular ridges lined by endocardial cells (118). The formation of this spongy, trabeculated pattern substantially increases the endocardial endothelial surface area.

Tissue interactions between the myocardium and endocardium in the atrioventricular canal and outflow tract regions lead to epithelial-mesenchyme transition (EMT) of endocardial cells, to participate in cardiac valves and membranous septa formation (119). Cushion formation is followed by the development of more compact myocardium in the periphery of the developing cardiac ventricles.

Vascular development initiates with the differentiation of endothelial precursors, or angioblasts, into endothelial cells (120). These cells assemble to form a primitive vascular plexus of uniformly sized vessels composed entirely of endothelial cells by the process of vasculogenesis. This primitive vascular plexus is then remodeled to form the veins, arteries, and capillaries through the process of angiogenesis.

Different Notch ligands (20, 61) and receptors (6, 10, 121, 122), as well as their downstream effectors and target genes (72), are expressed in the vascular system. Functional studies in zebrafish (123) and mice (61) showed that Notch is a critical regulator of cardiovascular development. Evidence for a crucial function of Notch in vascular development and homeostasis is the finding that the human disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarction and leukoencephalopathy), which involves the *NOTCH3* gene, causes stroke and vascular dementia (124). In addition, *JAGGED1* haploinsufficiency leads to the dominant inherited Alagille syndrome, which among other features is characterized by vascular anomalies (125, 126).

Analysis of mice with targeted mutations in *Notch1* or *RBPJk* revealed that these mutants have severe pericardial distension, indicative of a circulatory defect (53, 54, 98). Likewise, mutations of the *Delta1* (99) and *Jag1* (127) genes lead to vascular abnormalities and hemorrhage, indicative of an underlying vascular defect. Analysis of the *Notch1; Notch4* double mutants provided an insight into Notch function in the vasculature. Whereas *Notch4* mutants are viable and fertile, the *Notch1; Notch4* double mutants show normal vasculogenesis, but the process of angiogenesis is affected in the embryo proper, the yolk sac, and the placenta (61). This phenotype is more severe than that of *Notch1* mutants alone, suggesting that Notch1 and Notch4 have partially redundant roles during embryonic vascular development. From this study, it became clear that Delta4 was the key ligand for Notch1 and Notch4 receptors in the vasculature. In fact, *Delta4*-targeted mutants and endothelial-specific *RBPJk*-targeted mutants show a loss of arterial identity and arteriovenous malformations (128, 129), indicating that Notch signaling is required for arterial specification and patterning during development. These studies also suggested a relationship between Notch and EphrinB2/EphB4, another local cell signaling pathway involved in arterial-venous specification (130).

Targeted mutagenesis of potential Notch target genes in the cardiovascular system has helped to delineate the Notch signaling pathway that leads to arterial specification. *HRT1; HRT2* double mutants die after E9.5 and display severe angiogenic remodeling defects and massive hemorrhage, probably due to impaired arterial fate determination and maturation. This phenotype is very similar to that of zebrafish *gridlock* (*grl*) mutants, that show impaired aorta maturation (131). *Grl* encodes for the zebrafish homolog of mammalian *HRT2*. These report identified *HRT/CHF1/Hesr/Herp* genes as essential Notch effectors in vascular development. The view of Notch as a key regulator of arterial fate is consistent with

data indicating that ectopic activation of Notch signaling leads to repression of venous fate (132) and to severe vascular patterning defects (133). More recently, our understanding of how arterial-venous specification occurs has been refined by the study of an endothelial-specific mutant of the COUP-TFII transcription factor. Suppression of Notch signaling activity by COUP-TFII regulates vein identity, which may thus be genetically controlled and not derived by a default pathway (134, 135) (Fig. 4).

Specific Notch ligands and receptors are expressed in the heart from early developmental stages. *Delta4* (61), *Notch1* (6), and *Notch4* (10) are transcribed in the endocardial lineage from gastrulation onward, whereas other ligands and receptors show restricted expression in the myocardium from midgestation (136, 137). The Notch targets *HRT1* and *HRT2* are expressed in the endocardium and/or myocardium at different stages of cardiogenesis (72). Studies in *Xenopus* (138) and in mouse embryonic stem cells (139) indicate that cardiomyogenic commitment and differentiation require Notch signaling inhibition. *In vivo* studies in mouse and zebrafish nevertheless indicate that abrogation of Notch signaling does not affect primary cardiac cell fate determination and differentiation (139).

In *RBPJk*-targeted mutants, early cardiogenic differentiation and chamber identity is unaffected, but valve development is severely disrupted, presumably because of defective endocardial maturation and signaling (140). During valvulogenesis in the E9.5 mouse embryo, endocardial cells undergo EMT and form the cardiac cushions that later remodel to give rise to the thin, finely sculpted mature valve leaflets of the E14.5 embryo (141). *RBPJk* and *Notch1* mutants have a collapsed endocardium and lack mesenchymal cushion cells, indicating that endocardial EMT is defective in Notch

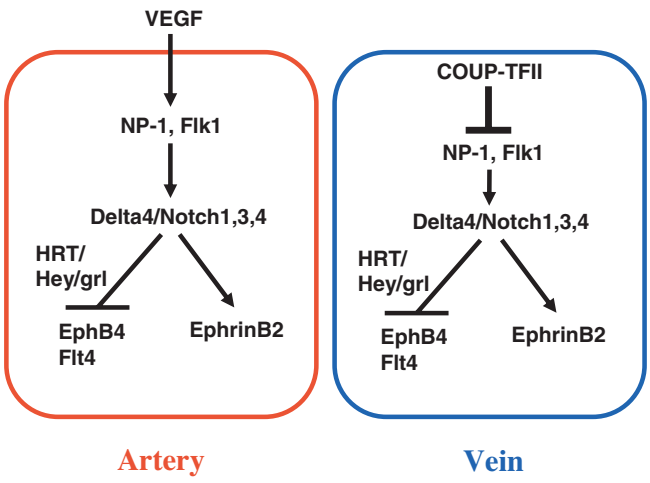

FIG. 4. Models of arterial-venous differentiation. In presumptive arterial endothelium, VEGF binds to its receptors neuropilin-1 (NP-1) and Flk1 that would in turn activate endothelial specific Notch signaling activity, leading to HRT2/gridlock-mediated repression of *EphB4* and *Flt4* receptor genes and activation of *EphrinB2* expression, triggering the arterial differentiation program. In presumptive vein endothelium, COUP-TFII suppresses NP-1 and Flk1 to down-regulate Notch activity and therefore release repression of *EphB4* and *Flt4* expression and down-regulate *EphrinB2* expression, leading to vein differentiation. [Adapted from L.R. You et al.: *Nature* 435:98–104, 2005 (134) with permission from Macmillan Publishers Ltd.]

pathway mutants. Ultrastructural analysis of E9.5 mutant atrioventricular canal endocardia reveals that cells remain in close association, abnormally maintaining adherens junctions, and do not invade the cardiac jelly, despite showing features of activated premigratory endocardial cells. These observations correlate with a specific reduction in the transcription of the snail repressor (snai1) (142) in the atrioventricular canal region. Concomitantly, *VE-cadherin* expression remains abnormally stabilized in the atrioventricular canal and outflow tract endocardium of the mutants, suggesting that the lack of snail expression prevents down-regulation of *VE-cadherin* in this tissue, blocking endocardial EMT. These findings demonstrate that Notch activity is required for endocardial EMT (140).

Explant assays with *Notch1* and *RBPJk* mutants demonstrate an impaired endocardial EMT, a phenotype reproduced in wild-type explants cultured with the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT). This finding is supported by Notch inhibition experiments in zebrafish that impair valve development and by gain-of-function experiments in which transient overexpression of *N1ICD* in the heart leads to formation of hypertrophic atrioventricular valves (140). This result contrasts with recent zebrafish data reporting that restricted *N1ICD* overexpression in the endocardium of atrioventricular canal inhibits EMT (143). Conditional activation of *Notch1* in the cardiac lineage of the mouse using a *MesP1-CRE* driver impairs atrioventricular myocardial differentiation and ventricular myocardium maturation of *MesP1-CRE; N1ICD* transgenic mice, but EMT occurs normally (144). This discrepancy may be explained by the fact that *N1ICD* RNA was ectopically expressed in both endocardium and myocardium (140), which may generate additional myocardial-derived EMT-inductive signals. In the case of the experiments by Beis *et al.* (143), *N1ICD* was overexpressed exclusively in the endocardium. Our own data infecting wild-type atrioventricular canal explants with a retrovirus expressing *N1ICD* are consistent with this possibility, because *N1ICD*-transduced explants transform more than controls (J. Grego-Bessa, L. Luna-Zurita, and J. L. de la Pompa, unpublished data). The relevance of Notch in human cardiac valve development and homeostasis was demonstrated by the recent finding that *NOTCH1* mutations cause aortic valve disease in humans (145). Studies in mice in the same report show that similarly to *Notch1*, its target genes *HRT1/Hey1* and *HRT2/Hey2* are expressed in the aortic valve leaflets at E17.5 where they repress *Runx2*, a regulator of osteoblast cell fate. These results suggest that *NOTCH1* mutations cause an early developmental defect in the aortic valve and a later derepression of calcium deposition that causes progressive aortic valve disease (145).

Jag1 and *Notch2* are also linked to cardiac development. Mice doubly heterozygous for *Jag1* and *Notch2* mutations exhibit developmental abnormalities characteristic of Alagille syndrome, such as jaundice, growth retardation, defective bile duct differentiation, and abnormal kidney, eye, and heart development. The cardiac defects include right ventricular hypoplasia, pulmonic valve stenosis, atrial and ventricular septal defect, and dextropositioning of the aorta (59). The Notch targets HRT1/Hey1 and HRT2/Hey2 have also been linked to cardiac development. *HRT2*-targeted mutant mice have several cardiac anomalies. Almost all *HRT2*

mutants show growth retardation and die within 10 d of birth. Surviving *HRT2* mutants have enlarged atria and ventricles, and echocardiographic analysis revealed abnormal cardiac hemodynamics, including stenosis and regurgitation of the tricuspid valve, mitral valve regurgitation, membranous ventricular septal defect, and secundum atrial septal defect (146). The phenotypic variation reported in *HRT2* mutants probably results from the use of different genetic backgrounds and/or functional redundancy between HRT2 and other HRT family members (147). Analysis of *HRT2* mutant mice suggests that HRT2 is required for formation of the atrioventricular valves. In addition, mice lacking both *HRT1* and *HRT2* die during embryogenesis due to severe cardiovascular malformations, including those in the development of the atrioventricular cushions. Few cells undergo EMT in the *HRT1; HRT2* mice (148), suggesting that HRT1 and HRT2 function synergistically in the EMT process. In addition, *HRT1; HRT2* mice show defects in trabecular myocytes (148). Although the ventricular chamber containing both the compact and trabecular zones forms initially, subsequent apoptosis of trabecular zone myocytes leads to poor trabecular formation. It is thus clear that HRT2 is important for correct cardiomyocyte development. The interaction between HRT1-expressing endocardial and epicardial cells and HRT2-expressing cells in the compact layer of the ventricle is also considered necessary to produce and/or maintain trabecular myocytes (149). Our own data (295) support a role for Notch in the development of ventricular myocardium, as the trabeculation-defective phenotype of standard and endocardial-specific *Notch 1* and *RBPJk* mutants indicates. We propose that Notch mediates an endocardium-myocardium interaction critical for trabeculation and ventricular chamber morphogenesis and identify two distinct Notch-dependent processes: 1) transition of primitive myocardial epithelium to trabecular and compact myocardium (EphrinB2- and NRG1-dependent); and 2) maintenance of a proliferating trabecular cardiomyocyte population (BMP10-dependent) during this transition. The defective ventricular phenotype of Notch mutants is reminiscent of a congenital disorder termed isolated ventricular noncompaction (IVNC) (296), which is characterized by altered myocardial structure. Thus, Notch signaling may be altered in infants with conditions including malseptation, abnormal valve development or conduction system defects, all of which are related to abnormal trabeculation.

Notch activity in the cardiovascular system functions via a mechanism termed lateral induction (83), by which Notch generates contiguous domains of cells that share the same fate, an embryonic field. This signaling mechanism also occurs in flies during wing margin boundary formation (25). Other examples of Notch-mediated lateral induction in vertebrates include induction of proneural domains in the ear (150), formation of the limb bud margin (151), and somite boundary formation (83). In these cases, Notch activation promotes ligand production via a positive feedback loop, so that signaling occurs simultaneously in a developmental field. Loss of Notch signaling leads to down-regulation of ligand expression throughout the embryonic field, indicating the existence of a positive feedback loop.

Figure 5 summarizes the processes in which Notch is involved during cardiogenesis. In a search for novel therapeutic approaches for cardiac disease, and in view of the role of Notch in the maintenance of an uncommitted state (152) and in inhibition of the cardiogenic fate *in vitro* (139), it will be of great interest to explore the role of Notch in adult cardiac homeostasis.

V. Notch in Endocrine Development: Pancreas, Gut, and Bone Endocrine Cells

A. Pancreatic development

The pancreas is an endoderm-derived organ formed by three main cell types, the exocrine acinar cells that produce digestive enzymes (*i.e.*, carboxypeptidase), the α-and β-endocrine cells (islets of Langerhans) that produce the hormones regulating nutrient homeostasis (insulin and glucagon), and the elaborate branched ductal tree that connects with the intestine (153, 154). Pancreas formation begins early in development (around E9.0 in the mouse) with the formation of two cell buds, ventral and dorsal. These buds express the Pdx1 transcription factor (155, 156) and arise from a specialized endodermal epithelium located in the foregut region that will give rise to the duodenum (157). During pancreatic organogenesis, the two buds undergo a series of morphogenetic, proliferative, and lineage specification events, grouped in three "developmental transitions," to give rise to the mature and functional pancreas (for a review, see Refs. 158 and 159). At E13.5, the developing pancreas is formed by a branched Pdx1-positive epithelial tree, without morphological signs of exocrine differentiation. Endocrine and exocrine precursors are marked by two members of the bHLH transcription factors family, Ngn3 and Ptf1-P48, respectively (160).

Specific Notch pathway elements and downstream effectors are expressed in the developing pancreas, suggesting a role for Notch in pancreatic development (161–163). Loss-of-function of various Notch pathway genes (*RBPJk*, *Delta1*, and *Hes1*) leads to up-regulation of the proendocrine gene *Ngn3* (164), a direct Hes1 target (165), and consequent accelerated and increased differentiation of pancreatic endocrine cells (161, 162). Conversely, forced Notch activity in the embryo blocks both endocrine and exocrine pancreas development (166, 167), and in pathological situations it leads to dedifferentiation of exocrine cell types in pancreatic epithelium (168). The physiological relevance of Notch in exocrine pancreas has been shown in the mouse, where Notch is active in committed exocrine progenitors cells and whose ectopic activation in pancreatic bud explants represses acinar cell differentiation (169). The available data indicate that Notch regulates the progressive recruitment of endocrine and exocrine cell types from a common precursor pool in developing pancreas. The inhibitory effect of Notch signaling in exocrine differentiation has been well characterized in zebrafish, where endocrine and exocrine cells arise independently (170). Thus, accelerated exocrine differentiation is observed in zebrafish *mind bomb* mutants or upon expression of a dominant negative *Su (H)* version in wild-type embryos (169).

Similarly to the CNS, Notch operates in the developing pancreas by lateral inhibition. This would explain the ini-

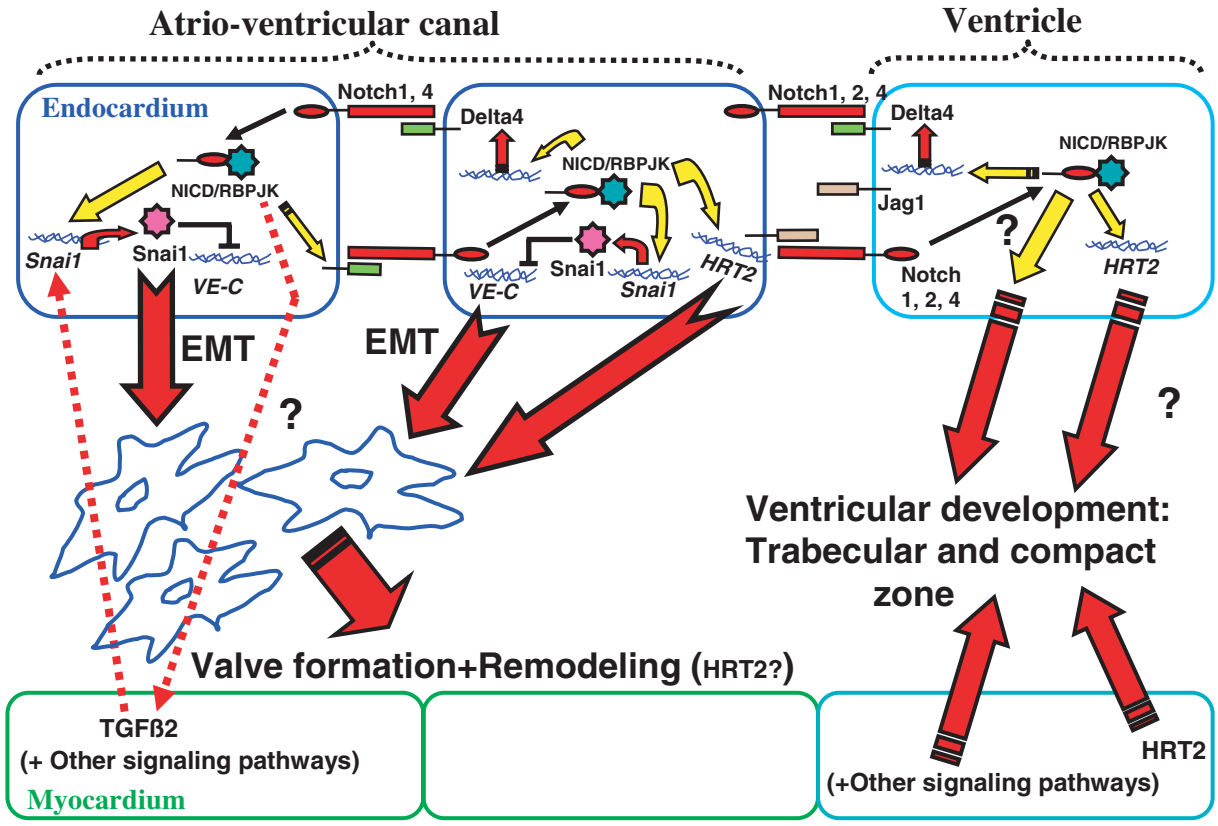

FIG. 5. Notch activity and cardiac development. The endocardial endothelium expresses specific Notch ligands and receptors simultaneously, behaving as an embryonic field. In the atrioventricular canal and outflow tract regions (data not shown), Delta4/Notch1 receptor interactions lead to *snai1* expression, which represses *vascular endothelial cadherin* (*VE-C*) expression, allowing endocardial cells to down-regulate adhesion, undergo EMT, and acquire a mesenchymal phenotype. Notch activity in the endocardium is also required for the production of a soluble signal that activates *TGFβ2* expression in the myocardium. TGFβ2 binding to its endocardial receptors would also lead to *snail* expression and EMT induction. In the ventricular endocardium, Notch signaling mediated by HRT2 (among other factors) is required for ventricular chamber development and trabeculation. Other signaling pathways active in the myocardium may cooperate with Notch in this process. In addition, myocardial HRT2 would also be involved.

tially dispersed distribution of endocrine cells within the pancreatic epithelium. Consistent with this idea, *Hes1* mutants show increased expression of the Delta 1 and Delta 3 ligands (162). Nevertheless, additional observations suggest that Notch signaling in the pancreas does not function only via lateral inhibition and perhaps respond to additional signals, emanating from the mesenchyme. Fibroblast growth factor 10 (FGF10) produced in pancreatic mesenchyme has been shown to be essential for precursor pool maintenance (171). Transgenic mice overexpressing FGF10 in pancreatic epithelia show pancreatic hyperplasia and a block in exocrine, ductal, and endocrine differentiation (172, 173). In these mice and in contrast to the wild-type situation (161, 162), *Notch1* and *Notch2* as well as *Hes1* are ubiquitously expressed in pancreatic precursor cells, whereas *ngn3* expression is abrogated (172, 173). Thus, ectopic FGF10 signaling is capable of maintaining Notch signaling activity in the pancreas. These observations have led to the suggestion that another mechanism different from lateral inhibition and termed "suppressive maintenance" sustains Notch signaling activity in the pancreas and suppress its differentiation (159, 172). The mechanism underlying suppressive maintenance is not well understood, but it may involve FGF10-dependent activation of the Jag 1 and Jag2 Notch ligands in pancreatic epithelium (172). It remains to be seen what is the effect of Notch signaling abrogation on the expression of its ligands.

A further refinement of our view of Notch in pancreatic development has come from the analysis of *Hes1*-targeted mutant mice. *Ptf1* is misexpressed in discrete regions of the primitive stomach and duodenum and throughout the bile duct of *Hes1* mutants. Ptf1-expressing cells are reprogrammed to multipotent pancreatic progenitors that differentiate into mature pancreatic exocrine, endocrine, and duct cells. These data demonstrate that Notch is required for proper regional specification of pancreas in developing foregut endoderm through *Ptf1* regulation (174). Because Notch is involved in the development and homeostasis of a variety of self-renewing tissues (175, 176), understanding Notch function in pancreatic development will help to design protocols to control β-cell development *in vitro* and thus treat diseases such as diabetes, in which the mass of insulin-producing β-cells is reduced.

B. Gut development

The gastrointestinal tract comprises the small intestine and colon. Small intestine epithelium is organized into finger-like villi and adjacent invaginations, the crypts of Lieberkühn; the

colon has a flat epithelial surface with no villi (177). In small intestine, the crypt compartment contains stem cells and progenitors, whereas the villus compartment is made up entirely of differentiated cells. Pluripotent stem cells, which reside at the crypt bottom, give rise to transit amplifying cells, which divide robustly before terminal differentiation. Four lineages can be distinguished in the gastrointestinal tract, *i.e.*, absorptive enterocytes, mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, and lysozyme- and cryptidin-producing Paneth cells (reviewed in 175). Enteroendocrine cells can be further subdivided on the basis of the hormones they secrete (178). Intestine homeostasis depends on a small number of evolutionarily conserved pathways, including bone morphogenetic protein (BMP)/TGFβ, sonic hedgehog (Shh), wingless (Wnt) and Notch (179).

The analysis of mutant zebrafish or targeted mice deficient for different Notch pathway elements or target genes has implicated Notch in the regulation of the earliest intestinal cell fate decisions. Thus, a recent study describes increases in secretory cells at the cost of absorptive cells in the intestines of zebrafish that are mutant for *DeltaD* and *mind bomb* (180). Different Notch pathway elements are expressed in murine crypts (181). The Notch target *Hes1* is expressed in crypts (162). Analysis of the developing fetal intestine of *Hes1* mutant mice revealed an increase in mucosecreting and enteroendocrine cells at the expense of absorptive enterocytes (162). The crypt progenitor pool in the small intestine seemed unaffected, as judged by an analysis of proliferative activity. Math1 is a target gene of Hes1-mediated repression in several organs, including the intestine (182). *Math1* mutant mice die neonatally. Although the crypt-villus architecture was essentially undisturbed in the mutant mice, commitment toward the secretory lineage had entirely halted (182). These results have been interpreted to indicate that Hes1 and Math1 are required to skew the fate of differentiating cells leaving the transit amplifying compartment toward an enterocyte or a secretory phenotype, respectively (162, 182).

Conditional deletion of the RBPJK effector in the epithelium of the small intestine and colon using different CRE-driver lines led to a decrease in *Hes1* expression, and *Math1* was up-regulated throughout the crypt compartment, whereas it is normally expressed only in secretory cells (183). In addition, the number of goblet cells was increased. In the small intestine, Paneth cells appeared in near-normal numbers at the bottom of the crypts. However, the rapidly dividing transit amplifying compartment, which normally occupies the remainder of the crypt, was entirely replaced by postmitotic goblet cells that expressed Math1 protein but not *Hes1*. Examination of proliferation by different techniques indicated that basically all epithelial cell division had halted. Essentially identical observations were made in the colon (183). As an explanation for the different severity of the phenotypes observed upon *Hes1* deletion and conditional *RBPJk* deletion in colon, it has been argued that other *Hes* genes such as *Hes6* are affected by conditional *RBPJk* deletion (183). This phenotype was confirmed using a highly efficient γ-secretase inhibitor. Cell proliferation had entirely halted and histological markers revealed that the tissue changes were indistinguishable from those observed after *RBPJk* deletion (183).

A reciprocal phenotype to that of *RBPJk* deletion in colon was obtained in transgenic mice overexpressing N1ICD in all cells of the intestinal epithelium, by virtue of the villin-CRE driver (184). N1ICD; villin-CRE transgenic mice have a complete lack of goblet cells in all intestinal tracts. In addition, enteroendocrine cells are markedly reduced, as well as Paneth cells (184). Microscopic examination of earlier developmental stages revealed that already at E18.5, N1ICD expression affects the architecture of the villi, and thus the differentiation of secretory cell lineages along the duodenal-ileal axis and the cranial-to-caudal wave of intestinal differentiation was inhibited (184). Transcriptional analysis revealed a direct correlation between N1ICD expression and elevated levels of *Hes1* transcription in the intestinal epithelium of N1ICD transgenic mice, although no other *Hes* genes were affected. Expression of the Hes1 targets *Math-1* (185) and *ngn3* (165), involved in secretory cell lineage specification, was reduced in N1ICD; villin-CRE mice. This finding was consistent with the idea that the expression of *Math-1* and *ngn3* is repressed by Notch activation (184). This intestinal phenotype is reminiscent of *Math-1* (lack of goblet and enteroendocrine cells) and opposite to that of *Hes1* mutant mice (an excess of secretory cells at the expense of enterocytes). Thus, the gain-of-function phenotype of N1ICD; villin-CRE mice provides direct evidence that Notch signals target *Hes1* in the intestine, explaining mechanistically the differentiation defects observed. Overall, these genetic data indicate that Notch-mediated *Hes1* expression regulates a binary cell fate decision between adsorptive and secretory cell fates. However, *Hes1*-deficient mice do not show a change in the proliferative status of the intestinal precursor pool (162), whereas Notch activation profoundly affects the proliferation potential of intestinal progenitors (184), suggesting that other Notch targets may be responsible for the increased in proliferation. The effect of deregulated Notch signaling in colon homeostasis and cancer will be discussed in *Section VI.B*.

C. Bone development

Bone is a dynamic tissue that is constantly renewed and degraded. Two main types of bone cells are responsible for these processes: bone-forming osteoblasts of mesenchymal origin, and bone-resorbing osteoclasts of hematopoietic origin. Bone formation and resorption are coordinated so that bone remodeling is balanced. When this equilibrium is altered in a way that bone resorption exceeds bone formation, osteoporosis occurs. This disease is prevalent in old age and is characterized by bone loss and a high risk of fractures. Therefore, knowledge about molecular events involved in osteoblast differentiation is crucial.

The *in vitro* potential of Notch pathway in osteoclastogenesis and osteoblastogenesis has been investigated in several reports. Evidence indicates that Notch down-regulates osteoclastogenesis activation, reduces the surface expression of c-Fms, which is a receptor for macrophage colony-stimulating factor, in osteoclast precursor cells and enhances the expression of osteoprotegerin in stromal cells, which results in the down-regulation of osteoclastogenesis (186). However, controversial results have been obtained with respect to os-

teoblastic differentiation. Continuous NICD expression inhibits bone morphogenetic protein 2-induced osteoblast differentiation in osteoblast precursor cells (187). In contrast, transient expression of N1ICD in osteoblast precursor cells leads to an enhanced bone mineral deposition (188). Notch1 is expressed in the mesenchymal condensation area and subsequently in the hypertrophic chondrocytes during chondrogenesis (189). Another study shows that Notch1, Delta1, and Jagged1 are expressed in cultured osteoblast precursor cells as well as in differentiating osteoblasts during bone regeneration, and that Notch1 is activated in these cells (190). These results suggest that Notch signaling plays an important role in the commitment of mesenchymal cells to the osteoblastic cell lineage (190). Concomitant expression of Delta1 and Jagged1 during *in vivo* bone regeneration suggests that there is a functional redundancy between Delta1 and Jagged1 and that these ligands direct osteoprogenitor cells to the differentiated status through identical signaling pathways (190). These data suggest a therapeutic potential for Notch in bone regeneration as well as osteoporosis.

Mammalian bone marrow architecture involves hematopoietic stem cells (HSCs) in close proximity to the endosteal surfaces of bone (191), with more differentiated cells arranged in a graduated fashion as the central longitudinal axis of the bone is approached (192), suggesting a relationship between HSCs and osteoblasts. Osteoblasts produce hematopoietic growth factors (193) and are activated by PTH or the locally produced PTHrP through the PTH/PTHrP receptor (PPR). Experiments involving transgenic mice expressing in osteoblastic cells a constitutively active PPR under the control of the alpha1(I) collagen promoter showed increased numbers of trabeculae and trabecular osteoblastic cells in the long bones of transgenic mice (194). In addition, the number of HSCs was also increased in these mice, but mature cell number was not changed (194). Because this phenotype was reminiscent of the mode of Notch action in hematopoiesis (195) and Jag1 is expressed by marrow stromal cells (196) and by murine osteoblasts (197), the levels of Jag1 protein were analyzed in the bone marrow of transgenic mice. col1-caPPR transgenic mice showed a marked increase in Jag1 expression in osteoblastic cells. The response of hematopoietic stem cells to increased Jag1 expression was measured by N1ICD staining, which was increased in transgenic mice. These data support a model in which PPR activation in the osteoblastic population increases cell number and the overall production of Jag1. This, in turn, may activate Notch on primitive hematopoietic cells, resulting in expansion of the stem cell compartment (194). In another study, these authors demonstrated that PTH treatment led to increased levels of Jagged1 in osteoblastic cells both *in vivo* and *in vitro*, in a protein kinase A (PKA)-dependent manner (198). Because Jagged1 is very important in stromal-HSC interactions and PTH regulates HSC expansion through osteoblastic activation, these studies suggested that stimulation of osteoblastic PKA activation may alter the HSC niche. This is of great therapeutic importance because this study suggests that alternative means to stimulate osteoblastic PKA activation may alter the HSC niche. In addition, Jagged1/Notch signaling modulates osteoblastic differentiation (189), and Jagged1 may play a critical role in mediating the effects of PTH on osteoblasts.

VI. Notch in Cancer

Experimental evidence supports the idea that signaling pathways essential for embryonic development also have a role in regulating self-renewing tissues (199, 200). Mutations in these pathways (such as TGFB, Wnt, and ErbB) often lead to tumorigenesis, as is also true for Notch (reviewed in Ref. 200). An interesting aspect of Notch is its apparently opposite functions in tumor development, because it can act as an oncogene or as a tumor suppressor. Although the mechanism underlying this dual Notch action is being explored, the outcome of Notch signaling activity depends on signal strength, timing, cell type, and context (reviewed in Ref. 201). The result of altered Notch signaling depends on its normal function in a given tissue. Notch thus acts as an oncogene if its normal function is as a gatekeeper of stem cells or as a regulator of precursor cell fate; its tumor suppressor activity is detected in tissues in which Notch signaling initiates terminal differentiation events (202). Table 1 summarizes the involvement of abnormal Notch signaling in cancer.

The oncogenic function of Notch is shown by the finding that truncated forms of all four Notch isoforms (Notch1-Notch 4), resulting in constitutively active Notch signaling, have transforming activity *in vitro* (203) and in various animal models (204–208). Furthermore, deregulated expression of wild-type Notch receptors, ligands, and targets is found in many human solid tumors (209, 210) and hematological malignancies (176, 210). Notch inhibition by antisense retrovirus or by pharmacological γ-secretase blockade has antineoplastic effects in Notch-expressing transformed cells *in vitro* and in xenograft models *in vivo* (Refs. 211–214; reviewed in Refs. 176 and 209). Notch alone may not be a very efficient oncogene, however, and it must associate with another oncoprotein to cause transformation. Although such partners have not yet been identified in naturally occurring tumors, transformation can be induced *in vitro* in various cell types by expressing NICD with certain oncoproteins (215, 216). The Notch tumor suppressor function may be a peculiarity of the mouse skin system, or it may also apply in man and include human keratinocytes as well as other human epithelial cell types (217). Available evidence thus suggests that, with the possible exception of some human epidermal malignancies, Notch signaling inhibition is a viable strategy for treatment of certain solid and hematopoietic tumors (176, 209). JAG1 expression in head and neck squamous cell carcinoma (HNSCC) cells triggers Notch activation in neighboring endothelial cells and promotes network formation (218). In xenograft models, HNSCC cells overexpressing JAG1 formed larger tumors with increased vascularization, and JAG1 protein levels were notably higher in HNSCC samples than in normal samples (218). These results offer a causal link between Notch signaling and tumor angiogenesis and describe a novel juxtacrine signaling mechanism from tumors to surrounding vasculature. As we saw in *Section IV*, Notch is essential for angiogenic remodeling in the embryo and for vascular homeostasis in the adult. This study shows that Notch is also involved in pathological angiogenesis (218), suggesting a possible direction for therapeutic intervention in tumors. In this regard, a recent report has shown that VEGF-induced Delta4 acts as a negative regulator of tumor

TABLE 1. Abnormal Notch signaling in tumorigenesis and EMT

Tumor type/process	Function	Human models	Animal models
Hematological tumors			
T cell malignancies (T-ALL)	Oncogenic Notch signaling	Constitutively active NOTCH1: t(7; 9) (q34:q34.3) (223), activating mutations (224, 289)	Activating mutations in Notch1 in mouse models of T-ALL (290). Constitutively active Notch2 (291). Constitutively active Notch3. Transgenic mice; lck promoter-driven intracellular Notch3 (205). DLL4 overexpressing mice (292).
B cell malignancies	Oncogenic Notch signaling	Notch1, Notch2, and Jagged1 (229–231)	
	Tumor suppressive Notch signaling	Intracellular Notch1–4, Jagged 1–2 (232)	Intracellular Notch1–4, Jagged 1–2 (232)
Solid tumors			
Breast cancer	Oncogenic Notch signaling	Notch1 and Jagged-1 (252, 253)	Constitutively active Notch4 in transgenic mice: MMTv insertion (239), MMTv-intracellular Notch4 (237); WAP- intracellular Notch4 (240). Constitutively activated Notch1 in transgenic mice: MMTv insertion (242), MMTv-intracellular Notch1 (207, 208). Constitutively activated Notch3 in transgenic mice: MMTv-intracellular Notch3 (208).
	Tumor suppressive Notch signaling	Notch2 (252)	
Gut cancer	Oncogenic Notch signaling		Notch signaling in APC min mice (183)
Skin cancer			
Keratinocyte-derived carcinoma	Tumor suppressive Notch signaling	Notch signaling in basal cell carcinoma (260)	Conditional ablation of Notch1 in murine epidermis (257). Conditional transgenic mice with epidermal inhibition of Notch signaling (SM22-Cre+/DNMAML1+ mice) (259).
Melanocyte-derived carcinoma	Oncogenic Notch signaling	Notch1 in primary melanoma (265, 266) NOTCH2 and HEY1 (269)	
Cervical cancer	Oncogenic Notch signaling	Notch1 in early disease stage (293)	
	Tumor suppressive Notch signaling	Notch1 in late disease stage (293, 294)	
EMT	EMT promoter	JAGGED1 in prostate cancer metastasis (284). MCF 10A cell line (212). Human keratinocytes (283).	PAE cell line (140).

angiogenesis (219). Thus, tumor-derived VEGF induces Delta4 expression in angiogenic endothelial cells to negatively regulate vascular growth, acting to restrain excessive vascular sprouting and branching and allowing angiogenesis to proceed at a productive rate. Thus, increasing Delta4/Notch activity resulted in decreased vascular density associated with reduced sprouting and branching of the vascular network. On the contrary, the blockade of Delta4/Notch signaling produced enhanced angiogenic sprouting and branching, resulting in a marked increase in tumor vessel density but decreased tumor vessel function (219). These data suggest that an alternative treatment of tumors may be based on the promotion of "nonfunctional" tumor angiogenesis.

A. Notch in hematological tumors

Notch signaling mediates hematopoietic cell fate determination in the embryo (220) and in the adult (221) and is also a critical factor in the maintenance of a pool of self-renewing HSCs (222). Deregulated expression of Notch pathway elements can thus lead to development of hematological malignancies. The prototypical Notch-associated cancer is human acute T cell acute lymphoblastic leukemia/lymphoma (T-ALL), which constitutes approximately 15–20% of ALL in children and adults. The *NOTCH1* gene was discovered due to its involvement in a chromosome translocation [t (7; 9)] seen in some human T-ALL; this leads to expression of N1ICD in a T cell receptor-β-regulated manner (Fig. 6A) (223). *Notch1* was later shown to be essential for normal T cell progenitor development (62). Although t (7; 9) is rare (less that 1% of T-ALL; Fig. 6A), the majority of human T-ALL have gain-of-function mutations in *NOTCH1*, leading to aberrant increases in downstream signaling (Fig. 6A) (224), placing the NOTCH pathway at the center of T-ALL pathogenesis. Weng *et al.* (224) found that more than 50% of human T-ALL without specific (7; 9) chromosome translocation, including tumors from all major molecular oncogenic subtypes, have activating mutations that involve the *NOTCH1* extracellular heterodimerization domain and/or the C-ter-

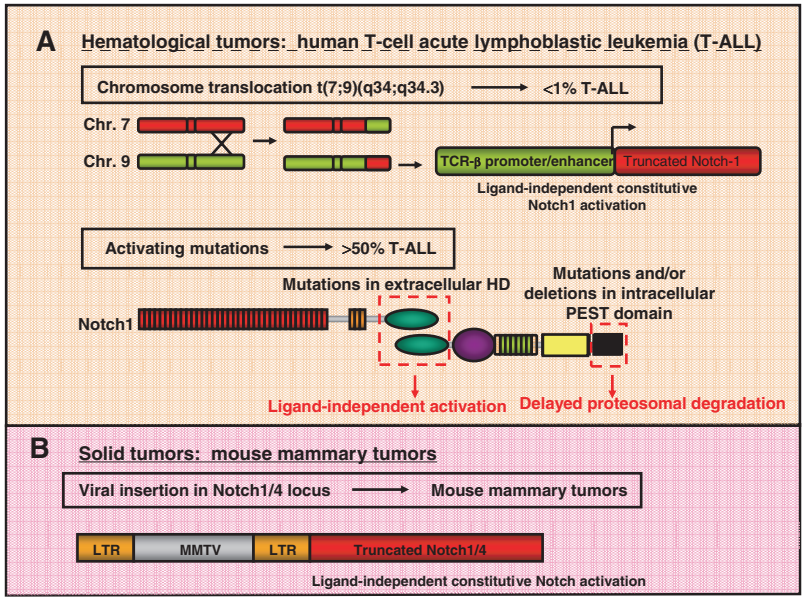

FIG. 6. Oncogenic Notch signaling in hematological malignancies and solid tumors. A, Chromosome translocations and activating mutations within the human NOTCH1 gene cause human T-ALL. The t (7; 9) translocation in T-ALL patients is characterized by juxtaposition of the 3′ portion of the human NOTCH1 gene with the T cell receptor β (TCRβ) locus. This leads to expression of truncated NOTCH1 transcripts and consequent production of dominant active, ligand-independent forms of the NOTCH1 receptor, causing T-ALL. This rare event occurs in less than 1% of all T-ALL patients. Schematic diagram of the full-length human NOTCH1 protein, showing "hot spots" of mutations found in more than 50% of T-ALL patients. B, Integration of the MMTV between the LNRs and the transmembrane domain of the Notch1 or Notch4 gene cause mammary tumors in the mouse.

minal PEST destruction box (Fig. 6A). The detection of mutations in multiple molecular subtypes of T-ALL is the basis for the conclusion that *NOTCH1* appears to collaborate with various other proteins that are also deregulated in T-ALL (176). A recent study also identified c-MYC as an important direct target of NOTCH1 in T-ALL and in a critical stage of normal pre-T cell development, showing that c-MYC inhibitors interfere with progrowth effects of activated NOTCH1 and that forced c-MYC expression rescues NOTCH1-dependent T-ALL cell lines from Notch withdrawal (225). Based on these findings, a phase I/II clinical trial was recently begun using a NOTCH pathway inhibitor to treat patients with refractory T-ALL. If this trial is successful, NOTCH pathway inhibitors may soon be considered therapeutic agents that target cancer-specific molecular lesions (reviewed in Ref. 176).

Although the Notch receptor is expressed throughout the hematolymphoid compartment, its transforming potential appears to be restricted to developing T cells. Several studies have explored this issue in malignant B cells, with conflicting results; three reports suggest that constitutive Notch signaling in malignant B cells leads to growth inhibition and/or apoptosis (226–228), whereas three groups found that Notch signaling promotes malignant B cell proliferation (229–231). A recent study of the effect of constitutive Notch signaling in malignant murine and human B cells showed generalized Notch-mediated growth inhibition and apoptosis in immature and mature human and murine B cell malignancies, including therapy-resistant subtypes (232). This suggests that Notch signaling may be of therapeutic use for certain B cell tumors, although more research is still required.

B. Notch as an oncogene in solid tumors: breast and gut cancer

1. Breast cancer. Stem cells thought to reside in the mammary gland are thought to renew mammary gland cells through cycles of pregnancy, lactation, and involution during a woman's lifetime (233). There is increasing evidence that stem cells might be targets of transformation during mammary carcinogenesis. The Notch signaling pathway is implicated in the self-renewal of normal mammary stem cells (234, 235), and recent work suggests a role for the Notch pathway in breast cancer (236). Notch4 was identified as a mouse mammary tumor virus (MMTV) insertion site in mammary tumors (Fig. 6B); provirus was inserted within the *Notch4* gene (originally known as the *int-3* locus) (237). This MMTV model system has proven useful for identification and characterization of genes involved in malignant transformation of normal mammary epithelium (238). In the case of the *Notch4* gene, provirus insertion leads to expression of a truncated Notch that lacks most of the extracellular portion of the protein but contains Notch4 transmembrane and intracellular domains (N4ICD) (239). Transgenic mice harboring this constitutively active *N4ICD* under the regulation of the *MMTV* promoter show arrested mammary gland development and eventually develop poorly differentiated adenocarcinomas (237). Additional evidence confirms the *Notch4/int-3* gene effect in mammary epithelial differentiation and mammary tumorigenesis; this is derived from studies in which *N4ICD* was expressed from the whey acidic protein (WAP) promoter in transgenic mice, which restricts its activity to secretory mammary epithelial cells of pregnant mice (240). As predicted, secretory lobule growth and differenti-

ation were inhibited, and mammary tumors were histologically identical to MMTV-*N4ICD* tumors. Other workers subsequently identified a 1.8-kb human NOTCH4/Int3 RNA species (designated h-Int3sh). *h-Int3sh* RNA encodes a protein lacking the CBF1-binding region (RAM23) of the NOTCH4/Int3 intracellular domain. Although WAP-Int3sh transgenic mouse lines develop mammary tumors, the latency period is long (241); the authors thus speculate that the N4ICD-induced block of mammary gland development and tumorigenesis is the result of an increasing CBF1-dependent NOTCH4/Int3 signaling gradient.

Notch1 involvement in mammary tumorigenesis is being studied extensively. The first evidence that aberrant Notch1 signaling has a role in mammary tumorigenesis came from studies in the MMTV model, which attempted to identify genes that collaborate with *Neu/erbB2* in mammary tumorigenesis (Fig. 6B). An MMTV insertion in the *Notch1* locus in MMTV-*Neu* mammary tumors causes N1ICD expression (242). HC11 mouse mammary epithelial cells expressing N1ICD-encoding cDNA are transformed, form colonies in agar, but are unable to form tumors in nude mice, indicating that acquisition of malignant characteristics requires additional genetic events (242). Other studies showed that transgenic activation of N1ICD in mammary glands leads to development of lactation-dependent tumors that regress at weaning (207, 208); with time, these regressing neoplasms apparently become nonregressing adenocarcinomas (207). Recent evidence shows that *c-myc* is a direct transcriptional target of aberrant Notch1 signaling, with a fundamental role in MMTV-N1ICD-induced murine mammary tumorigenesis (243).

The study of *NUMB* expression in human breast cancer also supports NOTCH signaling pathway involvement in breast cancer. Pece *et al.* (244) showed that NUMB-mediated negative regulation of NOTCH signaling is lost in 50% of human mammary carcinomas. This is due to specific NUMB ubiquitination and proteasomal degradation (244) and indicates that enhanced NOTCH signaling activity occurs in these mammary carcinomas. NOTCH1 is also implicated as a downstream effector of oncogenic Ras in human mammary tumorigenesis (245). H-Ras is thought to have a central role early in human mammary carcinogenesis (246). The Ras signaling network and its mediators in human neoplastic cells thus have considerable importance for the development of antineoplastic agents for breast cancer (247). Weijzen *et al.* (245) suggested that N1ICD mediates the tumorigenic effects of oncogenic Ras. Analysis of several human breast cancer cases showed high NOTCH1 expression in all Ras-positive tumors. The authors also show that *NOTCH1* down-regulation in Ras-transformed human cells led to a marked decrease in cell proliferation, and inhibition of Ras signaling blocked N1ICD up-regulation, indicating that Ras acts upstream of NOTCH1. Nonetheless, they showed that N1ICD overexpression in the absence of oncogenic Ras was consistently unstable in human cell lines that do not bear Ras mutations. In addition, expression of oncogenic Ras leads to higher DELTA1 and PRESENILIN1 protein levels, which may increase NOTCH1 processing (245).

Another study showed that Notch4 also requires Ras signaling to exert its oncogenic effect (248), whereas Notch antagonism by transgenic expression of *Deltex* affects H-Ras-induced mammary tumorigenesis (207). Crossing MMTV/v-H-Ras and MMTV-DTX1 mice and monitoring the bitransgenic progeny for tumor development showed that Deltex expression greatly inhibited the oncogenic effects of H-Ras expression in mammary glands (207). The authors also showed that in MMTV-H-Ras mice (which eventually will develop mammary tumors), H-Ras induced *cyclin D1* up-regulation, even in a precancerous state. In contrast, the H-Ras/hDTX1 bitransgenic mice showed a marked reduction in the amount of cyclin D1 compared with MMTV-H-Ras mice. These results would be consistent with the finding that N1ICD expression activates *cyclin D1* gene transcription in cultured human cells (249) and that mice lacking *cyclin D1* were resistant to development of H-Ras- or Neu/Erb2-induced mammary tumors (250). Recent work also suggests a relationship between WNT and NOTCH pathways in breast tumorigenesis. Ectopic WNT-1 expression in human mammary epithelial cells increases WNT signaling and produces a tumorigenic state via a NOTCH-dependent mechanism (251). This suggests that deregulation of WNT signaling is an early event in NOTCH-dependent mammary epithelial transformation.

Other groups are using gene expression profile analysis to test the correlation of NOTCH pathway components, clinical outcome, and tumor clinicopathological parameters in human breast cancer. In tissue samples from breast cancer patients, Parr *et al.* (252) quantified NOTCH1 and NOTCH2 expression in association with clinical outcome and showed aberrant NOTCH1 and NOTCH2 levels in breast cancer tissues compared with normal breast tissue. Examination of the clinicopathological parameters for breast cancer patients indicated that high NOTCH1 levels may be associated with poor prognosis, whereas increased NOTCH2 levels correlated with greater probability of survival (252). NOTCH1 may thus have tumor-promoting functions, whereas NOTCH2 could have a tumor-suppressive role in human breast cancer, supporting the suppression of NOTCH-1 activity as a therapeutic strategy. Tissue microarray studies showed high *JAGGED1* and/or *NOTCH1* expression levels in human breast cancer, associated with poor overall survival compared with patients with low levels of these genes (253). Because high-level *JAGGED1* and *NOTCH1* coexpression showed a synergistic effect on overall survival, this type of breast tumor could also benefit from γ-secretase inhibitor-based therapy.

Recent evidence suggests an antiapoptotic role for the NOTCH pathway in human breast cancer. An increase in RBP-JK-dependent NOTCH signaling in the normal human breast cell line MCF 10A thus protects them from drug-induced apoptosis by abolishing the p53-mediated response (212). Other authors show that NOTCH1 signaling confers chemoresistance by inhibiting p53 activation of transcription and thus, apoptosis (254).

2. Gut cancer. As we have seen in *Section V.B*, Notch is essential for gut development and homeostasis. The Wnt cascade is also implicated in crypt progenitor cell maintenance; it is considered a major force behind the proliferative potential of intestinal adenomas and adenocarcinomas (179). In

their study of the role of Notch signaling in intestine development and cancer, van Es *et al.* (183) showed that the Wnt signaling pathway remained active in *RBPJk*$^{floxed/floxed}$; *P450-Cre* mutant mice. Their data indicated that Notch might function downstream of Wnt in the intestine and that both pathways may synergize as gatekeepers in the intestinal epithelium. This study also showed that spontaneous adenomas in *Apc*^{Min} (multiple intestine neoplasia) mutant mice have high *Hes-1* expression levels, similar to the intestinal crypts. The Notch pathway is thus activated in intestinal adenomas in these mice, as is the Wnt cascade (175). Notch pathway inhibition by γ-secretase inhibitors in *Apc*^{Min} mice induces goblet cell differentiation and reduces adenoma proliferation (183). Taken together, these data indicate that maintenance of undifferentiated, proliferative cells in crypts and adenomas requires activation of the Notch pathway in concert with the Wnt cascade. They also suggest that Notch signaling could provide an alternative target-drug strategy for intestinal neoplastic disease therapy (183). Thus, NOTCH and WNT inhibitors could be combined in an approach for colorectal neoplasia treatment (211).

C. Differential roles of NOTCH in two types of skin cancer: keratinocyte-derived carcinoma and melanomas

In human primary keratinocytes, increased NOTCH1 activity promotes commitment of self-renewing stem cells to transit-amplifying populations that continue to proliferate, although only for a limited time (255). In primary mouse keratinocytes, Notch acts as a tumor suppressor gene, promoting exit from the cell cycle and entry into differentiation (256, 257). Conditional ablation of *Notch1* in murine epidermis results in epidermal hyperplasia, skin carcinoma (basal cell carcinoma-like tumors), and facilitation of chemical-induced skin carcinogenesis. This is explained in part by reduced p21$^{Waf1/Cip1}$ protein levels (257), because *p21*$^{Waf1/Cip1-/-}$ mice are also more sensitive to chemical-induced carcinogenesis (258). Indeed, *Notch1* mutant keratinocytes are highly susceptible to *ras* oncogene malignant transformation; *Notch1* mutant cells infected with a retrovirus transducing the *ras* oncogene and injected sc into nude mice form aggressive squamous cell carcinoma (SCC), whereas wild-type cells do not (257). Loss of Notch1 activity may thus cooperate with *ras* oncogene transformation in keratinocyte tumor development. Accordingly, conditional transgenic mice with epidermal-restricted inhibition of Notch signaling (SM22-Cre^{+}; DNMAML1^{+} mice) have a hyperplastic epidermis and develop both spontaneous SCC and dysplastic precursor lesions (259); these mice also show enhanced accumulation of nuclear β-catenin and cyclin D1 in suprabasilar keratinocytes and in lesional cells from SCC, as also observed in human SCC (259).

The antioncogenic effect of Notch1 in murine skin appears to be mediated by p21$^{Waf1/Cip1}$ induction and by repression of Shh and Wnt signaling (Fig. 7) (45, 257, 260). Notch1 induces *p21*$^{Waf1/Cip1}$ expression directly by targeting NICD/RBPJK to the *p21*$^{Waf1/Cip1}$ promoter (256) and indirectly through the calcineurin/NFAT (nuclear factor of activated T cells) pathway (Fig. 7) (261). Mice with skin-specific *Notch1* deletion develop spontaneous basal cell carcinoma-like tumors; human basal cell carcinomas also show reduced *NOTCH1*, *NOTCH2*, and *JAGGED1* expression (260). In mice and in man, this tumor type is frequently associated with aberrant Shh signaling. Consistent with this, *Notch1* deletion in the mouse epidermis leads to aberrant expression of *Gli2*, a downstream component of the Shh pathway. Loss of NOTCH in human epidermis could also lead to aberrant SHH signaling, thus contributing to the development of basal cell carcinomas (Fig. 7).

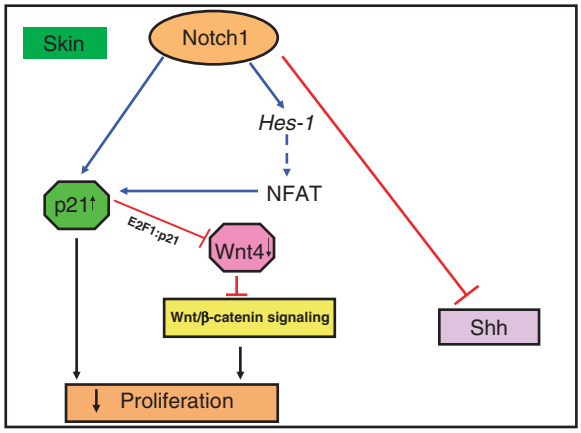

FIG. 7. Tumor suppressive Notch signaling pathway in the skin. Activation of Notch modulates signaling pathways that inhibit proliferation, preventing neoplastic transformation. The diagram shows functional and/or biochemical interactions (discussed in the text). NFAT, Nuclear factor of activated T cells.

Wnt signaling is suppressed by Notch1 activation and is elevated in keratinocytes and tumors as a consequence of loss of Notch1 function (257). Increased Wnt/β-catenin signaling is biologically important because it is associated with maintenance of keratinocytes in their stem cell compartment (262) and with keratinocyte-derived malignancies (263). Devgan *et al.* (45) recently established the mechanism for Wnt signaling suppression by Notch1 activation in keratinocytes, showing that Notch1 activation down-regulates this pathway by suppressing *Wnt-4* expression. p21$^{Waf1/Cip1}$ mediates this negative regulation; Notch1 activation increases levels of p21$^{Waf1/Cip1}$ protein, which subsequently associates with E2F1 transcription factors at the *Wnt4* promoter, down-regulating *Wnt4* expression (Fig. 7) (45). The function of Notch signaling in epidermis and in keratinocytes is thus to induce terminal differentiation processes, as well as to withdraw proliferating cells from the cell cycle. A long-term consequence of *Notch1* deletion in murine skin is the development of basal cell carcinoma-like tumors, suggesting that Notch exerts tumor-suppressive functions in this tissue.

Melanoma is a skin cancer that originates from melanocytes. In human skin, melanocytes are positioned at the epidermal-dermal junction and are interspersed among the basal keratinocytes (264). Melanocytes are tightly controlled by keratinocytes and maintain a nonproliferative status. Transformation to melanoma is the pathological consequence of disruption of cell control, which is environmentally initiated and is linked to specific genetic aberrations.

Recent data suggest that NOTCH signaling does not inhibit melanoma as it does in keratinocyte-derived carcinoma (260). *In vivo* and *in vitro* experiments show that NOTCH1 activation is insufficient to transform melanocytes but enables primary melanoma cells to gain metastatic capability (265). This oncogenic role of NOTCH is stage-specific. Thus, NOTCH signaling advances primary melanoma but has little effect on metastatic melanoma (265). A novel mechanism for NOTCH signaling in melanoma progression has been defined in which the oncogenic effect of NOTCH1 on primary melanoma cells is mediated by β-CATENIN, which is up-regulated after NOTCH1 activation. Inhibiting β-CATENIN expression reverses NOTCH1-enhanced tumor growth and metastasis. In primary melanoma cells, β-CATENIN thus acts as a downstream target of NOTCH1 signaling (265). Concurring with these findings, another report showed that NOTCH1 promotes primary melanoma progression by activating MAPK/PI3K-AKT survival pathways and up-regulating N-CADHERIN expression (266). NOTCH1 also down-regulates microtubule-associated protein 2 expression in primary melanoma cell lines (267). Abundant microtubule-associated protein 2 is often associated with longer disease-free survival rate in melanoma patients (268). Although previous work showed that NOTCH1 has a critical role in melanoma progression, the involvement of other NOTCH family members is not excluded. NICD are expressed in primary lesions of human malignant melanoma (reviewed in Ref. 209). *NOTCH2* and *HEY1* genes are also up-regulated in melanomas compared with melanocytes (269).

These data indicate that NOTCH signaling is implicated in primary melanoma progression and suggest that certain members of the NOTCH pathway are potential therapeutic targets in the development of new melanoma treatments. Qin *et al.* (270) showed that γ-secretase inhibitors can induce apoptosis in melanoma cell lines with markedly enhanced levels of activated NOTCH1 receptor. γ-Secretase inhibitor treatment triggers apoptosis in melanoma cells but only causes G_2/M growth arrest in melanocytes, without subsequent cell death (270); this treatment also induced the pro-apoptotic BH3-only protein NOXA in melanoma cells, but not in normal melanocytes (Ref. 270; reviewed in Ref. 271).

D. Notch in EMT and tumor progression

EMT is a fundamental process that implies loss of cell polarity and intercellular adhesion, as well as acquisition of a migratory phenotype, leading to mesenchymal cell formation from primitive epithelium. EMT takes place during critical phases of embryonic development such as gastrulation or the formation of the cardiac valve primordium (see *Section IV*). EMT also occurs during tumor progression when cells from a primary epithelial tumor change phenotype, become mesenchymal, and disseminate as single carcinoma cells, invading other organs and leading to tumor metastasis. EMT might also be involved in the dedifferentiation program that leads to malignant carcinoma (272, 273). The mechanisms underlying EMT are under intense study, and it is clear that developmental and metastatic EMT are governed by the same signaling pathways (272, 273).

Loss of expression of the epithelial adhesion protein E-CADHERIN indicates progression from an *in situ* to an invasive carcinoma (274). In most differentiated tumors, E-CADHERIN production is maintained, and there is an inverse correlation between E-CADHERIN levels and cancer grade or patient survival (274, 275). In accordance, a causal role has been established for E-cadherin loss in the transition from adenoma to carcinoma in mouse models (276). Transcription factors detected at EMT sites during embryonic development were recently identified as key transcriptional repressors of *E-cadherin* expression during tumor progression (277). One of these molecules is the zinc finger protein SNAIL (SNAI1), an *E-CADHERIN* repressor; carcinoma cell lines lacking E-CADHERIN produce considerable amounts of SNAI1, and E-CADHERIN-positive cell lines transfected with *SNAI1* undergo EMT (278, 279). We previously showed that Notch is critical for promotion of EMT during cardiac valve development via *Snai1* induction (see Ref. 140 and *Section IV*), and that N1ICD overexpression in immortalized porcine aortic endothelial (PAE) cells induces EMT, with induction of *Snail* and repression of *VE-cadherin* (140). The data indicated that Notch is upstream of Snai1 in the EMT process that generates the valve primordia and in the PAE system. Other authors showed that JAGGED1 activation of endogenous NOTCH receptors in human endothelial cells also promoted EMT as endothelial cells expressing activated NOTCH1 or NOTCH4 repressed VE-CADHERIN (280). NOTCH might thus be implicated in EMT during tumor progression, potentially via SNAI1 induction. After stable expression of N1ICD or an RBP-JK/VP16 fusion protein, the normal human breast cell line MCF 10A showed marked changes in cell shape associated with a reduction in E-CADHERIN protein levels (212). N1ICD induction in the human adenocarcinoma cell line MCF7 promotes migratory behavior associated with E-CADHERIN loss (our unpublished observations).

TGFβ is another well-known inducer of EMT during embryonic development and the later stages of tumor progression. During early tumor development, however, TGFβ inhibits the growth of most epithelial cell types, acting as a tumor suppressor (272). Another mechanism of NOTCH-induced tumor development and progression may involve modulation of the TGFβ signaling pathway. N1ICD was recently shown to suppress the growth-inhibitory effects of TGFβ by sequestering the transcriptional coactivator p300 from SMAD3, a downstream effector of TGFβ (281). Moreover, N4ICD binds directly to and inhibits SMAD2, SMAD3, and SMAD4 signaling activity, resulting in attenuated TGFβ signaling in MCF-7 breast cancer cells (282). Cells with activated NOTCH might thus be resistant to the growth inhibitory effects of TGFβ, promoting tumor development. Another recent study shows that RNA silencing of HEY-1 or JAGGED-1 or the chemical inactivation of NOTCH inhibits TGFβ-induced EMT in human keratinocytes (283). The ability of TGFβ to induce EMT may thus be Notch signaling-dependent. The authors also found that TGFβ induces HEY1 and JAGGED1 expression at the onset of EMT in epithelial cells from mammary gland, kidney tubules, and epidermis. The HEY1 expression profile is biphasic, consisting of immediate-early SMAD3-dependent, NOTCH-independent activation, followed by delayed JAGGED1/NOTCH-depen-

dent activation (283). In our PAE system, N1ICD did not appear to affect TGFβ signaling, suggesting that Notch signaling may be TGFβ-independent in this setting (140). Distinct pathways and/or transcription factors might thus participate in EMT via *E-cadherin* repression in distinct cell lines and tumor types.

A study based on immunohistochemical analysis of human prostate tumors suggests a role for the NOTCH pathway in prostate cancer metastasis (284). JAGGED1 expression is notably higher in metastatic prostate cancer than in localized prostate cancer or benign prostate tissues. This finding supports a model in which dysregulation of JAGGED1 protein levels plays a role in prostate cancer progression and metastasis and suggests that JAGGED1 may be a useful marker to distinguish slow-progressing from aggressive prostate cancers (284). Although genomic studies show that expression of NOTCH pathway components is increased in a variety of solid tumors (285), a systematic search comparing the expression status of NOTCH pathway elements and potential target genes in metastatic and nonmetastatic tumors remains to be carried out.

VII. Concluding Remarks

Notch is a key regulator of many developmental processes during embryonic and adult life. In the embryo, Notch activity via lateral inhibition or induction mechanisms generates molecular differences between adjacent cells. In the vertebrate CNS, Notch maintains the neural progenitor state and inhibits differentiation. In contrast, during gliogenesis Notch seems to have an instructive role, directly promoting the differentiation of different glial subtypes. More detailed analyses have also revealed that Notch regulates progenitor pool diversification and neuronal maturation. Emerging data suggest that Notch signaling has a role in neuronal function in the adult brain.

During somitogenesis, spatial and temporal changes in Notch signaling in the PSM are linked to segmental clock activity and are critical for segmental patterning. Oscillatory gene expression in the PSM is likely to involve Notch activation of *Hes* genes that negatively feed back on their own transcription, as well as the expression of ligands and/or Lfng. A major question is how the transition occurs between oscillatory Notch activity in the caudal PSM to fixed Notch activity in the anterior PSM.

During vascular development, Notch is dispensable for vasculogenesis but is essential to establish the arterial endothelial fate and for angiogenesis, suggesting that arterial/venous specification may be important for proper vascular remodeling. In addition, the vein phenotype is not achieved by default but rather requires the inhibition of Notch activity in presumptive vein territory. Studies in humans with vascular pathologies involving NOTCH suggest that this pathway may be required to maintain or stabilize the cells that contribute to blood vessels.

In the heart, Notch is not necessary to establish the primary cardiac fates *in vivo*, but its activity regulates crucial cell communication events between endocardium and myocardium during both the formation of the valve primordial and ventricular development and differentiation. Human studies indicate that NOTCH is critical for cardiac valve homeostasis, and more information about the implication of NOTCH in other human disorders involving the cardiovascular system is likely to come soon.

During pancreas development, Notch is required for timely cell lineage specification of both endocrine and exocrine pancreas.

In the gut, Notch and Wnt have a gatekeeper function for crypt progenitor cells, and Notch appears to influence binary fate decisions of cells that must choose between the secretory and adsorptive lineages. Deregulation of the Wnt pathway is central to the development of colorectal cancers in man. It remains to be shown whether Notch deregulation also follows the Wnt cascade in this respect.

In bone development, Notch may expand the HSC compartment and participate in commitment to the osteoblastic lineage, suggesting a potential therapeutic role for Notch in bone regeneration and osteoporosis.

Aberrant Notch activity is involved in hematological and solid tumor formation. The best-established role of Notch within the hematopoietic system is the ability to influence and/or specify cell fates of lymphoid progenitors. It has become clear that aberrant Notch signaling in humans due to activating mutations in the NOTCH1 receptor promotes T-ALL. Notch1 is thus an established oncogene in the hematopoietic system.

The evidence suggests that Notch signaling activity has an oncogenic role in human breast cancer, which may be facilitated by cooperation with other signaling pathways such as Neu/ErbB2 and WNT. Deregulated NUMB activity also supports NOTCH involvement in breast cancer, where it acts as a downstream effector of Ras.

This contrasts dramatically with Notch1 function in the skin, where Notch1 appears to induce terminal differentiation and also acts as a tumor suppressor. The observation that Notch can have such opposite functions in different self-renewing organs indicates that the outcome of Notch activation depends to a great extent on the cell context and factors such as the cell type in which Notch is activated, the specific growth factors in the microenvironment, and the level of Notch activity.

During embryonic development, the question is basically the same: How does Notch integrate its activity with other cell inputs to control specific developmental events? Another important matter is to understand how Notch activates distinct target genes according to cell type and time. Genomic studies will undoubtedly increase the spectrum of target genes and allow development of a systematic approach for understanding these different responses. Deregulated Notch signaling activity is involved in disease, but it remains to be determined whether modulation of such a pleiotropic pathway can be a therapeutic target for cancer and in regenerative medicine.

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