Review

Is there a role for Notch signalling in human breast cancer?
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Abstract

Aberrant Notch signalling has been observed in several human cancers, including acute T-cell lymphoblastic leukaemia and cervical cancer, and is strongly implicated in tumourigenesis. Unregulated Notch signalling in the mouse mammary gland leads to tumour formation. These results raise the possibility that Notch signalling might play a role in human breast cancer. There are currently few reports that address this question directly and this appears to be an area worthy of further investigation.

Keywords: breast cancer, Notch signalling

Introduction

Notch genes encode large transmembrane proteins that act as receptors for the Delta, Serrate, Lag-2 (DSL) family of ligands (Fig. 1a) [1]. There are four different Notch proteins in mammals and five known ligands: Delta-like 1, Delta-like 3 and Delta-like 4, and Jagged 1 and Jagged 2 [2–6]. Notch proteins are highly conserved, and they play crucial roles in cell fate decisions during the development of organisms as diverse as humans and sea urchins [7]. In addition, aberrant Notch signalling is associated with several human diseases. These include the autosomal dominant developmental disorder Alagille’s syndrome, the neural degenerative disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), and several cancers [8].

Experiments in Drosophila, Caenorhabditis elegans, and mammalian cell lines have provided a detailed model for DSL signalling via Notch receptors (Fig. 1b) (reviewed in [1,9]). The signal is initiated by the interaction of DSL ligands with the extracellular domain of Notch molecules on the surface of neighbouring cells. This leads to two proteolytic cleavages, one outside and one within the transmembrane domain, which release the Notch intracellular domain (NICD). The extracellular cleavage event is catalysed by an ADAM protease (a disintegrin and metalloprotease), while the intramembrane cleavage is mediated by a complex containing Presenilin and Nicastrin. The released NICD fragment then enters the nucleus, where it interacts with members of the CBF1, Suppressor of Hairless, Lag-2 (CSL) family of transcription factors. This interaction converts the CSL proteins from transcriptional repressors to transcriptional activators, and thus leads to elevated expression of specific genes. Several such target genes have been identified in mammals including Hes1 and Hes5, members of the Hairy and Enhancer of Split family of basic helix–loop–helix transcription factors [10,11].

While the mechanism of DSL Notch signalling via CSL factors has been extensively documented in a variety of biological settings, recent research indicates that Notch proteins can also signal via an alternative intracellular pathway. This pathway, which requires the cytoplasmic protein Deltex, appears to prevent cell differentiation. Although there are data that suggest a similar pathway may exist in mammals [12,13], it has so far been described only in Drosophila (Fig. 1c) (reviewed in [14]). With the excep-
tion of Deltex, the intracellular proteins required for this alternative pathway are currently unclear. It has been suggested, however, that signalling through this pathway may inhibit Jun N-terminal kinase signalling [15] or sequester the transcriptional coactivator CREB binding protein (CBP)/p300 [16]. Importantly, the domains of Notch required for this pathway are not the same as those needed for Notch signalling via CSL family members (Fig.1c) [17,18]. Recent experiments also suggest that Notch signalling via the Deltex-dependent pathway suppresses the expression of Wnt target genes [19,20], and that crosstalk between the two pathways inhibits Notch signalling via this pathway [18]. This crosstalk is likely to occur at the cell surface through interactions between either Notch and Wnt proteins themselves, or between Notch and Dishevelled (reviewed in [14]).

Perhaps the best understood biological role of Notch signalling is in the conserved developmental process of lateral inhibition (Fig.2a) [21,22]. For example, in both Drosophila and vertebrate embryogenesis, lateral inhibition is responsible for limiting the number of cells that will adopt a neural fate [23–27]. In both cases, neural development is initiated within small groups of cells. In each of these groups, however, only one or two cells will maintain their neural fate. As these cells differentiate, they produce an inhibitory signal that prevents their neighbours from also adopting the neural fate. It is the DSL/Notch/CSL signalling pathway that transduces this inhibitory signal. In addition, Notch signalling has been shown to play an important role in defining the two different cell fates that result from asymmetric cell divisions, and in inductive signalling events that cause cells to adopt a specialised cell fate at the boundary between two cell populations (reviewed in [22]) (Fig. 2b,c).

**Notch signalling in human cancer**

As with several other signalling pathways, aberrant Notch signalling has been observed in a number of human cancers, suggesting a possible role of Notch signalling in tumour formation. Moreover, the Notch1 gene was first identified in humans as a locus disrupted in a subset of T-cell acute lymphoblastic leukaemias by the (7;9)(q34;q34.3) chromosomal translocation characteristic of such tumours [28]. This rearrangement leads to expression of a Notch1 protein comprising the transmembrane and intracellular domains only. Similar Notch proteins have been shown to undergo spontaneous cleavage within the transmembrane domain in a ligand-independent manner, and hence to activate the Notch signalling pathway constitutively in experimental assays [1]. These data provide strong circumstantial evidence that unregulated Notch signalling contributes to tumour development.

This contribution to tumour development has been tested directly in two different mouse models in which a constitutively active Notch1 protein was expressed, either in T cells under the control of the lck promoter [29] or, more generally, in bone marrow cells [30]. The mice developed T-cell lymphomas in both cases. This observation strongly argues that uncontrolled Notch signalling is responsible for the development of T-cell acute lymphoblastic
leukaemia in human patients carrying the (7;9)(q34;q34.3) translocation. Activating Notch signalling by overexpressing the DSL ligand Delta-like 4 [31], or expressing truncated forms of Notch3 [32], also leads to T-cell lymphomas in mice. Moreover, only those forms of Notch1 protein that are able to activate a CSL-dependent reporter gene are able to induce T-cell leukaemia. This suggests that the leukaemias arise from excessive CSL-dependent signalling in T cells [33]. Similar conclusions about CSL-dependent signalling were reached in an analysis of epithelial cell transformation by Notch1 and E1A in rat kidney epithelial cells [34].

Activation of Notch signalling has also been observed in retrovirus-induced T-cell lymphomas in both mice and cats [35–39]. In the mouse models, retroviral proviruses are found inserted in the Notch1 locus just upstream of the exon encoding the transmembrane domain. This leads to expression of a novel cDNA that encodes the transmembrane and intracellular domains of Notch only. In cats, a subset of lymphomas that arise after feline leukaemia virus infection is caused by viruses that have acquired a Notch2 cDNA encoding a similar N-terminally truncated protein. In both cases, therefore, an activated Notch protein is expressed of the sort predicted to signal constitutively via CSL proteins.

In two of the aforementioned studies of T-cell lymphomas, a second class of retroviral insertions within the Notch1 gene has been described [38,39]. These insertions occur within the 3’ region of the coding sequence and result in truncated proteins lacking the region C-terminal to the cdc10/ankyrin repeats (see Fig.1). These deletions remove a PEST domain at the very C-terminus of Notch1 and are expected to increase the stability of the NICD fragment generated in response to DSL signalling. This

Roles of Notch signalling during development. (a) The best known role of Notch in development is in a process known as lateral inhibition. This is a conserved developmental mechanism by which a particular fate (navy blue cell) is adopted by one or two cells from a larger group that all have the potential to adopt the fate (mid-blue cells). As the selected cells adopt their fate (represented by the darkening of the shade of blue), they emit an inhibitory signal that prevents neighbouring cells from adopting the same fate (represented by lightening of the blue shade until it is white). The DSL/Notch/CSL signalling pathway transduces the lateral inhibition signal. (b) In addition to its role in lateral inhibition, CSL-dependent Notch signalling also plays an important role in defining the two cell fates that arise from an asymmetric cell division. Typically, the inheritance of the Numb protein (green) within one of the two daughter cells inhibits Notch signalling within that cell. As a consequence, one daughter cell receives a Notch signal while the other does not, causing the two cells to adopt different fates. (c) CSL-dependent Notch signalling has also been shown to play a role in the formation of a boundary (orange cells) between two different populations of cells (green and blue cells). In this situation, Notch signalling (arrows) is restricted to the cells at the interface between the two populations of cells, causing them to adopt a boundary cell fate.
may lead to a stronger and longer lasting CSL-dependent signal. On the other hand, Notch mutants have been isolated in Drosophila that encode similarly truncated proteins [18], and careful analysis indicates that their phenotype is due to increased Deltex-dependent signalling. The occurrence of this class of mutation in virus-induced lymphomas therefore suggests that unregulated Notch signalling via Deltex may also contribute to tumour development.

A role for unregulated Notch signalling has also been proposed in cervical cancer, largely because intense cytoplasmic and nuclear staining of Notch1 and Notch2 proteins has been observed in a majority of early stage cervical tumours [40]. Recent experiments with a human keratinocyte cell line, HaCaT, indicate that Notch signalling activates the phosphatidylinositol-3-kinase/protein kinase B signalling pathway and leads to increased resistance to anoikis [41]. In addition, HaCaT cells expressing the human papillomavirus (HPV) proteins E6 and E7 become transformed if Notch signalling is activated at the same time. Cooperation between HPV proteins and Notch signalling may thus lead to tumour initiation within the cervical epithelium. However, a survey of HPV-induced invasive cervical cancers and of established, malignant, HPV-positive cervical cancer cell lines found that Notch1 levels fall in late stage tumours [42]. This fall coincided with a rise in the expression of the E6 and E7 proteins, which is associated with neoplastic transformation. The results of this study indicate that Notch1 signalling represses E6 and E7 expression by suppressing the activity of the AP-1 transcription factor through upregulation of Fra-1 and suppression of c-Fos expression. Interestingly, this signalling is likely to occur through a CBF1-independent pathway as the repression of E6 and E7 expression is not affected by overexpressing a dominant-negative CBF1 protein. This suggests that a fall in Notch1 signalling may be necessary for progression of HPV-induced cervical tumours to later stages.

In cancers of many other tissues, the potential role of Notch signalling in tumour formation has not been extensively studied. However, overexpression of Notch pathway components has been observed in renal cell carcinomas [43], head and neck squamous cell carcinomas [44], endometrial cancer [45] and neuroblastomas [46]. In addition, activation of the Notch CSL signalling pathway appears to be mimicked during Epstein–Barr virus [47] and adenovirus infection [48]. Both viruses produce a protein that can interact with CSL proteins: Epstein–Barr virus nuclear antigen 2 in Epstein–Barr virus, and 13SE1A in adenovirus. Interaction of Epstein–Barr virus nuclear antigen 2 and 13SE1A with CSL proteins, like the effect of the Notch intracellular domain, converts the CSL proteins from transcriptional repressors to transcriptional activators. By analogy with the oncoproteins of DNA tumour viruses that subvert the key cell-cycle regulators RB and p53, these data suggest that activation of Notch signalling may be important for viral replication and may play an essential role in the immortalisation of cells by Epstein–Barr virus and adenovirus.

**What is the mechanism by which Notch signalling contributes to tumour formation?**

Altogether these data suggest a frequent role for Notch signalling in human cancer in much the same way as has recently become clear for Wnt signalling [49]. This raises the question of how unregulated Notch signalling might lead to tumour formation. Unlike components of the cell-cycle machinery or DNA repair proteins, aberrant Notch signalling does not obviously cause unregulated cell proliferation or genetic alterations associated with tumour progression. On the other hand, it can alter the developmental state of a cell and consequently maintain cells in a proliferative or undifferentiated fate.

Experiments examining the mechanism for T-cell lymphoma development in mice expressing an activated Notch3 protein under the control of the lck promoter strongly support this idea [32,50]. During normal thymocyte development, Notch3 is expressed at the transition between the CD4–CD8– double-negative and the CD4+CD8+ double-positive stages; its expression drops markedly after this. When Notch3 signalling is activated in transgenic animals, thymocytes fail to differentiate into CD4+CD8+ cells and remain as CD4–CD8– cells. This suggests that Notch3 signalling prevents thymocytes from making this transition in cell fate [32]. As a consequence of this maintenance of the CD4–CD8– cell fate, pre T-cell receptor signalling is sustained in the thymocytes of the Notch3 transgenic mice. Since pre T-cell receptor signalling causes both thymocyte survival and proliferation, constitutive signalling through this pathway may be responsible for lymphoma development. This notion has been confirmed by the failure of lymphomas to develop in Notch3 transgenic mice in which pre T-cell receptor signalling is abolished [50]. It is thus the change in cell fate caused by Notch3 signalling that leads ultimately to T-cell lymphoma.

Within the cervical epithelium, Notch signalling and the HPV E6 and E7 proteins are thought to cooperate to permit anchorage-independent growth [41], and thus to initiate tumour formation. Changes in cell fate may also play a role, however, as Notch signalling causes cells to differentiate during normal epithelial development [51,52]. HPV replicates within the differentiated cells and therefore its survival is dependent upon their continued differentiation [42]. The increased Notch signalling observed in early stage cervical tumours ensures continued differentiation of the tissue and may also lead to increased numbers of differentiated cells. Furthermore, Notch signalling will suppress E6 and E7 expression. This will limit viral replication
and prevent destructive lysis of host cells. An increase in Notch signalling will thus help to maintain a HPV infection.

If the mechanism of tumour formation in response to constitutive Notch signalling is indeed due to changes in cell fate decisions, there may be examples in which aberrant signalling causes cells to adopt a nonproliferative cell fate and, hence, does not lead to tumour formation. This possibility may explain recent experiments using small cell lung cancer cell lines [53]. In these experiments, the expression of active forms of Notch1 and Notch2 led to growth arrest and blocked the expression of Mash1, a transcription factor required for these cells to adopt their neuroendocrine fate.

A role for Notch in murine mammary gland development and tumourigenesis

The first indication that Notch signalling might play a role in normal and neoplastic development of the mammary gland came from the characterisation of a common insertion site for the mouse mammary tumour virus in Czech II mice [54]. In 20% of these tumours, the mouse mammary tumour virus provirus was inserted within the Notch4/int-3 locus. Since then, similar mouse mammary tumour virus insertions into Notch1 have been described [55]. At both loci, insertion of the provirus leads to expression of a Notch protein that consists of the transmembrane and intracellular domains only, again suggesting that unregulated Notch signalling leads to tumour formation.

To test this directly, transgenic mice were generated in which an activated Notch4 allele was expressed in the mammary gland [56–58]. In these animals, the mammary epithelium failed to branch or to penetrate the mammary fat pad, secretory lobules did not develop during pregnancy, and poorly differentiated mammary adenocarcinomas developed within 7 months. Furthermore, the epithelial cells in the virgin gland showed morphological features similar to cap cells, the proposed epithelial stem cells found at the ends of ducts. In addition, most cells failed to express casein during pregnancy, suggesting that the alveolar epithelial cells were unable to differentiate correctly.

The effects of Notch4 have also been studied in TAC-2 cells, a mammary cell system that can form ductal structures when grown in a collagen matrix. Expression of an activated Notch4 allele in these cells prevented ductal branching in response to hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β), and induced an invasive phenotype [59,60]. Taken together, these results suggest that Notch signalling may regulate ductal branching during normal mammary gland development and that unregulated Notch signalling prevents terminal differentiation of mammary epithelial cells. Aberrant Notch signalling may thus maintain the epithelial cells in a proliferative cell fate that leads to extensive ductal dysplasia and an associated risk of progression to carcinoma.

Notch signalling and human breast cancer

The involvement of Notch signalling in murine mammary tumourigenesis and its potential general role in human cancer clearly raise the possibility that aberrant Notch signalling might play a role in human breast cancer. Currently, however, there is very little direct evidence of this. The relevant published data are largely restricted to three expression studies. One of these studies examined Notch4 expression in several different tumour cell lines [61], while the others examined Notch1 and Notch4 expression in normal breast tissue and ductal carcinoma in situ [62], and Notch1 expression in breast cancers that overexpress H-ras [63].

In the first study, an mRNA transcript encoding the intracellular domain of Notch4 was detected in several tumour cell lines, including the breast cancer lines BT474 and Hs578T [61]. Importantly, expression of this activated form of Notch4 in the breast epithelial cell line MCF 10A enabled the cells to grow in soft agar, indicating that the transcript had transforming potential. Interestingly, the Notch4 mRNA isolated encodes an intracellular Notch4 protein that lacks the RAM23 domain required for the interaction with CBF1. This raises the possibility that the transformation of MCF 10A by this protein is due to Deltex-dependent Notch signalling. It should be noted, however, that Notch1 forms lacking the RAM23 domain can interact with CBF1 and activate a CBF1-dependent promoter in the presence of the transcriptional coactivator Mastermind [34]. Furthermore, a deletion analysis of the Notch1 protein indicated that only Notch1 proteins able to activate CSL signalling can transform the murine mammary epithelial cell line HC11 [55].

The survey of Notch1 and Notch4 expression in normal breast tissue and ductal carcinoma in situ indicated that expression of neither protein was detectable in normal tissue but that one or both proteins were expressed in the majority of ductal carcinoma in situ lesions [62]. In addition, Notch1 overexpression was observed in breast cancers that overexpress H-ras [63]. Finally, it has recently been shown that reducing the expression of the DSL ligand Jagged 2 in the breast cancer cell lines MCF7 and T47D induced caspase activity, suggesting that Notch signalling prevents apoptosis in these cells [64].

Conclusion

There is strong evidence to date to link unregulated Notch signalling with T-cell acute lymphoblastic leukaemia in both humans and mice, and with mammary tumourigenesis in mice. It is probable that Notch signalling can play a general role in tumour development in many different tissues by causing cells to adopt a proliferative cell fate. The initial results described suggest that unregulated Notch signalling may also play a role in human breast cancer. However, there is now a need to examine systematically whether aberrant Notch signalling occurs in breast cancer.
First, it will be important to determine whether rearrangements or mutations occur at Notch loci that lead to expression of activated Notch proteins in breast cancer. However, while Notch signalling is activated by such rearrangements in lymphomas and murine mammary tumours, it is entirely possible that other mechanisms of Notch signalling activation pertain to breast cancer. It may thus be necessary to look for either the aberrant activation of target genes of the Notch signalling pathway or for nuclear localisation of the NICD fragment. It may also be necessary to examine activation of the Notch signalling pathway at different stages in tumour progression, as this may vary as observed in cervical cancer.

Second, the effects of Notch signalling on mammary epithelial cell fate, proliferation and survival remain to be established, along with the target genes that regulate these effects.

Third, interactions between the Notch and Wnt signalling pathways during mammary gland development and tumourigenesis should be investigated. Constitutive signalling through each pathway has been linked to the development of tumours in the murine mammary gland, yet the interaction between the two pathways appears antagonistic.

Finally, it will also be important to determine which effects of Notch in mammary development or tumourigenesis are due to CSL-dependent signalling and which effects are due to Deltex-dependent mechanisms.

Competing interests
None declared.

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