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Current Perspective

Interplay between Numb and Notch in epithelial cancers: Role for dual oxidase maturation factor

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ABSTRACT

Numb and Notch signalling pathways are vitally important in cell fate and differentiation. The outcome of these signalling processes is determined by a delicate balance between opposing effects of Notch and Numb. Imbalance in Numb/Notch regulation was implicated in aberrant differentiation programme and epithelial cancer progression and metastasis. Recent identification of Numb-interacting protein (NIP), which is also known as dual oxidase maturation factor, and was shown to associate with Numb and DUOX and promote their translocation, sheds a new light on how Numb/Notch network may be coordinated in epithelial cancers. Here, a possible link between Numb, Notch and Dual oxidase maturation factor is examined.

1. Introduction

Metaplastic phenotype represents alterations in the expression of genotypic properties leading to depression of molecular mechanisms that contribute to the epithelial phenotype. Numerous studies have proven the capacity of epithelial cells to undergo metaplastic changes according to the mesenchymal growth pattern. This process is called epithelial to mesenchymal transition or transdifferentiation and is characterised by a loss of epithelial cell characteristics, including their polarity and cell–cell contacts, and acquisition of dysplastic morphology.[1] Many of polarity proteins behave as tumour suppressors and their loss leads to tumourigenic changes in epithelial cells.[2]

The mesenchymal conversion of epithelial tissue has been reported in normal embryogenesis.[3–5] In early development, primary epithelial–mesenchymal transition occurs in the primary epithelium and involves loss of epithelial phenotype or de-epithelialisation. Disaggregation of folding epithelium and reshaping cells for movement during gastrulation, neurulation and neural crest formation lead to formation of mesoderm.[4,6–8] Numb and Notch are among the major factors involved in the regulation of cell specification by controlling the balance between proliferation and differentiation in development and homeostasis.[9] Thus, during neurulation progress, apicobasal polarity is controlled by Numb, which is a major determinant in the asymmetric cell divisions.[10] The biological activity of Notch is antagonised by the cell fate determinant Numb.[11–14] During asymmetrical division, Notch segregates equally from the progenitor cells, whereas Numb segregates into only one of the daughter cells where it blocks the Notch signal.[13] Based on the roles of Numb and Notch in

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cell fate and differentiation, both Numb and Notch signalling pathways have been implicated in tumourigenesis. Cells expressing epithelial phenotype do not express Notch, whereas neural differentiation, associated with the loss of the polarised neuronal progenitor neuroepithelial phenotype, activates Notch.[15–17] Activation of Notch prevents differentiation by maintaining cell proliferation.[18]

2. Implication of Numb and Notch in epithelial cancer

The roles for Notch and Numb have been suggested in epithelial cancers including breast cancer.[19,20] The Numb-mediated control of Notch signalling is lost in a half of the breast carcinomas and salivary gland carcinomas as a result of downregulation or loss of Numb expression.[19,21] The Numb functions including the regulation of Notch signalling are defined by interaction with other proteins via N-terminal phosphotyrosine binding domain (PTB domain).[12,14,18] Numb may influence the Notch signalling by bringing multiple proteins to the Notch receptor. Furthermore, it has been shown that Numb is phosphorylated at Ser residues (Ser264), and that phosphorylation may play a role in regulation of the cell migration.[22] The oncogenic role of Notch derives from its ability to prevent differentiation and stimulate cell cycle progression. Silencing of Numb and an increase in Notch signalling in cancer cells lead to deregulated cell proliferation and metastatic-like phenotype.[20] Aberrantly high Notch signalling reduces $p27^{Kip1}$ leading to a more rapid cell cycle progression and inability of cells to terminally differentiate.[23–25] It was suggested that some tumour cells may derive from normal stem cells, or tumour cells may harbour cancer stem cells with impaired differentiation pattern.

Both oestrogen receptor (ER) positive MCF-7 and ER negative MDA-MB-231 cells, as well as non-transformed HMEC cells expressed Notch 1 and 2, whereas Notch 4 was highly expressed in highly metastatic MDA-MB-231 cells.[26–28] Similarly, Notch 4 was detected at very low level in normal mouse mammary gland epithelium, whereas its level was highly increased in carcinomas.[29] The expression of Notch 2 correlates with more differentiated tumours, whereas Notch 4 is associated with tumour aggressiveness, poor differentiation and poor survival outcome in human breast cancer.[30] Furthermore, the expression of Notch 1 and 2 isoforms correlates with the tumour grade. Thus, Notch 1 is abundantly expressed in grade 3 tumours with negative expression of oestrogen receptor, progesterone receptor and HER-2.[28] In MDA-MB231, Notch knockdown decreased cyclins A and B1, causing G(2) arrest, and death, whereas in oestrogen positive MCF-7 cells, Notch inhibition potentiated the effect of tamoxifen.[31] The activated Notch pathway in mammary epithelial cells probably blocks the cell differentiation by increasing the number of cells undergoing symmetrical cell division and leads to uncontrolled cell proliferation and impaired cell polarity. Since the effects of Notch are antagonised by Numb, the up-regulation of Notch signalling in epithelial breast cancer cells suggests that the expression of Numb responsible for regulation of Notch is suppressed or turned off.

3. Functional role of dual oxidase maturation factor, also known as Numb interacting protein (NIP), in epithelial cancers

Numb interacting proteins have been reported to affect the subcellular localisation of Numb suggesting that they may function as molecular anchors that localise Numb to the interactions sites with Notch.[32,33] DUOX maturation factor, DUOXA or NIP, was originally identified as a Numb-Interacting protein (NIP) in a screen for proteins that bind to Numb in *Drosophila*.[34] It has been demonstrated that NIP binds to Numb via the PTB domain and shows asymmetric localisation along with Numb in dividing neuroblasts. Based on these data, NIP was implicated in the polarised recruitment of Numb. Later, the same protein was identified by Grasberger et al. as a factor associated with dual oxidase enzymes, which require maturation factors to achieve their full enzymatic activity.[35] DUOX maturation factors (DUOXA1 and DUOXA2) recruit oxidases to the plasma membrane where they express superoxide generating activity.

Dual oxidases 1 and 2 (DUOX1 and DUOX2) are considered epithelial cell related NADPH oxidase isoforms. These oxidases are expressed in thyroid, epithelial cells in airway and in salivary glands, and gastrointestinal tract.[35–38] Both dual oxidases contain NADPH oxidase portion showing about 50% similarity to gp91phox (NOX2) and peroxidase-homology domains with high homology to peroxidases.[39,40] No mutations in DUOXA1 were identified as yet; however, a mutation of DUOXA2 was reported in a patient with congenital hypothyroidism.[41] However, in lung cancer cells and in most of the lung cancer specimen, both DUOX1 and DUOX2 are predominantly silenced by hypermethylation of both DUOX genes.[42] NADPH oxidases are among the primary oxygen sensors in the cell, and the H_2O_2 production rate positively correlates with pericellular PO_2.[43,44] Therefore, downregulation or silencing of DUOX in lung cancer specimen might be a response to hypoxic condition in the cell. The transcriptionally linked dual oxidases, maturation factors, DUOXA or NIP, were also downregulated in lung cancer cell lines and most of the lung cancer specimen. There is a strong similarity in the pattern of changes in DUOXA/NIP and Numb expression, as opposed to that of Notch in epithelial cancers. In almost half of the non-small cell lung cancers, up-regulation of Notch has been demonstrated,[45] which may also suggest that the expression of Numb was diminished or kept at very low level. Similarly, in epithelial breast cancer cells, the highest expressions of DUOX1 and DUOXA1/NIP1 were noted in well-differentiated MCF-7 (p53+/ER+) cancer cells, as compared to less differentiated MDA-MB-231 cells expressing inactive or mutated p53, in which the highest expression of Notch-4 was observed.[27,46]

The analysis of DUOXA/NIP showed that its expression was highly increased in embryo stage of Drosophila and in dividing Drosophila neuroblasts and corresponded to the expression of Numb.[34,47] Dual oxidases and their maturation factors were also linked to the formation of polarized epithelia.[48] Undifferentiated unpolarised foetal lung epithelial culture under differentiating condition showed formation of polarised epithelia associated with substantial up-regulation

of dual oxidase 1 and its maturation factor. The dual oxidases and their maturation factors localise to the apical plasma membrane of the airway epithelium.[49] Noticeably, DUOX and DUOXA show a similar distribution to Numb, which expresses at the apical membrane of non-skeletogenic mesoderm in the area vacated by Notch.[10,50] It has also been suggested that Numb forms a crescent on the apical membrane of mitotic progenitors during asymmetric division to inhibit Notch signalling.[10,51]

The DUOXA or NIP was implicated in the regulation of both Numb and dual oxidases. Such a diversity in the DUOXA/NIP response and the fact that Numb and NIP were implicated in regulation of cell polarity may also implicate dual oxidase in regulation of Notch or Numb activity. First of all, dual oxidase contains a NADPH oxidase portion and peroxidase-homology domains, which show high homology to peroxidases. The dual oxidase NADPH portion primarily catalyses the reduction of oxygen to form H_2O_2, while the peroxidase domain reduces H_2O_2 to water. Consequently, DUOX catalyses the reduction of both oxygen and hydrogen peroxide. Hydrogen peroxide signalling is involved in bidirectional regulation of phosphorylation resulting in stimulation and inhibition of phosphorylation.[52–54] Recently, Smith et al. reported that phosphorylation of Numb is required for its asymmetrical localisation in epithelial cells.[55] Based on these data, it is possible to speculate that DUOX may be involved in the regulation of Numb phosphorylation/dephosphorylation by maintaining redox regulation. Furthermore, these observations highlight the importance of DUOXA/NIP and suggest its important role in regulation of Numb/Notch signalling pathways.

4. Do tumour suppressors regulate Numb and Notch signalling?

It is crucial that tumour suppressors are involved in determining the cell fate and division, and the balance between asymmetric and symmetric divisions may be responsive to various factors including oncoproteins. It has been shown that p53 plays a role in differentiation by regulating proliferation of progenitor cells.[56] In epithelial breast cancer cells such as MCF-7 and MDA-MB-231 cells, silencing of Notch was associated with induction of apoptosis.[28,57] The key regulator of apoptosis is p53 tumour suppressor (TP53) that makes p53 a potential target of Notch signalling. Indeed, Notch signalling was reported to act upstream of p53, suppressing its function.[58,59] Up-regulation of Notch leads to chemoresistance by inhibiting p53 pathway through PI3K-Akt/PKB pathway.

The activation/inactivation of p53 signalling is regulated by a large number of post-translational modifications and multiple protein–protein interactions. Mdm2 oncoprotein, which is a well-known inhibitor of p53, interacts with the cell fate regulator Numb targeting it for degradation.[60,61] Similarly to the NumbNotch interaction, Numb binds to MDM2 via PTB domain.[61] Recently, Colaluca et al. showed that Numb interacts with MDM2 and p53 *in vivo* forming a trimeric complex between proteins.[62] Furthermore, formation of p53 complex with Numb regulates the stability of p53 by preventing ubiquitination and degradation of the tumour suppressor. Up-regulation of Numb expression not only results in stabilisation of p53 protein but also enhances the cellular level of p53. A loss of Numb correlates with a low steady-state level of p53 and is associated with high aggressiveness of breast cancer and poor prognosis.[62]

The above-mentioned data indicate that Numb-interacting proteins including DUOXA/NIP may also be responsive to p53 signalling. DUOXA1/NIP1 was detected in epithelial breast cancer cells with functional p53, whereas inactivation of p53 suppressed the level of DUOXA1/NIP1.[46] The restoration of p53 activity, which results in increased transcriptional activity, enhanced the cellular level of DUOX1/NIP1. Recently, Luxen et al. demonstrated that DUOXA1/NIP1 was downregulated in lung cancer cell lines and most of the lung cancer specimens.[42] However, the data reported by Luxen et al. do not support the idea that expression of DUOXA1 depends on the p53 status. The fact that investigations of p53 status and its role as prognostic factor have been the least conclusive in lung cancer, which is generally associated with extremely poor prognosis, may be an explanation for poor correlation between DUOXA1/NIP1 and p53 activity.

Collectively, these data suggest that Numb/Notch signalling pathways and DUOXA1/NIP are tightly linked to the p53 regulatory network. These observations lead to hypothesise that differential regulation of p53 may also regulate the cell fate by controlling asymmetric cell division.

5. The possible role for DUOX/NIP in interplay between Notch and Numb to maintain non-invasive phenotype

For most cancer types including breast cancer, acquiring of metastatic ability results in aberrant cell–extracellular matrix interactions and improper architecture of epithelia. Numb is required for maintaining the polarised structure of epithelium through regulation of junction components that maintain epithelial integrity.[51,63] The loss of Numb leads to improper adherent junctions and the loss of polarity.[64] Some studies suggest that Numb controls the integrity of the neural epithelium via interactions with E- and N-cadherins, and mutation of Numb leads to disruption of epithelium integrity.[64] On the contrary, activation of Notch destabilises normal epithelial morphogenesis and promotes invasion, which contributes significantly to tumourigenic potential.[65]

Asymmetric localisation of Numb is controlled by actin filaments.[66] In dissociated cell cultures, retinal neuroepithelial cells divide asymmetrically and distribute Numb to only one of the two daughter cells, suggesting that the dissociated cells can retain their polarity *in vitro*.[67] The polarised structure of the cells is due to asymmetrical spindle migration to the cortex controlled by actin, which ensures extrusion of small polar bodies in the two meiotic divisions.[68–70] Numb-interacting protein NIP or DUOXA has been implicated in recruitment of Numb to the plasma membrane during asymmetric division.[34] Preliminary MS analysis showed that DUOXA/NIP binds to actin and actin-binding proteins (unpublished data). These data suggest that DUOXA/NIP may control the asymmetric localisation of Numb during mitosis via filamentous actin and thus control the cell polarity. The primary epithelial tumour cell phenotype is associated with abrogation of the cell–cell contact and transition to mesenchymal pheno-

type.[71,72] Epithelial tumour cells show elongated morphology, continuous growth, suppression or complete loss of Numb and NIP/DUOXA, aberrant actin bundling, and a shift from epithelial cell markers to mesenchymal and endothelial cell marker expression.

Dedifferentiation and loss of polarity in epithelial cells lead to appearance of tumour cells with invasive phenotype. Members of tetraspanins or transmembrane 4 superfamily (TM4SF) are involved in tumour propagation such as cell activation, growth, adhesion and motility. They are often overexpressed in carcinoma cells causing the cellular phenotype changes that resemble epithelial–mesenchymal transition.[73] Stimulation of tetraspanin expression by Notch signals has been shown in the dorsal ectoderm during Xenopus gastrulation movements.[74] It has also been shown that tetraspanins play an important role in the regulation of cellular interactions required for gastrulation. CD9 or motility-related protein 1, which is the most common member of the family, was implicated in cell motility and metastasis.[75] Cells with high levels of CD9 exhibit an increased ability to proliferate and enhanced cell adhesiveness.[76] CD9 is highly expressed in microvascular endothelial cells and is located intracellularly and in the plasma membrane at the cell–cell contact sites.[77] In epithelial breast cancer cells, only highly metastatic MDA-MB-231 and MDA-MB-435 cells, but not non-metastatic MCF-7 cells, abundantly express surface CD9. Overexpression of DUOXA1/NIP1 resulted in downregulation of cell-surface CD9 expression.[46] It was already mentioned above that activation of Notch induces expression of tetraspanin whereas DUOXA/NIP reduces it.

These observations lead to the hypothesis that DUOXA/NIP is involved in the regulation of epithelial cancer either through its Numb or DUOX regulating ability, or via new pathways independent of Numb and DUOX. Furthermore, there are indications that dual oxidases might be involved in the regulation of the Numb/Notch signalling pathways. Moreover, regulation of DUOXA/NIP can lead to a new approach to cancer therapy.

Conflict of interest statement

None declared.

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