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Review

Dysregulation of Hedgehog, Wnt and Notch signalling pathways in breast cancer

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Summary. There has been a significant decrease in mortality from breast cancer in the last two decades. This has been attributed to the introduction of mammographic screening and to the development of specialised therapies, notably anti-estrogens such as tamoxifen in estrogen receptor (ER) positive tumours, and adjuvant chemotherapy. More recently monoclonal antibodies such as trastuzumab directed against Her2-overexpressing tumours show significant promise in improving outcome from this aggressive subtype. While there have been significant advances, a number of clinical challenges still remain, particularly development of targeted therapies for other forms of breast cancer lacking ER or Her2, such as the aggressive basal-like carcinomas. Identification of new therapeutic targets in poor prognosis groups will be critical to further improvements in breast cancer treatment. Proper functioning of the Hedgehog, Notch and Wnt signalling pathways is required for normal development during early life and these pathways also play a key role in regulation and maintenance of stem cells. Increasing evidence implicates dysregulation of these pathways in the development and progression of a number of malignancies, including breast cancer. This review presents the current evidence for aberrations in these pathways in breast cancer and proposes that the Hedgehog, Notch and Wnt signalling pathways may represent novel therapeutic targets.

Key words: Hedgehog, Wnt, Notch, Breast cancer

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Therapeutic challenges in breast cancer

Breast carcinoma is the leading female malignancy and cause of cancer death in developed countries. There has been a significant decline in breast cancer mortality over the past 20 years which has been attributed to screening, early detection and particularly the introduction of specialized treatment, most notably endocrine therapy such as Tamoxifen for oestrogen receptor positive cancers (Stylianou et al., 2006). Pathologists have been key team members in determining prognosis and suitability for targeted treatment of breast cancer patients. While traditional clinicopathological factors such as tumour size, grade, stage, ER, PR and Her2 status provide important information for patients and their treating clinicians, it is apparent these factors are not able to accurately predict and prognosticate for all patients. There are numerous instances of patients with "good" tumours who do poorly and vice versa and there is a pressing need to further refine our ability to predict the behaviour of cancers for individual patients in order to minimise their exposure to toxic therapies. This need to individualise treatment for patients has been assisted through gene expression profiling of breast cancer.

Perou et al. (2000) identified novel subgroups of breast cancer based on hierarchical clustering analysis of cDNA microarray gene expression from 76 breast cancers based on the similarities of expression of 456 genes. The authors of this study proposed a novel classification based on the molecular portraits of the tumours and grouped cancers into luminal epithelial-like, basal epithelial-like, HER2 amplified and normal breast-like phenotypes. These sub-groupings reflect the cells constituting breast ducts; luminal cells line the ducts while basal cells form the outer layer adjacent to

the basement membrane. The luminal epithelial-like tumours expressed luminal specific genes, such as ER· and were further sub-grouped into luminal sub-types A, B and C, based on the degree of ER expression. The basal-like subtype displayed high expression of markers characteristic of mammary duct basal myoepithelial cells such as CK 5, CK 17, and fatty acid binding protein. The HER2 amplified cancers expressed high levels of HER2 mRNA whereas the normal breast-like phenotype showed the highest expression of genes which are normally expressed by adipose tissue and non-epithelial cells. These groupings defined distinct prognostic groups, which have subsequently been validated in several larger cohorts (Sorlie et al., 2003).

Most notable is the sub-classification of tumours previously described as ER negative and which all displayed low to absent luminal specific genes. Of these ER-negative tumours, basal-like cancers and HER2 cancers were associated with a particularly poor prognosis (Perou et al., 2000; Sorlie et al., 2003).

Identification of new therapeutic targets in these poorer prognosis groups will be critical to further improvements in breast cancer treatment. The HER2 and basal-like subtypes have a significantly increased propensity to metastasise and even in ER positive tumours there has been relatively little improvement in survival in patients with metastatic breast cancer. Despite the apparent short-term response of distant tumour deposits to therapies, very few patients with metastatic disease survive long term. This resistance to therapy of many metastatic malignancies has been largely attributed to environmental selection of clones of tumour cells, which through mutation or epigenetic changes, acquire resistance to therapy. However this clonal evolution theory has been challenged more recently by the emergence of a greater understanding of the role of stem cells in normal tissue maintenance and in cancer.

Adult stem cells and the breast

Adult stem cells are slowly dividing, long lived cells, which reside in normal adult tissue and comprise a small proportion of the total cellular population. These cells are relatively undifferentiated and are thought to generate a fixed range of progeny which differentiate into mature functioning cells in a particular organ or tissue (Sell, 2004). A number of tissues, such as bone marrow, skin and the small intestine have relatively well defined stem cell populations, but stem cells in most other organs including the breast are, as yet, poorly defined. This is particularly due to the quiescent and relatively undifferentiated nature of adult stem cells which makes them difficult to isolate and characterise, particularly in non-haematopoietic systems, where the adherence of cells and admixture of a wide range of cell types in tissue samples poses significant technical challenges.

The breast undergoes cyclical growth and regression throughout much of women's lives. Puberty marks the onset of a rapid period of growth of the breast, with the expansion of blunt ended primary and secondary ducts which branch into a complex tree connecting with terminal ductal/lobular-alveolar units (TDLUs). From menarche until menopause with each menstrual cycle the breast shows significant fluctuation in growth. Pregnancy triggers a massive increase in the number of lobules and alveolar cells. This dramatic cycling of the breast has strongly suggested the existence of mammary stem cells (Williams and Daniel, 1983), with numerous experimental reports supporting this hypothesis (Deome et al., 1959; Kordon and Smith, 1998; Dontu et al., 2003; Shackleton et al., 2006; Stingl et al., 2006).

The cancer stem cell theory

A great deal of interest has focused on mutation or aberrant regulation in stem cells as a key factor in carcinogenesis. A link between stem cells and cancer is not a new concept; the famous pathologist Virchow commented on the morphological similarity between teratatocarcinoma and embryonic tissue over 150 years, while over 50 years ago Cohnheim and Durante proposed that cancer in adults develops from embryonic remnants (Sell, 2004). However, subsequently it was widely accepted that the initiation and progression of malignancy is a multi-step process, driven by numerous genetic changes that result in the transformation of normal cells into malignant cells. Environmental factors apply evolutionary pressure on the tumour, which leads to selection of clones with a greater capacity to survive, grow and metastasise – the clonal evolution theory of cancer development (Nowell, 1976). In this theory, any normal cell undergoing sufficient genetic alterations to result in its unregulated proliferation may become a tumour-initiating cell. The observed heterogeneity of many tumours is due to the development and expansion of numerous subclones. This clonal evolution theory is believed to explain the ultimate insensitivity of many tumours to chemotherapy, as clones with the ability to export the drug, or which lack key components of metabolic pathways targeted by the drug, are positively selected for their ability to evade death. One problem with this theory is that it requires the existence of a single long-lived cell able to accumulate numerous genetic "hits" in order to be transformed to a malignant cell. Genomic instability has been proposed as the mechanism by which this cell is able to undergo malignant transformation, characterised by insensitivity to growth inhibitory signals, evasion of apoptosis, almost unlimited capacity for proliferation and invasive growth. Although mutations in DNA repair pathways will promote the accumulation of further mutations, it is not clear how an incipient tumour cell accumulates the gross genetic changes observed in many malignancies, particularly as these changes are relatively uncommon in the normal and premalignant tissues of most tumours.

The identification of stem cells in a range of tissues

and organs and a greater understanding of their biology has again focused attention on the "stem cell theory of cancer" which proposes that malignancy arises from the transformation of a normal tissue stem cell. The cancer stem cell theory hypothesises that, analogous to stem cells in normal tissues, there are a small proportion of cells within tumours that have stem cell properties giving rise to progeny which may show heterogeneous patterns of differentiation and form the bulk of the tumour mass. The existence of cancer stem cells is thought to explain the failure of chemotherapy and other treatments to eradicate metastatic disease. Chemotherapeutic agents predominantly affect rapidly dividing cells, sparing relatively quiescent cancer stem cells, which often have high levels of ATP-binding cassette (ABC) transporters associated with multi-drug resistance in a wide variety of tumours (Sarkadi et al., 2004). Even the newer therapies which target growth factor pathways and angiogenesis are unlikely to directly affect stem cells.

Chemoresistance is explained by the protection of the cancer stem cells because of their slower rate of proliferation, as well as increased expression of ABC transporters. In this theory, chemotherapy will ultimately be ineffective if it fails to kill the cancer stem cell population, which can again repopulate the tumour with its more differentiated progeny and has significant implications for current anti-cancer therapy. Although there has been a significant improvement in the prognosis of early cancers in many organs, there has been very little change in the survival of patients with metastatic malignancy (Wicha et al., 2006). One possible explanation is that the cancer stem cell evades current therapies, and targeting these cells will be a critical step in making a therapeutic impact in advanced malignancy. Cancer stem cells have been identified in a range of solid tumours, including the breast, where Al-Hajj and colleagues (Al-Hajj et al., 2003) identified a subset of $CD44^+/CD24^-/Lin^-$ human tumour cells which reformed a tumour in a mouse xenograft with as few as 100 cells, while tens of thousands of tumour cells of other phenotypes were unable to form a tumour.

With the continuing identification of stem-like cells within increasing numbers of malignancies, it is apparent that a new approach to treatment is required, one which directly targets the cancer stem cells in association with more traditional approaches that affect tumour bulk. Central to this approach is the need to increase our understanding of the biology of normal stem cells and their malignant counterparts and the significance of dysregulation of three developmental pathways which play key roles in maintenance and self-renewal of normal and cancer stem cells. In the cancer stem cell theory, disruption of genetic pathways which regulate self-renewal of the normal stem cell is a key event in carcinogenesis. The genetic pathways reported to be important in regulating stem cell self-renewal are also pathways critical in embryonic and early development. It is possible that different stem cells or progeny give rise to the distinct genetic subtypes of breast cancer. The current data implicating three key pathways Hedgehog, Notch and Wnt signalling pathways in the pathogenesis of breast cancer and their association with particular breast cancer subtypes will be evaluated in this review.

The Hedgehog signalling pathway

The Hedgehog gene was first discovered by Nusslein-Volhard and Wieschaus in their Nobel prize-winning mutational analysis of segmental patterning in *Drosophila melanogaster* larvae. One of the mutations identified resulted in denticles, or spikes, covering the back of the larvae, with an appearance fancifully equated to a hedgehog (Nusslein-Volhard and Wieschaus, 1980). Three mammalian homologs of this gene were subsequently identified (McMahon et al., 2003); Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Epistatic studies in Drosophila identified other members of the Hedgehog signalling pathway, for many of which, such as patched (Ptch) and smoothened (Smo) there are also mammalian homologs (Wicking et al., 1999).

In mammalian Hedgehog signalling there are three Hh ligands which initiate the pathway. Shh is the most widely expressed of the ligands during development, and regulates development of the notochord, floorplate, developing mid- and forebrain, as well as the branchial arches, heart, and axial skeleton. Ihh stimulates endothelial cell formation in the yolk sac, and is involved in haematopoiesis and endochondral bone formation. Dhh plays a key role in male germline development (Cohen, 2003). Shh is the most widely studied and best characterised and is a 47 kD protein, which undergoes autocatalytic cleavage and dual lipid modification to form a 19kD active amino-terminal protein, which holds all known biological activity and a 25kD C-terminal form, of uncertain function and significance.

It seems that Hh signalling in adults is significantly reduced compared to the embryo and neonate, detected only in a few adult sites such as central nervous system stem cells (Machold et al., 2003) (which have detectable levels of Ptch and Gli) and the gut epithelium (van den Brink et al., 2004).

The Hh receptor Patched (Ptch) is a twelve-transmembrane protein that acts catalytically to inhibit the seven-transmembrane protein Smoothened (Smo), rendering the pathway inactive in the absence of Hh ligand. Binding of Hh ligand inactivates Ptch, de-repressing Smo resulting in positive Hh pathway signalling. When Smo is inactive, a multiprotein complex constitutively processes the Gli proteins to short, transcriptionally repressive forms. Activation of Smo decouples this complex from microtubule domains and leads to stabilization of full length, transactivating Gli proteins that initiate transcription of Hh target genes, including Ptch and Gli (Ingham and McMahon, 2001). A simplified diagram (Fig. 1) illustrates the mammalian Hh

pathway.

Hedgehog in cancer

Defects in Hedgehog signalling have long been known to be associated with human congenital disease with the loss of one copy of Shh resulting in holoprosencephaly, which at its most severe is a lethal condition characterised by fusion of the two forebrain hemispheres and defects of craniofacial development, such as cyclopia, (a single eye) (Roessler et al., 1996). However it has more recently been appreciated that aberrant Hedgehog signalling is associated with the development and progression of a wide range of human malignancies. This was first recognised with the discovery that a Ptch1 mutation was the cause of Gorlin's syndrome, a rare syndrome associated with a number of skeletal, skin and neural abnormalties as well as the development of multiple skin basal cell carcinomas (BCC), a significantly increased risk for medulloblastoma, an aggressive central nervous system malignancy and rhabdomyosarcoma, a sarcoma of muscle (Hahn et al., 1996). It has subsequently been shown that spontaneous Ptch1 mutations underlie the development of the majority of sporadic BCC (Unden et al., 1997), the commonest human malignancy with activating mutations of Smo accounting for approximately 10% of sporadic BCC (Xie et al., 1998). These malignancies caused by mutation in Hh pathway genes are referred to as being ligand-independent.

A second group of Hh pathway abnormalities called ligand-dependent were described first in lung (Watkins et al., 2003) and then in gastrointestinal tract and pancreatic carcinoma (Berman et al., 2003; Thayer et al., 2003), which show no mutation in Hh pathway genes but are characterised by upregulation of the expression of Hh ligand which is also thought to include *autocrine* and *paracrine* mechanisms of activation.

Hedgehog in breast carcinogenesis

There is emerging evidence that aberrant hedgehog signalling may be important in breast carcinogenesis. Some of the earliest evidence comes from studies in transgenic mice. Lewis et al. (1999) studying virgin mice with heterozygous disruption of Ptch1 found marked abnormalities in mammary ductal structures including hyperplasias and dysplasias similar to human breast lesions. More recently the same group (Moraes et al., 2007) studying mice with constitutive activation of human Smo under control of the MMTV promoter, found that mammary ductal cells showed increased proliferation, altered differentiation and developed ductal dysplasias.

Kubo and colleagues (Kubo et al., 2004) first reported an association between human breast cancer and aberrant hedgehog signalling, performing immunohistochemistry for components of the Hh signalling network in 52 invasive breast carcinomas. They found virtually all tumours expressed Shh, Ptch and Gli1 at levels significantly elevated compared to adjacent normal ducts. Gli nuclear staining was also associated with expression of estrogen receptor. This group also examined breast cancer cell lines and found that a significant proportion expressed Shh, Ptc and Gli and their growth could be inhibited by treatment with cyclopamine.

Although the mechanism of Hh activation in breast cancer is not yet clear, it does not seem that mutational activation is a common event. One group (Oro et al., 1997) reported a missense mutation, H133Y in Shh in 1 of 6 breast carcinomas, but Wicking and colleagues (Wicking et al., 1998) found no evidence for the H133Y missense mutation in Shh in 44 breast carcinomas and 8 breast cancer cells lines and Vorechovsky et al (Vorechovsky et al., 1999) found no evidence of this mutation in 84 breast cancers. Vorechovsky also found no evidence of mutations in Ptch in 45 breast carcinomas or of mutations in Smo in 48 breast carcinomas.

Although mutation may not be a common event triggering Hh pathway activation in breast cancer, it is possible that epigenetic events may play a role. A recent study (Wolf et al., 2007) demonstrated that treatment of two breast cancer cell lines with demethylating and histone deacetylating agents resulted in upregulation of the tumour suppressor Ptch. Re-expression of Ptch in MCF-7 cells resulted in inhibition of Hh activity. This group also demonstrated that in both DCIS and IDC

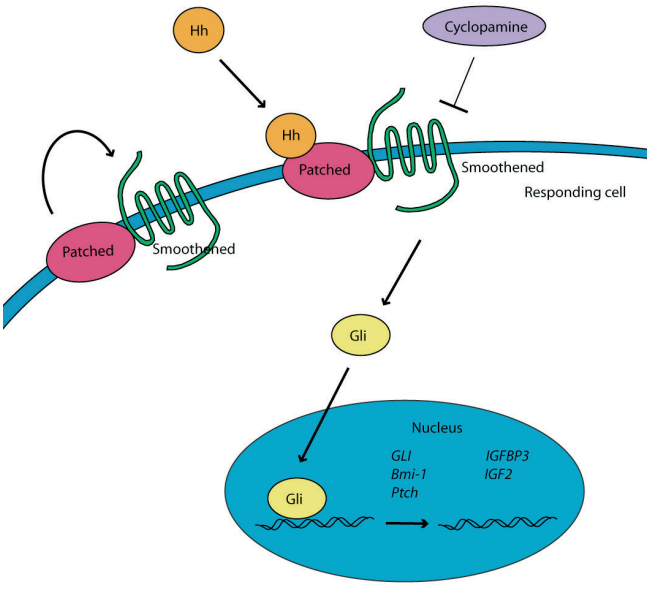

Fig. 1. The Hedgehog signalling pathway. In the absence of Hh ligand, Ptch represses Smo. Binding of hedgehog ligand (Shh) removes this repression allowing smoothened to activate the Gli family of transcription factors and other transcriptional targets. Cyclopamine is a specific inhibitor of Smo.

there was greater expression of Ptch by IHC in the associated normal tissues. They noted that Ptch was lost in 58% of invasive carcinomas and was associated with methylation of the promoter region.

Interestingly, Moraes et al. (2007) also characterized expression of Hh signalling components by IHC in a small human breast cancer cohort and found that Ptch1 was reduced in 50% of DCIS, in contrast to the work of Kubo et al. (2004), who found Ptch was overexpressed in 96% of invasive ductal carcinomas and Mukherjee et al. (2006) who reported increased Ptch1 expression in 33% of breast cancers. An association between a Ptch polymorphism, C3944T, and increased risk of breast cancer in patients using oral contraceptives was also reported in 2003, suggesting a possible role of the Hh pathway in hormone-induced breast cancers (Chang-Claude et al., 2003). It is also interesting that a positive association between ER expression and Ptch1 is reported (Wolf et al., 2007). Interpretation of these conflicting reports regarding Ptch1 expression is difficult but may be explained by several factors. First, activation of the Hh signalling pathway in a ligand-dependent fashion is associated with upregulation of both Ptch and Gli1, while ligand-independent activation is associated with loss of Ptch and upregulation of Gli1. It is possible that both mechanisms of pathway activation occur in breast cancer, suggested by the finding that although overall a positive association between Ptch1 mRNA with Gli1 was reported (Wolf et al., 2007) 40% of cases showed no correlation. In addition there is some concern in the scientific community regarding the reliability of commercially available antibodies for Ptch1, as well as variability arising as a result of different fixation of tissues and different immunohistochemical protocols.

However, there are many important unresolved issues to address in understanding the role of Hh signalling in the pathogenesis of breast cancer. There are few reports regarding the prognostic significance of aberrant Hh signalling pathway activation in breast cancer. Wolf and colleagues in a cohort of 104 patients with invasive ductal carcinoma reported that high expression of Ptch in invasive carcinoma was associated with a favourable prognosis (Wolf et al., 2007). In contrast, Bieche et al (Bieche et al., 2004) performed gene expression profiling of 36 patients with a particularly aggressive subtype of breast cancer, inflammatory breast cancer and found that a SHH, MYCN and EREG (epiregulin, a growth factor) expression signature was associated with a particularly poor prognosis even in this already poor prognosis tumour.

There is also no data regarding the association of dysregulation of the Hh signalling pathway with particular subtypes of breast cancer. However, there is evidence in other tissues suggesting that Hh may be important in the development of tumours of basal cells. Basal cell carcinoma of the skin was the first human malignancy associated with dysregulation of Hh signalling and this dysregulation mediates basal cell hyperplasia, leading to basal cell carcinoma in mice (Oro et al., 1997). Basal-like carcinomas of the breast are associated with expression of p63, a marker of myoepithelial/basal differentiation in a number of organs, including the breast and prostate, which has an important role in the maintenance of stem cells (Ribeiro-Silva et al., 2005). p63 overexpression can induce Hh pathway activity in a non-small cell carcinoma cell line (H1299) (Laurikkala et al., 2006), and Hedgehog signalling has also recently been implicated in the development of prostate basal cell hyperplasia and its tumourigenic progression (Chen et al., 2007). Another marker of basal differentitation, CK 14, commonly seen in basal-like breast cancer, is also associated with expression of Hh signalling pathway components in the prostate (Chen et al., 2007). Basal-like carcinomas have also recently been linked with the expression of SLUG, which plays an important role in mediating epithelial to mesenchymal transition, thought to be an important mechanism of metastasis (Storci et al., 2008). SLUG is a member of the snail family of transcriptional regulators, which are downstream targets of Hh signalling (Huber et al., 2005).

The Wnt signalling pathway

The Wnt signalling pathway was identified in the mouse as a tumour-promoting integration site of the mouse mammary tumour virus (MMTV) (named INT) and as a segment polarity gene in *Drosophila* (named *WINGLESS*). This gene was consequently named WNT (Rijsewijk et al., 1987). The Wnt signalling pathway is a highly complex signalling pathway with a central role in embryonic tissue patterning (Daniel et al., 2006). It is also involved in cell migration, maintenance of stem cells and progenitors in many tissues, epithelial-mesenchymal interactions and cell adhesion (Nelson and Nusse, 2004) and carcinogenesis (Polakis, 2000).

There are 19 WNT genes which encode proteins which bind to the frizzled (Fz) family of transmembrane receptors (Yang-Snyder et al., 1996). There are three Wnt pathways which are all activated through the frizzled family of receptors (Wong et al., 2003) but involve different Wnt proteins (Miller, 2002), activate different signal transduction pathways and utilise different effector complexes (Boutros et al., 1998). These pathways are the canonical pathway, planar cell polarity pathway and the Wnt/Ca^{2+} pathway. The canonical pathway, which involves the stabilization and translocation of ß-catenin to the nucleus and has been directly involved in a number of human malignancies, will be discussed in this review (Katoh and Katoh, 2007).

Wnt signalling mediates a variety of physiological functions including cell polarity, tissue patterning and control of cellular proliferation (Cadigan and Nusse, 1997). Aberrant canonical Wnt signalling has been implicated in many tumours, particularly breast and colon carcinoma. Wnt pathway deregulation in colon

cancer is often due to a single mutation, as loss of an Adenomatous Polyposis Coli (APC) allele causes the majority of colon carcinoma (Kinzler and Vogelstein, 1996). Other carcinomas in which aberrant expression of Wnt ligands and Fz receptors occurs include hepatocellular carcinoma (Shih et al., 2007), endometrial carcinoma (Bui et al., 1997) and head and neck squamous cell carcinomas (Rhee et al., 2002).

The canonical Wnt signalling pathway

Canonical Wnt proteins bind to the transmembrane receptors frizzled (Fz) (Yang-Snyder et al., 1996) and lipoprotein related protein 5 and 6 (LRP5/6) (Pinson et al., 2000; Tamai et al., 2000). This Wnt-LRP5/6-Fz complex binds to and activates the protein Dishevelled (Dvl) (Noordermeer et al., 1994). In the absence of Wnt ligands, Axin and APC form a cytoplasmic multi-protein complex with ß-catenin which initiates phosphorylation by Glycogen synthase kinase-3, (GSK-3,) and casein kinase-1α and subsequent degradation of ß-catenin (Fig. 2A) (Ikeda et al., 1998; Kishida et al., 1998; Nakamura et al., 1998; Sakanaka et al., 1998; Liu et al., 1999).

When Dvl is activated by canonical Wnt signalling, the ß-catenin-Axin-APC complex is disrupted and there is a resultant increase in free cytosolic ß-catenin (Mao et al., 2001; Tamai et al., 2004). ß-catenin then translocates to the nucleus and forms a transcription activation complex with T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors (Eastman and Grosschedl, 1999), Legless family docking proteins and co-activators (Fig. 2B) (Kramps et al., 2002; Katoh and Katoh, 2005). The key target genes of ß-catenin are FGF20 (Chamorro et al., 2005), DKK1 (Chamorro et al., 2005), WISP1 (Pennica et al., 1998), MYC (He et al., 1998) and CYCLIN-D1 (Tetsu and McCormick, 1999). ß-catenin driven transcription effects are only seen in canonical Wnt signalling and hence nuclear ß-catenin is considered to be a key readout of the activity of this pathway (Clevers, 2006).

The Wnt pathway is highly regulated. Negative regulators prevent ligand receptor binding (eg secreted frizzle-related proteins and Dickkopf proteins). There are further cytoplasmic antagonists of Wnt signalling such as APC, and nuclear factors which both promote or inhibit ß-catenin-LEF1 transcription (Hatsell et al., 2003). Canonical Wnt signalling is also negatively regulated by the Wnt/Ca^{2+} pathway (Ishitani et al., 2003).

Wnt signalling and breast carcinogenesis

Increasing evidence suggests a role for aberrant Wnt signalling in breast carcinogenesis. In vitro studies have

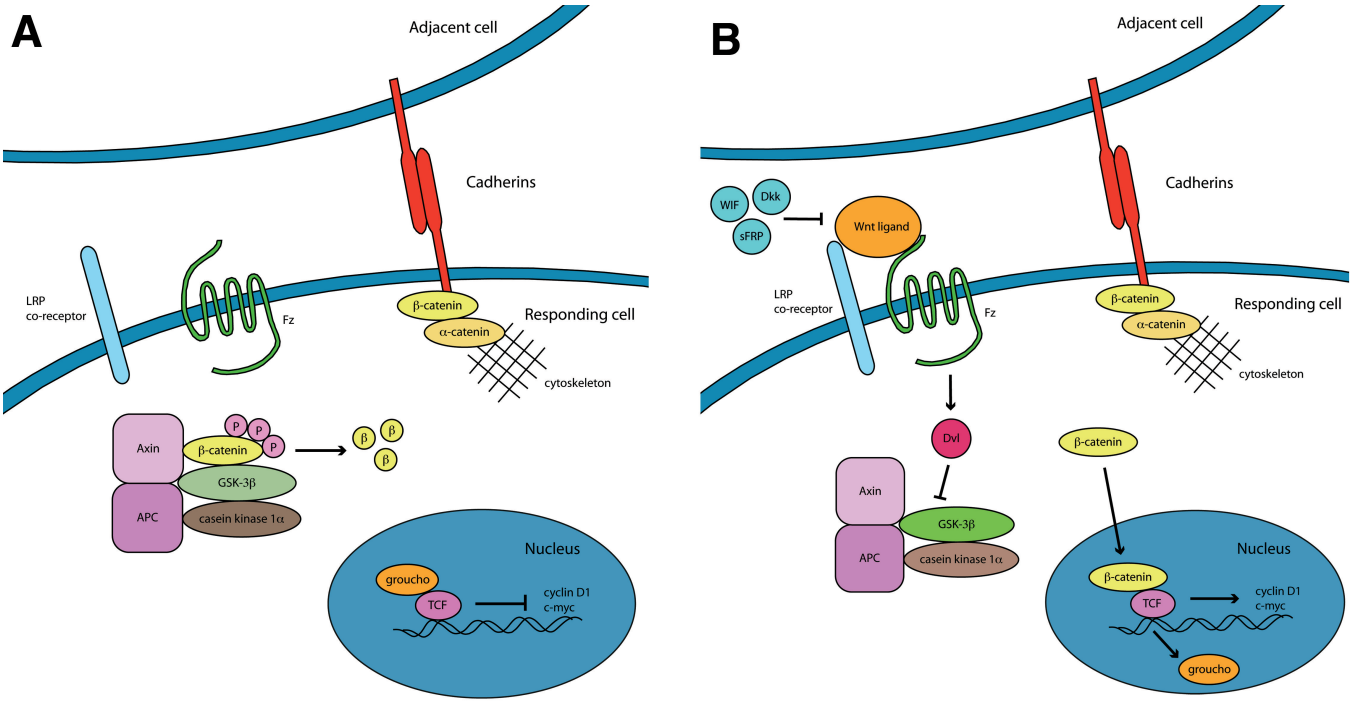

Fig. 2. The canonical Wnt signalling pathway. **A.** In the absence of Wnt ligands cytoplasmic ß-catenin forms a multi-protein complex with Axin, APC, GSK-3, and casein kinase 1α to cause phosphorylation and proteosomal degradation of ß-catenin. **B.** In the presence of Wnt ligands, Wnt, LRP5/6 and Fz activate Dvl which inhibits the activity of Axin, APC, GS3K and casein kinase 1α. This results in cytoplasmic accumulation of stabilized ß-catenin and its translocation to the nucleus. In the nucleus, ß-catenin binds with TCF to upregulate target genes, particularly CYCLIN-D1 and C-MYC.

shown the ability of Wnt ligands to transform breast cell lines in vitro to varying degrees. For example, in one study, *WNT1*, *WNT3A*, and *WNT7A* were highly transforming, while *WNT2*, *WNT5B*, and *WNT7B* induced transformation with a lower frequency and to a lesser degree (Wong et al., 1994). This initial study showed only partial transformation (Olson and Papkoff, 1994; Wong et al., 1994), but subsequent studies of *WNT1* transfected HMECS have shown complete transformation (Ayyanan et al., 2006). Similarly, human breast cancer cell lines show amplification of canonical Wnt genes whereas non-canonical Wnt genes were down-regulated (Benhaj et al., 2006).

Transgenic mouse models have shown that Wnt signalling is tumourigenic in both the luminal and basal cells of the mammary gland. *WNT1*, *WNT3* and *WNT10b*, which are not normally expressed in the mammary epithelium, all cause tumours in the luminal breast cells when transcriptionally activated by the MMTV promoter (Nusse and Varmus, 1982; Tsukamoto et al., 1988; Lane and Leder, 1997). MMTV *WNT1* and *WNT10b* mice rapidly develop precocious lobular-alveolar hyperplasia which progresses to focal carcinoma in the majority of animals by 12 months (Tsukamoto et al., 1988; Britto et al., 2000; Lane and Leder, 1997). These effects are thought to be due to canonical Wnt signalling as MMTV *ΔNß-CATENIN* mice with transcriptionally activated truncated forms of ß-catenin have a similar phenotype and the resultant tumours in these mice histologically resemble MMTV WNT adenocarcinomas (Imbert et al., 2001; Michaelson and Leder, 2001). Further, the oncogenic actions of Wnt1 may be independent of ovarian hormones (Tsukamoto et al., 1988; Lane and Leder, 1997; Bocchinfuso et al., 1999; Li et al., 2003) but the relationship between Wnt signalling and ovarian hormones is yet to be confirmed.

Abnormalities of various components of the Wnt signalling pathway have also been detected in human breast carcinomas. However there is as yet no evidence for activating mutations compared to other carcinomas such as colon carcinoma, where almost 100% of patients have ß-catenin mutations or deleted APC. Human breast carcinomas over-express a range of canonical WNT genes (Huguet et al., 1994; Dale et al., 1996; Bui et al., 1997; Katoh, 2001; Kirikoshi et al., 2001) and the protein DVL (Nagahata et al., 2003). There is also reduced expression of some Wnt-Fz binding regulators including *SECRETED FRIZZLED RECEPTOR 1* (Ugolini et al., 2001), *SECRETED FRIZZLED RECEPTOR 3* (Ugolini et al., 1999) and Wnt inhibitory factor 1 (Wissmann et al., 2003).

There are conflicting reports about the levels of stabilized ß-catenin in human breast tumours. In normal breast ducts and lobules, ß-catenin is primarily located at the plasma membrane bound to E-cadherin as part of cell adhesion complexes (Hatsell et al., 2003; Nelson and Nusse, 2004). In this form, ß-catenin is protected from degradation and does not contribute to Wnt signalling (Bankfalvi et al., 1999; Lin et al., 2000; Karayiannakis et al., 2001; Wong et al., 2002). The majority of studies report almost complete loss of membranous ß-catenin in invasive lobular carcinomas (ILCa), which is consistent with the loss of E-cadherin in these tumours (Bankfalvi et al., 1999; Karayiannakis et al., 2001). However, they do not describe ß-catenin seen in either the nucleus or membrane.

In IDCa, there is a generally observed loss of membranous ß-catenin with a translocation to the cytoplasm and nucleus (Lin et al., 2000). This increased cytoplasmic and nuclear expression of ß-catenin has been correlated with increased transcription of CYCLIN-D1 (Lin et al., 2000), increased expression of Wnt1 (Wong et al., 2002) and decreased APC levels (Wong et al., 2002) which suggests activation of the canonical pathway. Karayiannakis et al. (2001) and Bankfalvi et al. (1999) have also reported changing expression of ß-catenin with the progression of breast cancer. Normal, proliferative and pre-malignant lesions exhibited strong membranous expression of ß-catenin which was lost in high grade DCIS and Invasive ductal carcinoma (Bankfalvi et al., 1999; Karayiannakis et al., 2001) along with a parallel loss of E-cadherin (Bankfalvi et al., 1999). However, not all invasive carcinomas lose membranous expression of ß-catenin with approximately 30% of tumours retaining membranous expression (Lin et al., 2000; Ryo et al., 2001).

There are conflicting reports as to the clinicopathological implications of the altered localization of ß-catenin: while Lin et al. (2000) found that increased cytoplasmic or nuclear ß-catenin correlated with poor patient survival, subsequent studies by Jönsson et al (Jönsson et al., 2000) and Wong et al. (2002) report that increased cytoplasmic ß-catenin was associated with low stage, small, low proliferative rate disease. Loss of ß-catenin staining has also been associated with lymph node involvement but not with tumour stage, grade or clinical outcome (Bankfalvi et al., 1999; Karayiannakis et al., 2001). These inconclusive and conflicting results suggest that the sub-cellular localization of ß-catenin is important, but the clinical significance of aberrant ß-catenin expression and localization is as yet unknown.

As with Hedgehog signalling no association of Wnt signalling with specific breast cancer subtypes has been reported. However, there is some experimental evidence suggesting that aberrent Wnt/ß-catenin may be particularly associated with the basal-like subtype. Tumours and hyperplastic glands from MMTV WNT1, MMTV ΔNß-CATENIN and MMTV C-MYC mice express elevated levels of cytokeratin 6, a surrogate marker of undifferentiated, precursor cells as well as of basal-like carcinomas (Li et al., 2003), while Ayaanan and colleagues demonstrated that WNT1 transformed human mammary epithelial cells formed xenograft tumours which histologically strongly resembled human basal-like breast cancer (Ayyanan et al., 2006). Truncated ß-catenin is oncogenic when expressed solely

in the myoepithelial cells of the breast (Teuliere et al., 2005) and induces precocious ductal branching and undifferentiated basal hyperplasia which progresses to invasive carcinoma comprised almost entirely of basal cells.

The Notch signalling pathway

The Notch signalling pathway is a highly conserved developmental pathway first identified early in the 20th century through genetic mutation screens in *Drosophila*. Haploinsufficiency of the Notch gene resulted in 'notched' wing blades (Radtke and Raj, 2003). Notch signalling occurs through 2 pathways, CSL dependent signalling (CBP/RBP-jκ in vertebrates, Suppressor of Hairless in Drosophila and Lag-1 in *Caenorhabditis elegans*) and Deltex protein signalling. The majority of Notch signalling occurs through the CSL-dependent pathway (Brennan and Brown, 2003).

Notch signalling is critical in mammalian embryonic development, particularly in neurogenesis, angiogenesis, spermatogenesis and development of the heart and lymphoid system (Artavanis-Tsakonas et al., 1999). The physiological roles of Notch in embryogenesis centre around tissue patterning. Notch signalling causes lateral inhibition, whereby small difference between cells are amplified to cause cells to assume different cell fates; lineage specification where cells which have been committed to a particular tissue type are driven towards specific lineage pathways; and boundary formation, in which cells are organized by segregating cellular populations (Bray, 2006). Further, these effects of Notch signalling are dependent on signal strength, stage of development and the tissue on which it is acting.

Notch has also been recognised as an oncogene, particularly in acute T-cell lymphoblastic leukaemia, where more than 50% of patients have Notch1 mutations (Weng et al., 2004; Yehiely et al., 2006). Notch signalling has since been implicated in epithelial carcinomas, especially breast, colon, lung, cervical and skin carcinoma as well as central nervous system tumours (Radtke et al., 2006). Interestingly, Notch proteins also appear to have a tumour suppressive role in the epidermis (Nicolas et al., 2003; Proweller et al., 2006).

CSL Dependent Notch signalling

The Notch genes encode single-pass, transmembrane receptors which bind members of the Delta, Serrate, Lag-2 (DSL) family of transmembrane ligands. In mammals there are 4 Notch receptors (Notch1/TAN-1, Notch2, Notch3 and Notch4/Int3) and 5 DSL ligands (Delta-like1, Delta-like3, Delta-like4, Jagged1 and Jagged2) (Bray, 2006). DSL ligands bind to the epidermal growth factor repeats (EGFR) of the extracellular domain and cause receptor cleavage which is mediated by ADAM family proteases and γ-secretase (Callahan and Egan, 2004). Receptor cleavage releases the Notch intracellular domain (NICD) which translocates to the nucleus where it forms a tri-protein activation complex with the DNA-bound protein called CSL (Ong et al., 2006) and the transcription co-activator Mastermind. This complex displaces co-repressor proteins such as SMRT and SHARP (Bray, 2006) which are bound to CSL in the absence of NICD (Bray, 2006) and causes the assembly of active transcription complexes on the target promoters (Fig. 3) (Ong et al., 2006). Regulation of the Notch pathway occurs through receptor activation (Le Borgne et al., 2005), ligand activation (Chitnis, 2006), receptor endocytosis and ubiquitylation (Bray, 2006) and the cytoplasmic protein Numb (Chapman et al., 2006).

The best known target genes of Notch signalling are the Hairy enhancer of split (*HES*) (Iso et al., 2003) and Hes related repressor protein (HERP, also called *HEY*, *HESR*, *HRT*, *CHF*, *GRIDLOCK*) (Bray, 2006) families of transcription repressors. The Notch proteins have different promoter site selectivity and transcriptional actions. Notch1 is a potent activator of HES activity (Beatus et al., 2001). There are conflicting data about the selectivity of Notch3 and its activation of HES promoters (Fan et al., 2006). Other genes upregulated by Notch signalling are HER-2, CYCLIN-D1, NOTCH4 and NFÎB2 (Callahan and Egan, 2004).

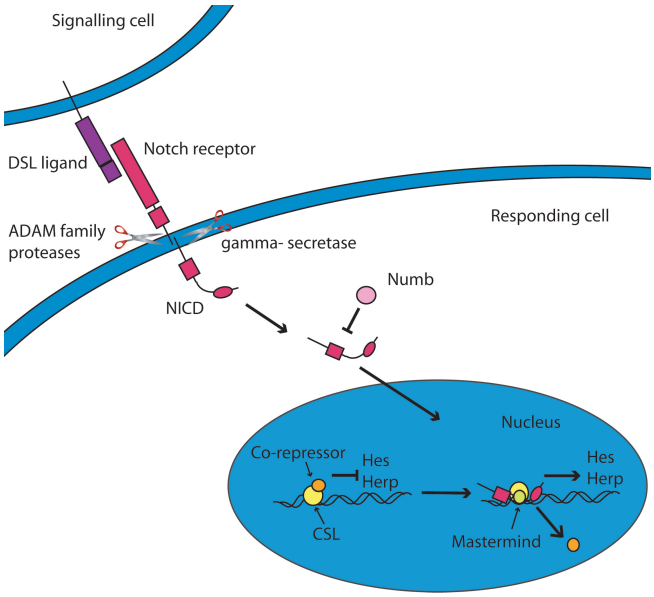

Fig. 3. The Notch Signalling Transduction Pathway, The Notch receptor is a transmembrane receptor. In the absence of Notch signalling transcription of Notch target genes is repressed as the transcription repressors inhibit the CSL DNA binding protein. DSL ligand binding initiates two proteosomal cleavages mediated by ADAM family proteases and γ-secretase. This liberates the NICD which then translocates to the nucleus were it binds to CSL and Mastermind to upregulate transcription of the HES and HER gene families.

Notch and breast carcinogenesis

There is accumulating evidence implicating Notch activity in breast carcinogenesis. Aberrant activation of the Notch signalling pathway in both mouse and human mammary cells causes the development of a malignant phenotype. These cells change shape and form disorganized, multi-layer cell masses in 3D culture and exhibit invasion of the underlying collagen matrix; an indicator of loss of cell adhesion molecules (Dievart et al., 1999; Imatani and Callahan, 2000; Soriano et al., 2000; Mungamuri et al., 2006; Stylianou et al., 2006). In human mammary epithelial cells (HMECs), this was specifically due to a loss of E-cadherin (Stylianou et al., 2006). Furthermore, over-expression of the Notch negative regulator Numb caused the cells to revert to a normal morphology (Stylianou et al., 2006). However, Ayyanan et al. (2006) demonstrated that this transformation was incomplete and Notch1 transformed cells were not able to form tumours when transplanted into the cleared mammary fat pads. These findings suggests that aberrant Notch signalling contributes to the malignant transformation of cells. Malignant transformation of cells exposed to aberrant Notch signalling also occurs *in vivo*. Transgenic mice with an activated Notch4 gene under mouse mammary tumour virus (MMTV) promoter regulation exhibited arrested mammary gland development, with poor ductal branching, and eventually developed poorly differentiated adenocarcinomas (Jhappan et al., 1992; Gallahan et al., 1996). Human lesions have also been examined for aberrant Notch signalling. Reedijk et al. (2005) examined two large cohorts of invasive breast carcinoma for Notch receptor and ligand status and found that Notch1 over-expression was related to increased tumour grade, with the highest expression of Notch1 in high grade tumours. High Notch1 expression was also associated with significantly poorer prognosis. Expression of the Notch1 intra cellular domain (N1ICD) in DCIS has also been linked to patient prognosis, as expression of the N1ICD predicted earlier recurrence in DCIS (Farnie et al., 2007). Reedijk also found that high levels of the Notch ligand Jagged1 was associated with poorer patient outcome. Notch2 expression has also been suggested to play a role in human breast carcinoma. An initial study found that Notch2 expression was highest in well differentiated tumours, correlating with a higher chance of survival (Parr et al., 2004) however these results were not replicated by Reedijk et al. (2005).

Work focussing on downstream Notch pathway components also suggests pathway activity in breast carcinogenesis. Transfection of HMECs with the N1ICD caused incomplete cellular transformation as these cells were not tumourigenic *in vivo* (Ayyanan et al., 2006), yet Stylianou found elevated N1ICD in both breast cancer cell lines and primary human breast tumours (Stylianou et al., 2006). Stylianou also found decreased levels of the Notch cytoplasmic inhibitor, Numb, in breast cancer cell lines and primary breast tumours. The loss of Numb-mediated Notch antagonism was specifically shown by Pece et al. (2004) who found this antagonist was lost in 50% of breast carcinomas due to ubiquitination and proteosomal degradation. The mechanism of this loss of Numb activity has been suggested to be through Mushashi1 which inhibits the production of Numb, and hence promotes Notch signalling in side populations (Clarke et al., 2005).

Increasing attention is being focussed on the putative downstream gene targets of Notch activity, such as HES and HEY genes. Stylianou et al (Stylianou et al., 2006) found elevated HEY1 in primary breast carcinomas and Leong et al (Leong et al., 2007) found up-regulation of HEY1, HEY2 and HEYL in HMECs transfected with the NICD as compared to controls. Further examination of two independent breast cancer microarray datasets confirmed positive correlations between expression of Jagged1, HEY1, HEY2 and HEYL (Leong et al., 2007). These results suggest intact Notch pathway signalling in breast cancer.

To date, little work has been done to link our new understanding of breast carcinoma molecular subtypes with the likely role of developmental pathways in breast carcinogenesis. In their analysis of human breast carcinomas, Reedijk et al observed that tumours with high levels of Jagged1, Notch1 and Notch3 were almost exclusively of the triple negative subtype (ER-negative, PR-negative, Her-2 negative (Reedijk et al., 2005)). Furthermore, 46% of basal-like tumours (as defined by expression of cytokeratin 5/17) expressed high levels of Jagged1. A subsequent study by Reedijk et al. (2007) in a large patient cohort found an association between high levels of JAGGED1 mRNA and the basal-like subtype which was not seen for high levels of Jagged1 protein. Further, the study by Dontu et al which demonstrated that Notch pathway activity drove early progenitor cells towards development of myoepithelial cells also indicates the need to examine the role of Notch in the basal-like subgroup (Dontu et al., 2004).

However, tumours with high levels of Jagged1 were associated with expression of ER and PR (Reedijk et al., 2005) suggesting that high Jagged1 expression is not exclusively limited to the basal-like subtype. Although these data are not entirely clear, they do suggest that aberrant Notch signalling may play a role in the basal-like and luminal subtypes. Further work is needed to address these conflicting reports.

There is also conflicting evidence linking Notch activity with the Her-2 positive subtype. Her-2 is an oncogene and high expression of Her-2 in breast carcinomas has been associated with poorer prognosis (Perou et al., 2000). Initial studies identified that the Her-2 gene binding protein was structurally identical to the Notch DNA binding protein CSL. Further, CSL activated by Notch intracellular domain resulted in Her-2 transcription (Chen et al., 1997). However, when Dievart et al examined mouse mammary breast carcinoma cells

with overactive Notch1 signalling, they did not observe over-expression of Her-2 (Dievart et al., 1999). Recent abstracts have linked Notch activity with Her-2 over-expression, suggesting both positive and negative interactions between these oncogenes (Sakanaki et al., 2005; Osipo et al., 2007). These conflicting findings indicate the need to clarify the role of Notch signalling in Her-2 positive subtype of breast carcinomas.

Conclusions

There is increasing evidence that dysregulation of developmental pathways plays an important role in the development and progression of breast cancer. Identification of novel targets will be a critical step in improving outcome from breast cancer. The data presented in this review provides extensive evidence that dysregulation of three critical developmental pathways, Hedgehog, Wnt and Notch, is a common event in breast cancer and furthermore that they each likely play a role in breast carcinogenesis and tumour progression. There is also some evidence to suggest that aberrations in each of these pathways may be associated with particular breast cancer subtypes; Hedgehog and Wnt signalling with the basal-like subtype and Notch possibly with Her-2 and basal-like subtypes. This is of significant clinical interest, as basal-like carcinomas lack specific targeted therapies. These pathways all contribute to stem cell regulation and self-renewal, thus targeting these pathways in cancer may specifically impact cancer stem cells, which in combination with more standard therapy is a concept worthy of further investigation. There is a need for research which specifically examines these associations in patient cohorts and for more mechanistic studies investigating the potential for inhibition of these pathways to influence tumour growth and progression.

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