Combining Notch inhibition with current therapies for breast cancer treatment

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Abstract: Over recent years, there has been an increasing interest in targeting Notch signalling for the treatment of breast cancer. This has stemmed from the realization that many Notch pathway components display altered expression in breast cancer, and that Notch signalling impacts on many of the cellular properties associated with tumour initiation and progression. Consequently, Notch pathway inhibitors are now entering the initial stages of clinical trials. However, there is a definite need to consider how best to use these inhibitors and therefore which treatment strategies are likely to yield the most promising results. In particular, recent studies suggest that the greatest success will come from combining Notch pathway inhibitors with current breast cancer therapies.

Keywords: Notch, breast cancer, Notch inhibitors, combination therapy

Aberrant activation of Notch signalling in breast cancer

Several groups have now reported altered expression of Notch pathway components in breast cancer [Farnie *et al.* 2007; Lee *et al.* 2008b; Li *et al.* 2010; Mittal *et al.* 2009; Pece *et al.* 2004; Reedijk *et al.* 2005; Reedijk *et al.* 2008; Rizzo *et al.* 2008; Stylianou *et al.* 2006; Yao *et al.* 2011; Zardawi *et al.* 2010]. These studies have primarily focused on the Notch receptors themselves and the ligands that activate the pathway, Jagged1&2 and Delta-like1,3&4, in invasive carcinoma. For example, elevated expression of Notch1 and Jagged1 has been reported and shown to associate with poor prognosis and basal breast cancer [Reedijk *et al.* 2005, 2008]. Similarly, downregulation of Numb, a negative regulator of the pathway, has been associated with high-grade breast cancers [Pece *et al.* 2004]. There is, however, no clear indication that upregulation of Notch pathway components associates with a particular breast cancer subtype, with different groups demonstrating an upregulation of Notch1 in oestrogen receptor positive (ER+ve) [Rizzo *et al.* 2008], human epidermal growth factor receptor 2 positive (Her2+ve) [Zardawi *et al.* 2010] and triple-negative/basal breast cancer [Lee *et al.* 2008b; Reedijk *et al.* 2008].

There are also several studies showing that Notch pathway components are upregulated in early

noninvasive stages of breast cancer, including usual ductal hyperplasia (UDH) and ductal carcinoma *in situ* (DCIS) [Farnie *et al.* 2007; Mittal *et al.* 2009; Zardawi *et al.* 2010]. These studies indicate that changes in the Notch pathway occur early in breast cancer progression and therefore changes in Notch signalling may play an important role in the initiation of the disease. Interestingly, one study also demonstrates that in DCIS, activation of the Notch pathway correlates with disease recurrence within 5 years, suggesting that pathway activation may associate with more aggressive and treatment resistant forms [Farnie *et al.* 2007].

Although all of this work strongly suggests that Notch signalling is activated in breast cancer, there are many ways by which signalling through the pathway is regulated [Bray, 2006]. For example, modification of Notch by the Fringe glycosyltransferases can both increase and decrease the ability of a Jagged or Delta-like ligand to activate Notch [Rana and Haltiwanger, 2011; Yang *et al.* 2005]. Therefore, overexpression of Notch ligands and receptors do not necessarily indicate that the pathway is activated. However, several studies have looked at the γ -secretase mediated cleavage of the Notch1 protein or the expression of Hes/Hey genes, both of which are indicative of Notch signalling [Farnie *et al.* 2007; Mittal *et al.* 2009;

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Stylianou *et al.* 2006]. Also several genetic rearrangements have been identified very recently in breast cancer samples and cell lines within the *Notch1* and *Notch2* genes that lead to the production of an active form the receptors [Robinson *et al.* 2011]. From these studies it is clear that Notch signalling is aberrantly activated in a broad range of breast cancer subtypes compared with normal breast tissue. However, it is interesting to note that all but one of the genetic rearrangements within the *Notch1* and *Notch2* genes were found within triple-negative breast cancer samples, suggesting that sustained Notch signalling is more important within this subtype [Robinson *et al.* 2011].

Notch signalling alters many cellular properties in breast cancer

Notch signalling has long been known to play a major role in cell fate specification during embryonic development [Andersson *et al.* 2011; Bolos *et al.* 2007]. However, it has become increasingly clear that Notch signalling affects many other cellular properties, including proliferation [Lee *et al.* 2008a; Rizzo *et al.* 2008], apoptosis [Meurette *et al.* 2009; Stylianou *et al.* 2006], epithelial-mesenchymal transition (EMT) [Leong *et al.* 2007], stem cell self-renewal [Farnie *et al.* 2007; Harrison *et al.* 2010] and angiogenesis [Zeng *et al.* 2005]. This is not really surprising, and simply reflects the multifaceted roles for Notch in development. The effect of Notch signalling, however, can vary between tissues or within an individual tissue between disease states. For example, Notch signalling has been shown to promote stem cell self-renewal in intestinal stem cells [Pellegrinet *et al.* 2011], whilst it promotes the differentiation of mammary gland [Bouras *et al.* 2008] and epidermal stem cells [Lowell *et al.* 2000]. Similarly, Notch signalling induces p21 expression and slows the cell cycle in normal keratinocytes and mammary epithelial cells ([Rangarajan *et al.* 2001] and Stylianou and Brennan, unpublished observation), but promotes proliferation in breast cancer cell lines by upregulating cyclin A and B expression [Rizzo *et al.* 2008]. This apparent inconsistency is almost certainly due to Notch signals being interpreted differently in different tissues resulting in the expression of distinct groups of target genes [Bernard *et al.* 2010]. This said, there are cases where Notch signalling consistently affects a particular cellular property. For example, Notch signalling promotes an EMT in mammary gland, kidney, endocardial and

epidermal cells by inducing the expression of members of the Snail family of transcription factors [Leong *et al.* 2007; Timmerman *et al.* 2004; Zavadil *et al.* 2004]. This difference in the regulation of cellular properties may reflect how directly Notch signalling controls these processes [Schwanbeck *et al.* 2011]. In the case of EMT, Notch signals directly regulate the expression of Snail and Slug and binding sites for CSL proteins have been identified in the promoters of both genes [Leong *et al.* 2007; Sahlgren *et al.* 2008]. On the other hand, the effects on cell cycle are likely to be indirect and therefore more influenced by the other signals and transcription factors present within the cell leading to different responses in different cell types.

Within breast cancer, Notch signalling has been shown to affect all of these cellular properties. It induces expression of cyclin A and cyclin B to promote cell division [Rizzo *et al.* 2008]. It activates Akt signalling to provide protection against a wide range of apoptotic stimuli, including commonly used chemotherapeutic agents [Meurette *et al.* 2009; Stylianou *et al.* 2006]. It induces the expression of Slug and therefore promotes an EMT in breast cancer cells [Leong *et al.* 2007]. It induces angiogenesis in the surrounding stroma [O'Neill *et al.* 2007]. It also promotes the self-renewal of breast cancer stem cells [Farnie *et al.* 2007; Harrison *et al.* 2010]. However, there does appear to be some receptor specificity as Notch4 is much more important than Notch1 for the renewal of breast cancer stem cells [Farnie *et al.* 2007; Harrison *et al.* 2010], whilst increased Notch2 signalling appears to prevent tumour formation [O'Neill *et al.* 2007]. Similarly, Notch3, but not Notch1, appears to be essential for the anti-apoptotic effect of Notch signalling in Her2-ve breast cancers [Yamaguchi *et al.* 2008]. This said, elevated Notch signalling clearly plays many roles in breast cancer making it a very attractive pathway to target [Arcaroli *et al.* 2011; Pannuti *et al.* 2010]. Importantly, it plays a role in both bulk tumour cells and cancer stem cells, ensuring that targeting the Notch pathway will not only reduce tumour bulk but should also reduce the likelihood of recurrence. It is also worth noting that Notch pathway inhibitors have effects with the surrounding tumour stroma that curtail tumour growth by inducing haemorrhage and hypoxia through their effects on endothelial cells [Cook *et al.* 2012; Dufraine *et al.* 2008; Rizzo *et al.* 2008].

Many ways to inhibit the Notch pathway

Notch signalling is activated by the interaction of Jagged1&2 and Delta-like1,3&4 transmembrane ligands with Notch receptors (1–4) on adjacent cells [Bray, 2006]. This leads to the endocytosis of ligands into the signal-sending cell, placing a physical strain on the bound Notch protein within the signal-receiving cell [Musse *et al.* 2012]. This causes a conformational change within the extracellular domain of the Notch proteins that exposes a cleavage site for the ADAM proteins Kuzbanian (ADAM10) and TACE (ADAM17) [van Tetering *et al.* 2009]. Cleavage at this site leads to endocytosis of the Notch extracellular domain into the signal-sending cell [Parks *et al.* 2000] and the generation of a signalling intermediate known as NEXT (Notch extracellular truncation) in the signal-receiving cell [Mumm *et al.* 2000]. NEXT is a substrate for γ -secretase, a protease that comprises Presenilin1 or 2, Nicastrin, APH-1 and Pen-2. γ -secretase cleaves NEXT twice within the transmembrane domain to release the Notch intracellular domain (NICD) that translocates to the nucleus [Okochi *et al.* 2002]. Within the nucleus, NICD interacts with members of the CSL family of DNA-binding proteins, displacing transcriptional corepressors associated with the CSL proteins and recruiting members of the Mastermind-like (MAML) family of transcriptional co-activators [Arnett *et al.* 2010]. The genes transcribed differ greatly between tissues, and are influenced by the number of receptors activated [Bernard *et al.* 2010; Mazzone *et al.* 2010; Schwanbeck *et al.* 2011]. However, members of the Hes/Hey family of transcriptional corepressors are frequently induced and can be seen as common targets [Fischer and Gessler, 2007].

Over recent years many approaches have been taken to inhibit the Notch pathway. The first Notch pathway inhibitors, used both experimentally and clinically, were the γ -secretase inhibitors which prevent cleavage of NEXT and therefore the release of NICD from the plasma membrane [Arcaroli *et al.* 2011; Pannuti *et al.* 2010; van Es *et al.* 2005]. More recently, monoclonal antibodies that recognise specific ligands (Dll4) [Hoey *et al.* 2009] or receptors (Notch1-3) [Aste-Amezaga *et al.* 2010; Aveo Pharmaceuticals; Li *et al.* 2008; Wu *et al.* 2010] have been developed. These either prevent ligand/receptor interaction or the conformational change within the extracellular domain required to expose the Kuzbanian or TACE cleavage site. Agents that target the RBPjk/NICD/MAML transcriptional activator complex have also been generated. These

agents, SAHM1 [Moellering *et al.* 2009] and TR4 [Epenetos *et al.* 2009], compete with MAML for binding to RBPjk/NICD. Consequently, if SAHM1 or TR4 are present in excess, the RBPjk/NICD/MAML complex will fail to form. As the transcription of Notch pathway target genes is dependent upon the transcription activation domain present within the MAML proteins, treatment with SAHM1 or TR4 will inhibit Notch signalling.

The γ -secretase inhibitors, SAHM1 and TR4 have the benefit that they can disrupt signalling by all four Notch receptors. However, there are also some advantages to targeting individual Notch pathway receptors and ligands, as pan-inhibition of Notch signalling with γ -secretase inhibitors has been associated with goblet cell dysplasia in the gut [Arcaroli *et al.* 2011; Pannuti *et al.* 2010; van Es *et al.* 2005; Wu *et al.* 2010]. This said, changes in the treatment regime for γ -secretase inhibitors have helped to reduce these problems. Also, both SAHM1 and TR4 seem to be well tolerated [Epenetos *et al.* 2009; Moellering *et al.* 2009]. It is also worth noting that long-term treatment with Notch pathway inhibitors is associated with development of vascular tumours [Liu *et al.* 2011; Yan *et al.* 2010].

Notch pathway inhibitors are unlikely to be useful as a monotherapy

Initial work with γ -secretase inhibitors suggested that their use alone may be sufficient to reduce the growth of xenograft tumours derived from breast cancer cell lines by inhibiting proliferation and inducing apoptosis [Lee *et al.* 2008a; Rizzo *et al.* 2008]. Subsequent work, however, suggests that cytotoxic effects seen in the xenograft tumours are due to off-target effects of the γ -secretase inhibitors used on proteasome function [Han *et al.* 2009]. In an elegant series of experiments, the Allalunis-Turner laboratory has shown that the γ -secretase inhibitor Z-Leu-Leu-Nle-CHO (GSI I) can inhibit proteosomal function unlike the γ -secretase inhibitors DAPT (GSI IX) and L685,458 (GSI X) [Han *et al.* 2009]. They also found that only Z-Leu-Leu-Nle-CHO treatment can induce apoptosis in breast cancer cell lines and that this effect can be rescued by restoring proteosomal function with the antioxidant edaravone. In keeping with this, others have shown that inhibiting Notch signalling genetically by overexpressing the pathway inhibitor Numb or a dominant negative form of MAML fails to induce apoptosis

in the absence of additional pro-apoptotic signals [Meurette *et al.* 2009; Stylianou *et al.* 2006].

Notch pathway inhibitors increase the effectiveness of current therapies

Despite the apparent ineffectiveness of Notch pathway inhibitors on their own, several lines of evidence indicate that they will be very useful in combination with the current therapies used for ER+ve, Her2+ve and triple-negative breast cancers. In fact, they may prove to be particularly useful for resensitizing therapy-resistant ER+ve [Haughian *et al.* 2011; Rizzo *et al.* 2008] and Her2+ve [Kondratyev *et al.* 2012; Osipo *et al.* 2008; Pandya *et al.* 2011] breast cancers to anti-oestrogen and anti-Her2 therapies, respectively.

In cell-line models of anti-oestrogen therapy resistance, Notch signalling is elevated compared with the parental therapy-sensitive cell lines [Haughian *et al.* 2011; Rizzo *et al.* 2008]. Furthermore, work with these models demonstrated that combining Notch pathway inhibitors with anti-oestrogen therapies enhances the response of therapy-sensitive cell lines, and resensitizes therapy-resistant cell lines to the anti-oestrogen therapy. Interestingly, a very recent paper indicates that the increase in Notch signalling observed in therapy-resistant ER+ve cell lines does not occur in all cells but rather within an ER-ve/cytokeratin 5 positive (CK5+ve) subpopulation of cells [Haughian *et al.* 2011]. Expression profiling indicates that these ER-ve/CK5+ve cells are similar to basal or triple-negative breast cancer cell lines and tissue samples. Given the recent suggestion that basal breast cancers are derived from progenitor cells [Lim *et al.* 2009; Prat and Perou, 2009], it is possible that these ER-ve/CK5+ve cells represent a stem or progenitor cell-like population within the cell lines [Liu and Wicha, 2010]. Interestingly, the presence of ER-ve/CK5+ve cells also correlates with the development of resistance to anti-oestrogen therapies, and their number is regulated by Notch signalling [Haughian *et al.* 2011]. Consequently, inhibiting Notch signalling reduces the number of ER-ve/CK5+ve cells and resensitizes therapy-resistant ER+ve cell lines to anti-oestrogen therapies.

Similarly in cell line models of anti-Her2 therapy resistance, Notch signalling is seen to increase in therapy-resistant cell lines [Kondratyev *et al.* 2012; Osipo *et al.* 2008; Pandya *et al.* 2011]. Again, inhibiting Notch signalling resensitizes

therapy-resistant cell lines to anti-Her2 therapy. Importantly, this has also been shown with *in vivo* xenograft tumours, indicating that this approach is likely to be successful therapeutically [Pandya *et al.* 2011]. Interestingly, a recent paper shows that combined GSI/anti-Her2 therapy can prevent recurrence of Her2 overexpressing xenografts [Pandya *et al.* 2011]. In these experiments, therapy-sensitive Her2 overexpressing xenograft tumours were established. These were then shrunk through treatment with anti-Her2 therapy or a combination of GSI and anti-Her2 therapy. When treatment was ceased, recurrence was seen following anti-Her2 therapy but not the combination therapy. Similarly, treatment of a mouse model of Her2+ve breast cancer with a GSI shrank the tumours and no recurrence was observed [Kondratyev *et al.* 2012]. As recurrence is thought to be driven by breast cancer stem cells [Liu and Wicha, 2010], this result, like those with the oestrogen-resistant cell lines, suggests that Notch pathway inhibitors target stem or progenitor-like cells within a tumour.

Furthermore, Notch pathway inhibitors have been shown to potentiate the effect of DNA-damaging chemotherapeutic agents, including doxorubicin, in a wide range of different breast cancer cell lines [Cho *et al.* 2011; Hoey *et al.* 2009; Meurette *et al.* 2009; Stylianou *et al.* 2006; Zang *et al.* 2010]. This suggests that Notch pathway inhibitors will prove useful for the treatment of triple-negative breast cancers which are treated primarily through surgery and chemotherapy. Interestingly many triple negative breast cancers initially respond well to chemotherapy but soon develop resistance [Carey *et al.* 2010]. It would be interesting to determine whether this resistance is due to activation of Notch signalling, and whether combined chemotherapy and Notch pathway inhibitors will improve treatment of this breast cancer subtype. Notch pathway inhibitors may also prove useful for the treatment of triple negative breast cancers because many of these tumours display enhanced EGFR signalling, and synergy has been seen when between anti-EGFR treatment and Compound E (GSI XXI) in this context [Dong *et al.* 2010].

Conclusion

Work over the last decade has shown that Notch signalling is aberrantly activated in breast cancer and that it regulates many of the cellular properties associated with transformation. This has led

to significant interest in the use of Notch pathway inhibitors for breast cancer treatment, especially as they are expected to have effects in bulk tumour cells, cancer stem cells and the surrounding tumour stroma. The question now is how best to use these inhibitors in clinical trials. Current pre-clinical work indicates that Notch pathway inhibitors are unlikely to be effective on their own, but that they should significantly increase the efficacy of current therapies. This said, there is still a need to identify patients that are likely to respond to Notch pathway inhibitors [He *et al.* 2011; Watters *et al.* 2009]. Finally, the preclinical studies have raised one possible note of caution to the pan-inhibition of Notch signalling for breast cancer treatment, as a couple of studies have indicated that Notch2 signalling may have a tumour-suppressive role in breast cancer [O'Neill *et al.* 2007; Parr *et al.* 2004].

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Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

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