Bringing androgens up a NOTCH in breast cancer

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Abstract

While it has been known for decades that androgen hormones influence normal breast development and breast carcinogenesis, the underlying mechanisms have only been recently elucidated. To date, most studies have focused on androgen action in breast cancer cell lines, yet these studies represent artificial systems that often do not faithfully replicate/recapitulate the cellular, molecular and hormonal environments of breast tumours in vivo. It is critical to have a better understanding of how androgens act in the normal mammary gland as well as in in vivo systems that maintain a relevant tumour microenvironment to gain insights into the role of androgens in the modulation of breast cancer development. This in turn will facilitate application of androgen-modulation therapy in breast cancer. This is particularly relevant as current clinical trials focus on inhibiting androgen action as breast cancer therapy but, depending on the steroid receptor profile of the tumour, certain individuals may be better served by selectively stimulating androgen action. Androgen receptor (AR) protein is primarily expressed by the hormone-sensing compartment of normal breast epithelium, commonly referred to as oestrogen receptor alpha (ERa (ESR1))-positive breast epithelial cells, which also express progesterone receptors (PRs) and prolactin receptors and exert powerful developmental influences on adjacent breast epithelial cells. Recent lineage-tracing studies, particularly those focussed on NOTCH signalling, and genetic analysis of cancer risk in the normal breast highlight how signalling via the hormone-sensing compartment can influence normal breast development and breast cancer susceptibility. This provides an impetus to focus on the relationship between androgens, AR and NOTCH signalling and the crosstalk between ERa and PR signalling in the hormone-sensing component of breast epithelium in order to unravel the mechanisms behind the ability of androgens to modulate breast cancer initiation and growth.

Introduction

Development of the adult mammary gland and its function are dependent upon oestrogen and progesterone acting via the oestrogen receptor alpha (ERa (ESR1)) and progesterone receptor (PR (PGR)) respectively. During reproductive cycles, ERa-positive mammary epithelial cells (MECs) respond to the rise in circulating oestrogen at the time of ovulation by up-regulating expression of the PR. If pregnancy occurs, the ERa- and PR-positive cells (hereon referred to as hormone-sensing MECs (HS-MECs)) detect increasing levels of circulating oestrogen and progesterone over the course of the pregnancy. The HS-MECs produce paracrine factors that stimulate the
proliferation and differentiation of adjacent MECs that lack ERα and PR in preparation for milk production during lactation. ERα expression is a hallmark of the majority (>70%) of early-stage breast cancers and therefore ERα is the main target of adjuvant hormone therapy in women with this disease. Aberrant ERα signalling can also contribute to continued tumour growth following resistance to anti-oestrogenic therapy (i.e. ER antagonists or aromatase inhibitor – Robinson et al. (2011), Toy et al. (2013), Haricharan et al. (2014), reviewed in Oesterreich & Davidson (2013)). Women with breast cancers who lack ERα or PR expression do not gain benefit from current forms of adjuvant hormone therapy. Some of these breast cancers are driven by the human epidermal growth factor receptor 2 (HER2/neu) oncogene and are eligible for treatment with anti-HER2 therapeutics. Currently, there are no available targeted therapies for the remaining tumours, which are classified as triple-negative (i.e. ERα-, PR- and HER2-negative) breast cancers (TNBC) and represent the most aggressive form of this disease. In addition to women with TNBC, women with other forms of breast cancer commonly acquire or have de novo resistance to anti-oestrogenic or anti-HER2 therapies necessitating the development of new target therapeutics for TNBC. In recent years, targeting the androgen receptor (AR) has entered the clinical trial arena for treating women with TNBC or ERα-dependent disease that has progressed following failure of standard of care therapeutic regimes.

Although women produce less androgen hormone than men and metabolize a higher proportion of their androgen to oestrogen, direct androgen activity mediated via the AR influences development of the female phenotype and is important for the healthy functioning of multiple organs in women, including the ovaries (Lebbe & Woodruff 2013, O’Reilly et al. 2014, Sen et al. 2014), uterus (Wood et al. 2009, Cloke & Christian 2012), bone (Watts et al. 1995), muscle (Smith et al. 2014), adipose tissue (Barbosa-Desongles et al. 2013, Chazenbalk et al. 2013), and brain (Shifren 2002, Lobo et al. 2003, Ryan et al. 2009, Zarrouf et al. 2009). However, less is known about the mechanisms of AR signalling in female tissues compared with either AR signalling in male target tissues (e.g. prostate and testes) or ERα and PR signalling in the breast. This is somewhat remarkable, particularly with respect to the breast, as it has long been known that androgen hormones inhibit pubertal and post-pubertal breast growth, and that treatment with androgen can cause regression of breast tumours (Lamar & Rezek 1958, Thomas et al. 1962, Tormey et al. 1983, Forsbach et al. 2000, Rose et al. 2000). Indeed, AR is the most frequently expressed sex steroid receptor in both primary and secondary (metastatic) breast cancers, making it a highly prevalent therapeutic target (McNamara et al. 2013, Santagata et al. 2014, Tsang et al. 2014). We have recently reviewed the role of AR signalling in normal and malignant breast tissues (Hickey et al. 2012), and McNamara et al. (2013) provide an update on that theme in this issue including a section on intracrine androgen metabolism in the breast and the implication of this for AR signalling in breast cancer. In this review, we focus on new knowledge about AR and NOTCH signalling in the normal breast and the potential for integration with NOTCH signalling in the context of breast tumourigenesis.

**Where and how do androgens act in the mammary epithelium?**

**Mammary epithelial organisation and hierarchy**

The mammary epithelium is composed of three principal cell lineages: myoepithelial (basal) MECs and two types of luminal MECs (Figs 1 and 2). Myoepithelial cells have contractile properties and form a network around the mammary ducts and lobules that assists in ejecting milk from the glands at lactation. Compelling evidence demonstrated that mammary stem cells, which can individually give rise to an entire mammary gland, reside within this basal MEC compartment (Shackleton et al. 2006). The luminal cells are comprised ERα-negative alveolar MECs that produce milk when terminally differentiated, and ductal HS-MECs that express ERα, PR and prolactin receptors (PRLR). An additional population of hormone receptor-negative ductal cells exists, but it is currently unclear how such cells are distinct from HS-MECs. The ERα-positive HS-MECs serve as a hub of hormonal information, integrating reproductive cues and translating them into paracrine instructions for adjacent cells that result in growth and differentiation of the mammary lobules and acini through oestrous/menstrual cycles and at pregnancy.

**AR expression in mammary epithelium**

The highest levels of AR mRNA in the mammary epithelium of mice and humans have been found within the HS-MEC-enriched population isolated by fluorescence-activated cell sorting (FACS; Lim et al. 2010, Kannan et al. 2013; Table 1). Another mouse study reported a higher level of Ar mRNA within the FACS-sorted basal MEC population compared with the HS-MEC-enriched population.
population (Kendrick et al. 2008). Despite this discrepancy, these studies found that ERα-negative alveolar MECs consistently had the lowest levels of AR transcriptional activity. These patterns of AR mRNA expression are consistent with that of the AR protein, which has been investigated in mouse, human and non-human primate tissues. In monkey breast tissue, AR protein is expressed exclusively in the luminal layer and the pattern of AR expression did not alter with cyclic changes in circulating ovarian hormones (Kawaguchi et al. 2009). We have observed that AR positivity in histologically normal human breast tissue occurs predominantly in luminal, not basal, MECs (Hickey et al. 2012). In addition, other cell types (fibroblasts and adipose cells) within breast tissue often had strong AR immunoreactivity. Immunoreactive AR was found in the luminal layer of mammary ducts and acini and in a subset of basal MECs, in reduction mammoplasty tissue obtained from pre-menopausal women (Li et al. 2010).

A recent detailed analysis of multiple common and specific MEC antigens in pre-menopausal human breast tissue from reduction mammoplasties has found that all AR-expressing cells belonged to the luminal lineage based on co-localisation with luminal-specific cytokeratins (Santagata et al. 2014). It is possible though that AR expression in basal MECs occurs within very specific structures (e.g. inter-lobular ducts – Lim et al. (2010)) or at a certain developmental stage (e.g. over the oestrous cycle) and therefore it is feasible that both basal and luminal MEC lineages can directly respond to androgens.

The vast majority of ERα- and PR-positive breast cancers also express AR (Ogawa et al. 2008, Park et al. 2011, Alshenawy 2012, Secreto et al. 2012, Santagata et al. 2014, Sultana et al. 2014, Tsang et al. 2014), and AR expression is observed in up to 50% of ERα- and/or PR-negative breast cancers (Gucalp et al. (2013), Tsang et al. (2014); reviewed in Hickey et al. (2012), McNamara et al. (2013) and Nahleh (2008)), making AR an attractive target for the treatment of multiple types of breast cancer. However, it is currently unclear how to stratify individual breast cancers based on whether they will respond favourably to androgen treatment or not. In different breast cancer cell lines, modulation of androgen signalling can have growth-inhibitory or growth-promoting effects (Birrell et al. 1995, Ortmann et al. 2002, Greeve et al. 2004, Lyu et al. 2014, reviewed in Fioretti et al. (2014)), and this extends to the applicability of AR expression as a prognostic marker in specific subtypes of breast cancer (Kuenen-Boumeester et al. 1996, Agoff et al. 2003, Peters et al. 2009, Kraus et al. 2010, Gasparini et al. 2014).

**Figure 1**
Organisation of the mouse mammary epithelium. The mammary gland is composed of a ductal tree composed of blind-ended lobules (A). The glandular lining is composed of a bi-layered epithelium comprising basal and luminal layers (B and C). The luminal layer is composed of hormone-sensing mammary epithelial cells (HS-MECs), which serve as hubs for reproductive signals, as they possess receptors for all reproductive steroid hormones (including the androgen receptor (AR), as well as milk-producing alveolar MECs).
Such conflicting reports/data raise concerns for the clinical efficacy of androgen modulation in breast cancer. To address this, we propose that studies using more clinically relevant models of breast cancer are essential for better understanding the nature of androgen action in the promotion and inhibition of breast cancer initiation, growth and survival. This is particularly relevant to current clinical trials that aim to inhibit AR signalling in ERα-negative and ERα-positive breast cancers (Gucalp et al. 2013; NCT00468715; NCT01990209; NCT02000375 and NCT02007512).

Role of AR in normal mammary gland function

Signalling via the ERα and PR is critical for the growth of mammary epithelium during puberty. These two sex steroid receptors are also required for side branching of the ductal network, expansion of lobular tissue and terminal differentiation of alveolar MECs to generate the milk-producing lobular units during pregnancy (Lydon et al. 1995, Hewitt et al. 2002, Feng et al. 2007, LaMarca & Rosen 2008, Aupperlee et al. 2013, Lain et al. 2013, Quaynor et al. 2013, Sampayo et al. 2013). In the absence of pregnancy in rodents and humans, a small degree of glandular expansion and differentiation occurs with every menstrual cycle (Robinson et al. 1995). In addition, all mammalian species only achieve terminal breast differentiation during pregnancy and lactation. Ductal branching, lobular growth and alveolar differentiation during the menstrual cycle or at pregnancy are thought to be principally directed by the actions of progesterone, though oestrogen signalling is required for progesterone action, which induces reorganisation and expansion of the mammary epithelium at ductal branch-points (Sampayo et al. (2013); reviewed in Macias & Hinck (2012)). Recent studies have shown that this growth process involves MECs from both basal and luminal cell lineages (Lain et al. 2013, Chang et al. 2014), rather than the activity of a common stem/progenitor cell population that gives rise to all cells within the newly developed lobules. Therefore, coordinated cell–cell signalling multiple MEC lineages is critical to the normal growth and differentiation of the adult mammary epithelium.

During puberty in humans, rodents and non-human primates, various androgenic compounds have been shown to inhibit ductal elongation (Casey & Wilson 1984, Jayo et al. 2000, Peters et al. 2011) and proliferation of MECs (Pashko et al. 1981, Peters et al. 2011), suggesting that AR signalling acts to antagonise the stimulatory actions of ERα in the regulation of this developmental process. The broad growth inhibitory, anti-oestrogenic influence of androgens in breast development is exemplified by cases of pubescent girls with adrenal hyperplasia, where breast growth is suppressed due to abnormally high-circulating androgen levels in the presence of normal levels of oestrogens (Forsbach et al. 2000). One proposed
mechanism for the ability of androgens to reduce MEC proliferation in adult primates is via inhibition of ERα expression and its consequential effect on expression of critical ERα-target genes (Zhou et al. 2000). In normal female mice, inhibition of AR signalling by administration of the AR antagonist flutamide had no effect on ductal extensions or MEC proliferation during pubertal growth, but significantly increased the number of branch-points, degree of MEC proliferation as well as the level of apoptosis in adulthood (Peters et al. 2011).

Several transgenic and mutant mouse models of AR modulation have been generated, including global and tissue-specific knockouts (reviewed in Walters et al. (2010), Chang et al. (2013) and Cheng et al. (2013)). Where mammary growth and function have been assessed in global knockout mice, knockout of Ar was achieved using Cre–loxP technology driven by ubiquitous promoters, such as β-actin (Yeh 2003, Hu et al. 2004), to excise exons 1 or 2, exhibit defects in ovulation that reflect broader modification of steroid hormone action and confounds specific analysis of androgen action in the mammary epithelium (Yeh 2003, Hu et al. 2004, Shiina et al. 2006, with detailed review of different knockout mouse models in Cheng et al. (2013)). In addition, actions of AR within the mammary stroma are known to be critical for androgen-induced regression of the mammary rudiment in male foetal development (Dürnberger & Kratochwil 1980, Cunha et al. 1997), raising the possibility that a lack of AR in the mammary stroma during adulthood may also influence adult mammary development and function in

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Table 1  AR expression in different breast cancer/cell types of primate and murine mammary epithelium
global knockout mice. Irrespective of the potential for such confounding defects in global Ar-knockout mice, ductal and lobuloalveolar growth, branching, MEC proliferation and terminal-end bud size were all significantly reduced in homozygous knockout mutants (Yeh 2003, Shiina et al. 2006), contrary to the predicted role of AR in the inhibition of oestrogen-mediated MEC proliferation. More specifically, defects in insulin-like growth factor receptor 1 and MAPK signalling were identified along with reduced response to oestrogen (Yeh 2003); therefore the mammary phenotype of these mice likely reflects a broader neuroendocrine defect. In contrast to the global Ar-knockout mouse models described above, a global CMV promoter-driven excision of exon 3 using Cre–loxP technology resulted in an in-frame deletion of Ar. Female homozygous mutant mice exhibited a less striking ovarian phenotype, and little change in circulating serum hormones, and steroid target gene transcription compared with WT littermates, though with a persistence of defective late-follicular development (Walters et al. 2007, 2009). In this model, the mammary gland phenotype is characterised by increased proliferation in the adult epithelium (Simanainen et al. 2012), observations that are consistent with the findings of systemic AR-inhibition studies outlined above (Peters et al. 2011). To date, only one study has reported on generation of a MEC-specific Ar-knockout (MARKO) mouse. In this model, the mouse mammary tumour virus (MMTV) promoter was used to drive CRE expression in all MECs and used in combination with an AR-containing loxP sites flanking exon 2, resulting in an unstable partial fragment completely lacking a DNA-binding domain. Currently available MMTV–CRE lines exhibit CRE expression at early stages in mammary development and therefore excise AR from all MEC subtypes. While subtle variations in CRE expression exist between lines, the specific line used by Hodgson et al. (2013) was not reported. Owing to the breeding strategy employed in the generation of the MARKO mouse, only heterozygous female knockout mice were generated. These mice had a 50% reduction in the proportion of Ar-expressing cells, due to the normal process of X-inactivation that naturally silences one Ar allele in female cells (Hodgson et al. 2013). No defects in mammary development or function were reported in this study. There was also no change in the transcription of ERα, PR or their classic downstream targets Areg (Amphiregulin), Tnfsf11 (Rankl) or Wnt4 in digested mouse mammary gland tissue (Hodgson et al. 2013). The lack of changes in ERα, PR or their target genes is surprising and would suggest that AR does not significantly interact with ER or PR activity in the normal state in mice, or that modification of AR activity results in relative changes in other factors that mask absolute changes in ER and/or PR signalling in MEC. However, relative differences in ER/PR may be detectable in pure FACS-sorted MEC subsets or in a complete mammary-specific Ar knockout. Nonetheless, MARKO mice were resistant to HER2/neu tumour formation, and this is further supported by contemporary studies in which carcinogen-induced breast cancer was significantly advanced in female mice that lacked a functional AR (Simanainen et al. 2012). In the absence of overt changes in the global expression of other reproductive steroid receptors and their targets, the lack of steroid signalling effects yet tumour resistance of MARKO mice suggests appreciable segregation of AR action from that of ERα and PR. This prompts us to look elsewhere for a mechanism to explain the ability of AR to modulate tumour growth in this model. Recent studies have implicated NOTCH pathways in the process of mammary outgrowth at puberty and lobuloalveolar differentiation at pregnancy (Lafkas et al. 2013, Šale et al. 2013). The fact that AR modulation (whether by genetic or hormonal manipulation) has specific effects on the same processes (Walters et al. 2007, Peters et al. 2011) indicates the potential for integration between AR and NOTCH in the HS-MEC population as being important to the tumour suppressive action of androgens.

**The mammary epithelium is a developmentally dependent organ**

In an analysis of 140 breast tissue samples of 70 deceased women (over the age of 70 with an average age of 79) who died of causes other than breast cancer, severe intraductal hyperplasia and ductal carcinoma *in situ* were identified in 43 and 6% of the 70 individuals tested respectively (Kramer & Rush 1973). Thus, like the prostate (where 66% of 80- to 89-year-old men possess occult prostatic carcinoma (Franks 1954), the breast is a hormone-dependent organ that demonstrates some degree of hyperplastic growth during normal ageing. This phenomenon suggests a persistent activation of developmental programmes in the breast throughout life. Moreover, the mammary epithelium is the final tissue to complete differentiation in women, is actively being modified through reproductive cycles and is required to regress and reform over multiple pregnancies. Thus, like other reproductive organs (and unlike most tissues not associated with reproductive function), normal mammary gland function maintains a dependency on developmental
programmes, such as those controlled by NOTCH, well into adulthood, and changes in these developmental programmes are likely instrumental to breast tumourigenesis (Tian et al. 2013). Such notions are supported by the powerful ability of the mammary microenvironment to modify the cell fate and differentiation of diverse cell types in co-transplantation studies (e.g. neuronal and testicular cells – Booth et al. (2008) and Boulanger et al. (2013)). One of the most important developmental programmes to occur in the mammary gland is lobuloalveolar differentiation at pregnancy. It has been known for decades that a single full-term pregnancy early in adult life affords lifelong protection against breast cancer in mice and humans (Beral 1983, Russo et al. 2005, Siwko et al. 2008, Deearth et al. 2010). In an attempt to uncover the mechanism behind this parity-induced protection, micro-array analysis of FACS-isolated MECs from virgin and parous mice was undertaken. Among the largest gene changes observed in virgin vs parous samples was down-regulation of the luminal, HS-MEC-secreted factors WNT4 and AREG (Meier-Abt et al. 2013). The relevance of HS-MECs in parity-induced breast cancer protection was validated in human breast tissue assessed for the greatest transcriptional and methylation changes after parity (Choudhury et al. 2013). The latter study implicated a subset of HS-MECs expressing the cell cycle regulator P27 as a principal effector of the breast cancer protective effect of pregnancy. The importance of HS-MECs in this effect was supported in non-human primates, in which the bulk of transcriptional changes occurring in the mammary epithelium over the lifespan of animals could be related to factors involved in luminal, HS-MEC function, including AR, ERA, PR and PRLR signalling (Stute et al. 2011). Altogether, these studies point to HS-MECs exerting a powerful developmental influence on the normal MEC population. Accumulating evidence indicates that HS-MECs also influence breast cancer susceptibility (Choudhury et al. 2013, Hodgson et al. 2013, Meier-Abt et al. 2013, Tarulli et al. 2013), an effect that may extend to both ERA-positive and ERA-negative breast cancers, and supports the use of androgen modulation as a form of breast cancer therapy as it principally targets the HS-MEC population.

Emergence of a new lobule-restricted progenitor

The concept of lobule- and duct-restricted mammary progenitors, responsible for the maintenance of ductal and lobular regions of the mammary epithelium respectively, was first proposed in the mid-1990s (Smith 1996). However, a lobule-restricted model fell from favour with the identification of a true mammary stem cell (Shackleton et al. 2006) and the development of powerful in vitro assays of stem/progenitor activity that identified multipotency in cultured MECs (Dontu et al. 2003, Dontu & Wicha 2005, Liao et al. 2007, Grimshaw et al. 2008, Lindeman et al. 2008). Therefore, a classic hierarchical model of mammary epithelium prevailed (Fig. 2A). In this model, mammary stem cells actively replace bipotent, basal- and luminal-restricted progenitors that give rise to differentiated basal and luminal subtypes, in a linear fashion. Such bipotency contributes significantly to mammary outgrowth during foetal development (Van Keymeulen et al. 2011). In contrast, recent lineage-tracing studies provided evidence that the adult mammary epithelium is largely maintained by basal- and luminal-restricted progenitors, rather than basal stem cells (Van Keymeulen et al. 2011; Fig. 2B). The finding of basal- and luminal-restricted turnover in the adult mammary gland has recently been questioned by lineage-tracing studies using the confetti transgene (Rios et al. 2014). This approach provided evidence of bi-potency in adult MECs, making it unclear at present if bi-potency occurs in all cases, or if the differences between the studies of Rios and Van Keymeulen merely reflect differences in the transgenic systems employed, or strain-specific differences. Further studies using more stringent models and later time-points of lineage marking are required to settle the issue of bi-potency in the adult mammary epithelium. More recently, additional layers of complexity in MEC turnover have emerged using lineage-tracing technologies, identifying previously unrecognised luminal progenitor populations characterised by the activity of NOTCH2 (Sale et al. 2013) or NOTCH3 (Lafkas et al. 2013). Initial evidence indicates a luminal, HS-MEC identity of these cells but clearly demonstrates, at least in the case of NOTCH2, that such cells are restricted to mammary ductal patterning during development and the coordination of lobule growth associated with pregnancy. While these recent findings demonstrate that a lobule-restricted progenitor may indeed exist, it appears to be more lineage-restricted than the lobule progenitor originally posited by Smith (1996), and rather than serving as a ‘stem cell’ for milk-producing lobules, it is involved in the co-ordinated action and integration of all three MEC lineages that develop together as part of lobule growth during pregnancy (Lafkas et al. 2013, Lain et al. 2013, Chang et al. 2014) and that in mouse results in an expansion of basal MECs in early pregnancy (as identified by FACS markers (Fig. 3)). Overall, data from NOTCH
lineage-tracing studies have fundamentally changed our view of the mammary epithelial hierarchy, indicating the existence of hitherto undefined restricted progenitors, and in the process providing new cellular players to better understand breast cancer initiation and growth, which may prove to be important targets of androgen action.

**NOTCH**

**Notch expression in MECs**

NOTCH genes are part of an evolutionarily conserved signalling network found in most multicellular organisms and principally function to coordinate cell differentiation and response to signals emanating from adjacent cells (reviewed in Andersson et al. (2011)). In their inactive form, NOTCH receptors exist as transmembrane precursors that undergo cleavage when engaged by ligands (such as Delta-like and jagged) on adjacent cells to release an intracellular portion of the receptor (NOTCH intracellular domain (NICD)) that acts as a transcriptional activator. This limits the range of communication by NOTCH to juxtacrine signalling. Once cleaved, the NICD translocates to the nucleus where it associates with recombination binding protein for immunoglobulin kappa J region (RBPI – also known as C-promoter-binding factor 1 (CBF1)), converting it from a transcriptional repressor to an activator via the recruitment of coactivators such as CREB-binding protein (CBP), mastermind-like proteins (MAML) and histone acetyltransferases (Chillakuri et al. 2012). Classic NOTCH transcriptional target genes include the transcriptional repressors, hairy and enhancer of split/related proteins (HES and HEY), while NOTCH targets relevant to the mammary gland include ERBB2 (HER2), CDKN1A (P21), CDKN1B (P27) and CCND1 (Cyclin D1) (reviewed in Bray & Furriols (2001) and Al-Hussaini et al. (2011)). Expression of NOTCH receptors and the intracellular machinery responsible for responding to NOTCH is predominantly found in the luminal lineage, with ligands mostly found in basal MECs (Fig. 4). This is with the exception of NOTCH4, where transcripts are expressed equally between basal and luminal MECs in mice (Bouras et al. 2008), with transcript and protein identified within basal cells of human breast (Harrison et al. 2010a, Kannan et al. 2013). The widest spectrum and highest levels of NOTCH machinery are found within sorted mature luminal MECs in mouse and human (containing HS-MECs – Bouras et al. (2008), Kendrick et al. (2008) and Lim et al. (2010)). The transcription of NOTCH ligands, Jagged and Delta-like, is enriched in basal human MEC populations (Raouf et al. 2008, Lim et al. 2010, Kannan et al. 2013), whereas protein expression studies demonstrate a broader pattern of JAGGED expression (Stylianou 2006). Overall, the direction of NOTCH signalling appears to be from basal to luminal MECs, in particular HS-MECs. This is in contrast to the direction of WNT signalling, where
prevailing evidence indicates a luminal MEC source, directed at basal MECs. Such a restricted pattern of paracrine/juxtacrine WNT–NOTCH signalling between MECs may be akin to the emerging integrated nature of WNT and NOTCH activity in Drosophila that forms a single signalling unit during development (Descalzo & Arias 2012), reviewed in Muñoz-Desca1zlo et al. (2012).

NOTCH function in mammary epithelium

In general, NOTCH signalling is associated with promoting a luminal phenotype in MECs. For example, inhibition of NOTCH activity with gamma-secretase inhibitors promotes basal colony formation, while lentiviral introduction of cDNA for the NOTCH coactivator, MMSML1, in sortd primary human bipotent progenitor MECs facilitated the formation of in vitro colonies with a luminal phenotype (Raouf et al. 2008). NOTCH3 was transcribed to a greater extent by human luminal MECs relative to bipotent progenitors, and lentiviral-mediated NOTCH3 expression also promoted luminal colony formation (Raouf et al. 2008). This is expected given the luminal-specific identity of NOTCH3 lineage-traced cells in vivo (Lafkas et al. 2013). One exception to the luminal-promoting nature of NOTCH receptor engagement is NOTCH4, expression of which is more restricted to basal MECs (Harrison et al. 2010b, Kannan et al. 2013) and implicates a role in maintenance of basal stem/progenitor MECs in this sub-population. This was demonstrated using mammospheres to measure stem cell self-renewal of primary human MECs in vitro, where secondary sphere capacity was increased tenfold when cells were cultured in the presence of a NOTCH-activating synthetic peptide (Dontu et al. 2004). Alternatively, blocking NOTCH4 using an inhibitory antibody or gamma-secretase inhibitors reduced self-renewal of normal human primary MECs and DCIS in mammosphere assays (Dontu et al. 2004; reviewed in Farnie et al. (2007) and Al-Hussaini et al. (2011)). Together, such in vitro experiments give rise to a model where NOTCH4 is important in the maintenance of basal stem/progenitor cell characteristics, while expression of other NOTCH factors is associated with commitment to the luminal lineage. However, mammosphere and in vitro colony-formation assays represent an artificial system and may be a read out, at least in part, of the effect of NOTCH on luminal and basal progenitor activity as well as ‘stem cell’ self-renewal. Therefore, the results of the aforementioned studies may be more applicable to cancerous states where spatial restrictions on the expression of developmental factors likely break down. Nonetheless, such results, in combination with the transgenic studies outlined below, set the scene for inhibiting NOTCH signalling in women with breast cancer. This is predicated on NOTCH-induced aberrations in MEC differentiation causing breast cancer initiation and growth, and that self-renewing ‘cancer stem cells’ are sensitive to such therapies (reviewed in Harrison et al. (2010a)).
Mouse mammary models of NOTCH action in mammary epithelium

Multiple mouse models of mammary-specific NOTCH expression or knockout have been generated, under the control of promoters expressed either in all MEC types, or restricted to alveolar MECs (outline of different NOTCH models and their phenotypes given in Table 2).

The expression of activated NOTCH4 (termed Int3 or NOTCH4 intracellular domain (NOTCH4ID)) within the entire mammary epithelium (using the MMTV promoter), or specifically within the alveolar compartment (using the whey acidic protein (WAP) promoter to express the transgene only in alveolar MECs) arrested lobuloalveolar differentiation (Gallahan et al. 1996) or the differentiation

Table 2 Transgenic NOTCH mouse models and their respective phenotypes

<table>
<thead>
<tr>
<th>Model</th>
<th>Promoter</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMTV-Int3 (mNotch4)</td>
<td>Expression of NOTCH4 in all MECs</td>
<td>Lack of full ductal development Full ductal development with pregnancy but no full alveolar differentiation Focal, poorly differentiated mammary tumours between 2 and 7 months of age</td>
<td>Jhappan et al. (1992) Smith (1996)</td>
</tr>
<tr>
<td>WAP-Int3 (mNotch4)</td>
<td>Expression of NOTCH4 in alveolar MECs</td>
<td>Normal ductal development (occasional ductal hypertrophy) and failure of differentiation at late pregnancy No WAP, little b-casein and reduced STAT5 at 14 days of pregnancy By full-term strong casein but little WAP isolated normally differentiated lobules</td>
<td>Gallahan et al. (1996)</td>
</tr>
<tr>
<td>MMTV-hNOTCH1ID</td>
<td>Expression of human NOTCH1 in all MECs</td>
<td>Elongated ductal structures, reduced side-branching and increased proliferation at 5 weeks of age, DCIS in some animals Pregnancy-dependent papillary in situ carcinomas (high but incomplete penetrance) Forced weaning or blocking oxytocin-induced tumour regression Mice generated invasive, non-regressing tumours after several rounds of pregnancy</td>
<td>Kiaris et al. (2004)</td>
</tr>
<tr>
<td>MMTV-mNotch1ID and mNotch3ID</td>
<td>Expression of NOTCH1 or NOTCH3 in all MECs</td>
<td>Normal development until 12 weeks of age Reduced alveolar growth at early and late pregnancy with a delay of proliferation Involution slowed Reduced b-casein expression Rapidly growing, non-metastatic tumours manifest at 7–10 months of age, independent of pregnancy Non-regressing tumours</td>
<td>Hu et al. (2006)</td>
</tr>
<tr>
<td>MMTV-Cre;RbpJfl/fl</td>
<td>Knockout of RBPJ in all MECs</td>
<td>Failure of basal differentiation into luminal population Profound defects in epithelial organisation at day 7 of pregnancy Failure in secretory differentiation Paucity of luminal cells and accumulation of multi-layered basal cells at pregnancy Luminal cells acquire p63 expression Dominance of HS-MECs in luminal compartment Animals allowed to undergo involution showed a return to normal MEC proportions</td>
<td>Yalcin-Ozuysal et al. (2010) Buono et al. (2006)</td>
</tr>
<tr>
<td>WAP-mInt3;WAP-Cre;RbpJfl/fl</td>
<td>Expression of NOTCH4 and knockout of RBPJ in alveolar MECs</td>
<td>Incomplete rescue of the alveolar defect in WAP-Int3 mice by knockout of Rbpj in alveolar MECs Tumour formation is independent of Rbpj, though latency increased</td>
<td>Raafat et al. (2008)</td>
</tr>
<tr>
<td>MMTV-tTA/tet-op-mNotchIC</td>
<td>Inducible expression of NOTCH1 in all MECs</td>
<td>Modified ductal branching (mainly tertiary) at pregnancy ERA-negative luminal adenocarcinomas by 12 months of age with 90% penetrance Tumours regress rapidly after removal of NOTCH1 expression</td>
<td>Simmons et al. (2012)</td>
</tr>
</tbody>
</table>
of MECs during transplant outgrowth (Jhappan et al. 1992, Gallahan et al. 1996, Robinson et al. 1996). Both systems resulted in the formation of mammary adenocarcinomas with 100% penetrance, demonstrating the relevance of NOTCH4 signalling to breast tumour development. Similarly, overexpression of mouse NOTCH1ID or NOTCH3ID using the MMTV promoter resulted in a similar phenotype to NOTCH4ID models and to one another (Hu et al. 2006), and more recently the NOTCH3ID model was found to expand a luminal progenitor population (Ling et al. 2013). In a separate model that expressed human NOTCH1ID under the control of the MMTV promoter (Kiaris et al. 2004), tumours rapidly appeared only upon pregnancy-associated differentiation whereas pubertal outgrowth was only associated with variable hyperplasia. In this model, pregnancy-associated tumours regressed upon the initiation of involution, illustrating that mouse tumours maintain acute sensitivity to developmental programming when expressing human NOTCH1ID (Simmons et al. 2012).

Removing the central transcriptional mediator of NOTCH signalling, RBPJ, from the entire mammary epithelium (Buono et al. 2006) demonstrated that NOTCH activity was not required for the establishment of basal and luminal cells during puberty or after transplantation. Akin to the specific NOTCH overexpression models described above, deletion of NOTCH signalling resulted in defects in MEC differentiation during pregnancy. In addition, profound defects in normal basal and luminal MEC differentiation were observed, with a striking accumulation of basal cells and ectopic expression of the basal MEC-defining marker P63 in a subset of luminal cells (Buono et al. 2006). This phenotype was attributed to a block in basal MEC differentiation that was amplified with hormone treatment and pregnancy. Basal MEC specification relies on the expression of P63, and this was recently shown to aid in the regulation of luminal cell growth by promoting the secretion of paracrine factors such as neuregulin (Forster et al. 2014). This is a potential point of integration between AR and NOTCH signalling that is discussed in section ‘Integration of NOTCH and AR’ below. A recent application of the same Rbpj-knockout mouse as utilised by Buono et al. (2006) has suggested that the basal MECs expansion observed during pregnancy in these mice was due to MECs deficient in NOTCH signalling being out-competed by WT MECs, supporting the idea that NOTCH signalling is critical for basal bipotent progenitor MECs to take on a luminal fate through suppression of P63 (Yalcin-Ozuysal et al. 2010). A role for AR in antagonising the effects of P63, via the cell cycle regulator P21, has been identified in prostate epithelium (Mirochnik et al. 2012), and it possible that a similar mechanism is at play in the mammary epithelium.

**NOTCH as a target in endocrine resistance breast cancer**

Breast cancer is a heterogeneous disease, even within specific breast cancer subtypes (Armakolas et al. 2012, Kim et al. 2012, Burrell et al. 2013, Pinto et al. 2013). In an analysis of more than 72 primary ERa-positive human breast cancers, small subpopulations of hormone receptor-negative breast cancer cells (with a basal-like phenotype) are present and can be positively selected for when using oestrogen-inhibition therapies for cancer that is ERa positive. This promotes the emergence of hormone therapy-resistant basal-like cancer cells that can be inhibited by concomitant reduction in NOTCH signalling (Haughian et al. 2012). In the absence of oestrogen activity (i.e. after anti-oestrogen therapy), NOTCH signalling can stimulate ERa-associated transcription, therefore substituting for the presence of ERa ligand via the activity of shared transcriptional coactivators p300 and CBP (Rizzo et al. 2008, Hao et al. 2010) that are also involved in AR co-activation. NOTCH4 is able to stimulate oestrogen-independent growth in T47D and MCF7 breast cancer cells that is dependent on suppression of the NOTCH transcription factor HES1 (Ström et al. 2000, Muller 2002, Yun et al. 2013). These observations could in part explain the development of resistance to oestrogen-targeted therapies and suggest a potential benefit of combining NOTCH modulation with current endocrine therapies, including AR-targeting therapy. Studies have also implicated the activation of the NOTCH pathway as an important response to HER2-targeted therapies (Fitzgerald et al. 2000, Pandya et al. 2011), further supporting the idea of using NOTCH inhibition in combinatorial therapies to overcome/minimise resistance to targeted therapy.

**Integration of NOTCH and AR**

Insights into the potential mechanisms of AR interacting with NOTCH signalling in breast tissues can be inferred from studies on the prostate, particularly in the lineage-restricted patterns of cellular turnover in the normal adult prostate gland (Van Keymeulen et al. 2011, Ousset et al. 2013). Using prostate-specific Rbpj-knockout (ARR2PB–CRE/RBPJfl/fl) mice, it was demonstrated that inhibition of NOTCH signalling did not affect prostate development but...
induced an expansion of basal cells during recrudescence after testosterone withdrawal and replacement (Valdez et al. 2012). This is comparable to the mammary-specific Rbpj-knockout mouse, in which expansion of basal MECs occurs after increased hormone signalling at pregnancy (Buono et al. 2006, Yalcin-Ozuysal et al. 2010), illustrating that androgen signalling is able to engage and modify NOTCH signalling in androgen-dependent tissues such as the prostate, and raising the possibility of a similar integration of androgen action in the mammary gland.

While limited information regarding the interaction of AR and NOTCH signalling pathways exist in the mammary gland, an antagonistic relationship is emerging in several epithelial tissues. Examples of interacting AR and NOTCH molecular pathways are outlined in Fig. 5. Studies on prostate tissue and cancer cell lines elucidated a role for the downstream NOTCH transcriptional repressors, HEY1 and HEYL, in the inhibition of AR-mediated gene transcription via interactions with the AF1 region of AR and direct competition with the AR coactivator SRC1 (Belandia et al. 2005, Belandia & Parker 2006, Lavery et al. 2011). This was proposed as a mechanism by which aberrant prostate growth develops androgen independence. In prostate cancer tissue, HEY1 was found to be

![Diagram of Endocrine-Related Cancer](image)

excluded from the nucleus and this exclusion was proposed as a mechanism of sensitising prostate cells to androgen signalling and evading the anti-proliferative function of NOTCH signalling (Belandia et al. 2005). Another NOTCH-induced transcription factor, HEYL, was shown to be a more potent repressor of AR activity and relevant to human breast as its repressive activities also occur in ERα-positive human breast cancer cells.

NUMB is an important negative regulator of NOTCH signalling (Pece 2004, Stylianou 2006) and expression of NUMB is up-regulated by androgen signalling in myeloblast and muscle cells (Kovacheva et al. 2010, Liu et al. 2012, 2013), further highlighting integration of NOTCH and androgen pathways. Loss of NOTCH control by NUMB has been implicated in promoting a basal-like phenotype in human breast cancer (Pece 2004, Rennstam et al. 2009), as has the stabilisation of p53 in response to NUMB (Colaluca et al. 2008) (Fig. 5). It is tempting to speculate that not only do androgens play a role in the inhibition of global molecular programmes that promote mammary growth and differentiation (e.g. through antagonising the actions of oestrogen and/or progesterone in luminal MECs), they may also act to modify the state of differentiation in MECs via interactions with principal factors dictating the balance of mammary lineage specification, such as NOTCH and p63. The function of AR to suppress NOTCH activity in normal HS-MECs could serve to maintain quiescence within the epithelium in the presence of hormonal signalling associated with active reproductive cycling. One study that supports such an idea is the dose-responsive nature of growth-promoting vs growth-suppressive responses to NOTCH activation observed in an immortalised normal human MEC cell line, MCF10A (Lindsay et al. 2008, Mazzone et al. 2010). When AR is introduced into MCF10A cells, either a growth inhibitory or stimulatory response to AR engagement occurs in the presence or absence of EGF signalling (Garay et al. 2012).

At the same time, AR acting on factors such as NOTCH could also amplify luminal differentiation signals through other pathways important for normal mammary function at pregnancy such as augmentation of prolactin-induced protein (PIP), an interaction demonstrated in the T47D breast cancer cell line that expresses similar levels of ERα and AR (Baniwal et al. 2012, Naderi & Meyer 2012). Prolactin signalling is essential for lobuloalveolar differentiation at pregnancy (Grimm et al. 2002, Naylor et al. 2003, Ormandy et al. 2003) and central to the function of HS-MECs during early pregnancy to promote the expression of paracrine growth factors associated with alveolar cell differentiation (Dontu et al. 2004, Mukherjee et al. 2010, Tarulli et al. 2013), and significant cross-talk between prolactin and progesterone pathways has been reported (Lee & Ormandy 2012, Obr et al. 2013). Importantly, expression of PIP has been associated with breast tumours with apocrine features, a subtype with an important emergent role for AR regulation of tumour growth (Mazoujian et al. 1983, 1989, Farmer et al. 2005, Doane et al. 2006).

Overall, there is sufficient evidence to indicate that AR engages multiple developmental pathways associated with normal adult breast differentiation, including NOTCH signalling, to suppress breast growth, differentiation and transformation. In some breast cancers, these suppressive effects may be circumvented or hijacked to promote breast cancer growth, and is still unclear how to specifically identify these cancers and thus whether to consider androgen activating or inhibiting therapies in a clinical context.

**Future perspectives**

Given the dependence of adult mammary function on developmental programmes and the broad, integrated actions of oestrogen, progesterone and androgen, the successful application of androgen-modulation therapy likely lies within an individualised approach to breast cancer management by identifying the developmental processes that each cancer is dependent on. By better understanding the role of AR in normal breast development and function, and the genetic, cellular and architectural characteristics of an individual tumour, clinicians will be able to better select individuals who likely will respond favourably to AR activation, and conversely those in which such therapy would be contra-indicated. The successful historical application of androgen therapy for advanced breast cancer is compelling evidence that there are pathways engaged by AR signalling which can be identified and successfully targeted in breast cancer patients. The identity of NOTCH-expressing/responsive cells in mammary epithelium implicates the HS-MEC population, which are the predominant AR-expressing MEC, as the principal luminal NOTCH-expressing lineage. Therefore, it is worthwhile to focus research on this population and the ways in which AR signalling integrates with the NOTCH pathway therein, to develop a greater understanding of how to harness the breast cancer modulating actions of androgens in breast cancer.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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References
Bellido B & Parker MG 2006 Nuclear receptor regulation gears up another Notch. Nuclear Receptor Signaling 4 e001. (doi:10.1621/nrs.04001)


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