Wnt Signaling in Mammary Glands: Plastic Cell Fates and Combinatorial Signaling

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The mouse mammary gland is an outstanding developmental model that exemplifies the activities of many of the effector pathways known to organize mammalian morphogenesis; furthermore, there are well-characterized methods for the specific genetic manipulation of various mammary epithelial cell components. Among these signaling pathways, Wnt signaling has been shown to generate plasticity of fate determination, expanding the genetic programs available to cells in the mammary lineage. It is responsible first for the appearance of the mammary fate in embryonic ectoderm and then for maintaining bi-potential basal stem cells in adult mammary ductal trees. Recent technical developments have led to the separate analysis of various mammary epithelial cell subpopulations, spurring the investigation of Wnt-dependent interactions. Although Wnt signaling was shown to be oncogenic for mouse mammary epithelium even before being identified as the principle oncogenic driver for gut epithelium, conclusive data implicating this pathway as a tumor driver for breast cancer lag behind, and we examine potential reasons.

There are several excellent recent reviews that cover aspects of Wnt signaling and mammary gland development and transformation (Boras-Granic and Wysolmerski 2008; van Amerongen and Nusse 2009; Incassati et al. 2010; Roarty and Rosen 2010; Wend et al. 2010; Jarde and Dale 2011). The aim of this article is to focus attention on the open questions in this area. There are a remarkable number of tools available to assist with this, given the focus on breast cancer research in the past 10 years. They include large collections of human breast cancer cell lines (Neve et al. 2006; Hoeflich et al. 2009; Hollestelle et al. 2010) and dozens of strains of mice that are useful for analysis of different aspects of Wnt signaling and biology (van Amer-

ongen and Berns 2006). This battery of genetic tools includes transgenic mice that express (or induce conditional ablation of) genes in either of the two main mammary epithelial cell types that comprise the mammary gland (the organization of the mammary gland is shown in Fig. 1). These are the luminal cells—typically targeted by one of three drivers, namely, MMTV LTR (mouse mammary tumor virus long terminal repeat), WAP (whey acidic protein promoter, expressed during milk production/terminal differentiation), or BLG (β -lactoglobulin, another milk whey protein) promoters—and the basal cells (typically targeted using keratin-5 or -14 promoters, also expressed in other stratified epithelia, notably skin). The definitions of luminal

Editors: Roel Nusse, Xi He, and Renee van Amerongen
Additional Perspectives on Wnt Signaling available at www.cshperspectives.org

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Cite this article as *Cold Spring Harb Perspect Biol* 2012;4:a008037

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Figure 1. The mouse mammary gland, organization, and cell types. The (10) mouse mammary gland(s) comprise fat pads attached to the ventral mouse skin, colonized by a branched tree of hollow, epithelial mammary ducts (stained with carmine red) that are connected to the nipple. During pregnancy, there is massive proliferation of lobuloalveolar side branches to colonize the interstitial spaces between ducts, becoming filled with milk, expressed by oxytocin-induced contraction of myoepithelial cells. (*Inset*) An immunofluorescent stain of a longitudinal cross section of a non-pregnant duct, stained with a basal cell anti-cytokeratin (CK5) together with a luminal cell anti-cytokeratin (CK8), to show the bilayered structure of the epithelium.

and basal cells vary between studies because they are based on the analytical method used. These may be (1) location in tissue sections (facing the lumen or adherent to the basement membrane); (2) expression of molecular markers usually associated with basal cells (e.g., expression of basal-specific cytokeratin 5 [CK 5] or p63, or luminal-specific CK8 or Muc1); or (3) copurification with subgroups of cells isolated by flow cytometry (e.g., using Lin/CD29/CD24 or Lin/EpCAM/CD49f). These latter cell populations are often subsequently typed using their expression of histological markers or mRNA profile to generate a “luminal” or “basal” descriptor. The cell groups described by these means are not always the same, and this becomes important to experimental interpretation.

Not all cells in a given lineage are identical. For luminal cells, perhaps one of their most obvious differences is their expression of ER α (estrogen receptor- α). Thus, for both mouse and human, $\sim 15\%$ of luminal cells express ER α (at any one time), irrespective of their stage

of development (Clarke 2003; Mastroianni et al. 2009). ER α -positive and -negative cells can be purified by flow cytometry and analyzed separately (Kendrick et al. 2008; Lim et al. 2010). The ER α -negative luminal cell subpopulation contains a proliferative activity, leading it to be labeled “luminal progenitor cells” (a heterogeneous group), whereas the ER α -positive group is non-clonogenic in vitro and therefore labeled “mature” (Table 1).

Using lacZ reporter strains, the heterogeneity of expression of MMTV- and WAP-driven transgenes is obvious, but the basis for this is unknown (e.g., Wagner et al. 2001). Indeed, the MMTV LTR is a remarkable 1200-bp gene expression motif that condenses all of the key elements of mammary regulation, to include the specification of mammary fate (expression starting early in the ectoderm of embryogenesis), together with the hormone inducibility that appears during puberty in females (Rouault et al. 2007), with further up-regulation during pregnancy and lactation (Mink et al. 1990). Cre expression has effects on mammary morphogenesis and lactation; therefore, experiments that use this tool need to be interpreted with caution (Chan et al. 2007; Robinson and Hennighausen 2011).

There are several Wnt reporter lines that show highly regulated (but slightly different) expression patterns in mammary gland (including a number of strains based on Wnt response elements [WRE] driving lacZ [for review, see Barolo 2006], and two newer strains based on WRE-GFP, perhaps more useful for purifying Wnt-responsive cell types [Currier et al. 2010; Ferrer-Vaquero et al. 2010]). Because of the redundant expression of many Fzd (8/10), both Ror receptors (2/2) and Wnt ligand types (11/19) in mammary gland (Table 1), initial experiments have focused on producing gain or loss of function for canonical Wnt signaling. Thus, overexpression of the forerunner Wnt ligand species, Wnt1- (using an MMTV-Wnt1 construct) (Table 1) (Tsukamoto et al. 1988) induced ductal hyperplasia (not correctly described as mid-pregnant equivalent) and basaloid tumors (Li et al. 2003a; Liu et al. 2004; Vaillant et al. 2008), whereas the cell-autonomous Wnt signaling effector

$\Delta\beta$ -catenin (using MMTV-LTR) induced later onset hyperplasia (luminal in origin) and luminal-type tumors (Imbert et al. 2001) or ductal hyperplasia and basaloid tumors when expressed by a CK5 construct (Teuliere et al. 2005). Loss of function for β -catenin, induced by overexpression of Axin or a dominant-negative β -catenin effector in luminal cells, has implicated β -catenin in lobulo-alveolar development during pregnancy (Hsu et al. 2001; Tepera et al. 2002). This is despite a lack of clear evidence for activation of canonical Wnt reporter activity during pregnancy (Chu et al. 2004; Boras-Granic et al. 2006a). If a canonical β cat/TCF signaling is indeed activated in luminal cells, this presents a paradox. Cell surface presentation of Lrp is considered to be the limiting factor for canonical Wnt signaling (Brennan and Brown 2004), and Wnt1 responses are confined to Lrp5-positive cells (Badders et al. 2009; Teissedre et al. 2009; Baker et al. 2010; Kim et al. 2011). Only basal cells appear to be competent to generate a canonical response in response to Wnt ligands (they express the obligate canonical Lrp5 and Lrp6 receptors). Therefore, the identity of the cell surface receptor able to mediate β -catenin/TCF-induced luminal cell division is not yet known.

Lrp6 knockout (KO) mice die at birth, and the functionality of Lrp6 KO mammary rudiments has not yet been evaluated, although Lrp6 hypomorphs (Lrp6^{+/-}) are underbranched (Lindvall et al. 2009). Although dominant-negative TCF molecules have been used effectively in other cell types (van de Wetering et al. 2002), their use has not yet been reported for mammary gland. Table 1 summarizes the expression data and functional information that are available to describe Wnt signaling effectors from studies of normal and transgenic mice and human tissues (including some observations from cell lines).

The vast majority of data that describe the molecular basis of signaling via Lrp5/6 derives from ectopic expression of Wnt components in HEK293T cells (Brennan et al. 2004; Cong et al. 2004; Zeng et al. 2005, 2008). Thus, the specific molecular complexes that form in mammary epithelial cells have yet to be described (Fig. 2). For example, there are convincing data for the

activation of LRP (at the PPSP motif) by a range of different kinases, including GSK, PKA, Pfk1, Grk5/6, and CK1 (Niehrs and Shen 2010), and for the control of kinase recruitment by local phospho-lipid effectors such as P(4,5)IP2 (Tanneberger et al. 2011); however, the relative significance of each of these mechanisms for mammary epithelial cells is not yet known.

PLASTICITY OF MAMMARY EPITHELIAL CELL FATE REQUIRES A WNT SIGNAL

When mammary precursor cells are set aside from the embryonic ectoderm, there is a pulse of Wnt signaling that can be visualized by the expression of Wnt reporter genes (the lacZ Wnt reporter TOPgal) (Chu et al. 2004). If dkk1 is overexpressed in the basal keratinocyte layer (using a CK14 promoter) or when Lef1 function is missing (Boras-Granic et al. 2006b; Boras-Granic and Wysolmerski 2008), mammary specification is inhibited. Wnt10b is expressed at the earliest time during differentiation of the mammary placode (Veltmaat et al. 2004; Boras-Granic and Wysolmerski 2008), suggesting that Wnt10b could be the placode specifier. Thus Wnt signaling is essential for increasing the plasticity of embryonic skin cells so that they initiate mammary-specific programs. The induction of plasticity is a typical function associated with pulses of Wnt signaling during mammalian developmental processes (Wend et al. 2010; Domyan and Sun 2011).

Somatic stem cells are different from the majority of cells in mammalian organs, owing to their plasticity and pluripotency. For mammary gland, ductal mammary stem cells are defined by their capacity to regenerate a new mammary gland comprising a bilayered epithelial ductal network following transplantation to cleared fat pads. These stem cells have been shown to comprise a specific subpopulation of basal epithelial cells (with high expression of integrins $\alpha 6$ and $\beta 1$, and other cell surface-associated antigens such as epithelial cell adhesion molecule [EpCAM] and CD24, a GPI-linked sialoglycoprotein). When ectopic Wnt signals are present, mammary stem cells comprise a larger fraction of the total population (Li et al. 2003b; Liu et al.

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Figure 2. Cell surface Wnt signaling components shown to function during various phases of mammary gland growth and development. Structural and function information is drawn from data from He et al. (2004), Liepinsh et al. (2006), Kikuchi et al. (2007), Green et al. (2008), Bourhis et al. (2010), and Weis (2011). The information presented here can be cross-referenced to Table 1 for expression data. Physiological activities are detailed in the text. The Lgr4/5/RSpO component of embryonic placode formation is likely but has not yet been shown (hence it is grayed out). Although both Lrp5 and Lrp6 are required to maintain mammary stem cells, the Lrp5-6 heterodimer shown here is implied from our data (Goel et al. 2012a). It is not known whether the inhibitors shown (dkks, WIF, and sFRPs) are functional, but several are expressed. When components are illustrated with ribbon diagrams, their structure is known in some detail. (Wnt ligands are shown in pink.)

2004; Shackleton et al. 2006), and undifferentiated cells (lacking expression of marker proteins) are evident in mammary glands (Liu et al. 2004). There are data to support a role for Wnt proteins as inhibitors of differentiation. Analysis of a mouse mammary cell line (HC11 cells) showed that Wnt3a inhibited differentiation (assayed as β -casein expression) in response to endocrine effectors (including prolactin/dexamethasone/insulin), and that was reversible by sFRP4 expression (Constantinou et al. 2008). Furthermore, cells from Wnt1-induced glands show higher expression of the Δ N-isoform of the p53 family member, p63 (Badders et al. 2009), and less TA-p63; a profile typically

associated with dividing progenitors of basal epithelial cell types and functionally implicated in tissue senescence and aging (Wu et al. 2003).

Ductal mammary stem cells require an Lrp5-dependent, Wnt-associated signal for their induction or maintenance, and their activity is much reduced when the Lrp5 receptor species is missing (Lindvall et al. 2006; Badders et al. 2009). After the embryonic phase of development, there is little evidence for the expression of TCF-consensus concatamer-based Wnt reporters, including BAT-gal (Chu et al. 2004; Boras-Granic and Wysolmerski 2008; Lindvall et al. 2009). This is somewhat of a conundrum, given the importance of canonical Wnt signaling to

adult stem cells. However, more recently, Zeng and Nusse (2010) and R van Amerongen and R Nusse (pers. comm.) identified rare lacZ⁺ cells in postnatal glands of Axin2^{LacZ} mice. These cells were quantified using flow cytometric analysis, separated using a substrate loading approach, and found to be enriched in basal stem cells (Zeng and Nusse 2010). Furthermore, the addition of high concentrations of Wnt3a ligand sustained a long-lived mammary stem cell activity (measured by clonogenicity in vitro and in vivo) when added to cultures of primary mammary epithelial cells.

Note that the absence of Lrp5 does not significantly affect embryonic mammary placode development (even though that too is Wnt dependent), nor does it affect lobulo-alveolar development during pregnancy. The Wnt factors that regulate these processes are therefore likely to be different and Lrp5 independent. The role of Lgr4/5 and RSpO proteins in the maintenance of other stem cell types is described later. This together with the expression pattern of RSpO proteins during embryogenesis (Table 1) suggests that Lgr/RSpO may be the embryonic counterpart of the Lrp5-Lrp6 complex that is essential to the adult gland (Fig. 2).

Furthermore, in the absence of stem cells (due either to the absence of Lrp5 or β 1 integrin), ductal outgrowth is almost normal, as indeed are all other functions associated with mammary gland growth and differentiation (Lindvall et al. 2006; Taddei et al. 2008; Badders et al. 2009). This may appear to be counterintuitive. However, recently, it has emerged that bi-potential stem cell activity is not activated during normal mammary development. Thus, Van Keymeulen et al. (2011) genetically tagged CK14-expressing or CK5-expressing mammary epithelial basal cells and showed that this cell population contained bipotent stem cell activity when transferred to cleared fat pads. When tagged during puberty, the bi-potential activity (stem cell activity) of these basal cells was not expressed throughout ductal extension or pregnancy, deduced from the lack of tagged luminal cell daughters (Van Keymeulen et al. 2011). Vice versa, CK8-expressing or CK18-expressing tagged luminal cells did not appear in the basal

cell compartment. In fact, the bipotency of basal cell stem cells could be suppressed during fat pad transfer if luminal cells were included at a cell ratio of one per five basal cells. These investigators propose that there are long-lived monopotent basal and luminal stem/progenitor cell types that organize the morphogenesis of mammary gland after birth. It is implied that the activity of each stem/progenitor activity cell activity would primarily affect the directly related lineage. Indeed, the loss of basal stem cell activity (by targeted gene mutations) typically results in a decrease in the proportion of basal cells (compared with luminal cells), without affecting overall mammary gland proliferation (Taddei et al. 2008; Badders et al. 2009). Using other constructs and reporters, some of these results have been reproduced by other investigators (R Van Amerongen, R Nusse, and J Jonkers, pers. comm.). This represents a profound change in our view of stem cell function during adult organogenesis.

What, then, is “stem cell” activity scored by fat pad transfer, and is it a relevant assay? Stem cell activity is scored by assaying the outgrowth of mammary ductal trees after inoculating limiting doses of dissociated, singlized cells from mammary glands. The regenerative activity must therefore be able to survive cell isolation, to subsequently differentiate in vivo, and to create both basal and luminal mammary epithelial cells. Without ectopic endocrine support (e.g., pituitary extract), outgrowths of purified luminal cells are limited in extent (Kamiya et al. 1998; Visvader and Smith 2011). Given the evidence for luminal stem/progenitor cells provided by these studies, it is important to be able to assay this activity somehow, preferably in vitro. Perhaps it is this activity that is measured by the mammosphere assay (spheres contain no dividing basal cells) (S Kim and C Alexander, unpubl.).

IS THERE A WNT SIGNAL THAT CREATES THE MAMMARY STEM CELL NICHE?

Given that mammary stem cells are dependent on Wnt signaling, the question then turns to the identity of the ligand that interacts with Lrp5/

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Fzd to maintain these cells. Lrp5 is expressed and presented, alongside Lrp6, on all basal mammary epithelial cells (within the limits of detection) (Badders et al. 2009), meaning that the stem cells are not alone in being able to respond to Wnt ligands. Indeed, it is interesting that Lrp5 is specifically required as a maintenance factor for stem cells, given that Lrp6 is often described as the “better,” more potent receptor (MacDonald et al. 2008). However, we have shown that a subgroup of Wnt ligands (including Wnt1 and Wnt9b) requires both Lrp5 and Lrp6 to signal in some cell contexts (Goel et al. 2012b). One of this subgroup is presumably responsible for adult MaSC function (Fig. 2).

It is likely that there is a localized source of a Wnt ligand that is responsible for the stem cell niche. The phenotype of $Lrp5^{-/-}$ mammary glands maps to the mammary epithelial cells, and not the $Lrp5^{-/-}$ fat pads (NM Badders and CM Alexander, unpubl.). Most of the Wnt ligands expressed in mammary gland (Table 1) are known to have a short range of effect in vivo (due to binding to heparan sulfates or their lipidation). In fact, the diffusion range of a Wnt ligand has been visualized in mouse limb bud, where Wnt is produced by the surface ectoderm. In this case, the range appears to be approximately five cells, through the relatively loosely packed cells of the limb bud (and assuming no influence of cross gradients of other molecules) (ten Berge et al. 2008). The ligand responsible for stem cell maintenance may be expressed by other epithelial cells, or by a stromal host cell (for candidates, see Table 1).

WNT RESPONSES: A SUM TOTAL

Given the variety of Wnt signaling receptors (illustrated in Fig. 2) together with the fact that these receptors are commonly coexpressed, any one Wnt ligand is likely to generate several different signals in any one cell. The signaling outcome will depend on the absolute concentration of each ligand and receptor species, along with their relative affinity. Whether these pathways synergize with one another, or antagonize each other, depends on the context and molecules involved (Semenov et al. 2007; van

Amerongen and Nusse 2009). Thus, for melanoma cells, non-canonical Wnt signaling, mediated by Wnt5a/Ror2, promotes malignant changes in the presence of high levels of canonical signaling, and these pathways are considered to be synergistic during tumor development (O’Connell and Weeraratna 2009).

For mammary gland, the “old-fashioned” distinction of canonical and non-canonical Wnt ligands appears to hold up, presumably because of the specific repertoire of cell surface receptors expressed (van Amerongen et al. 2008). Thus, Wnt5a, although it can acquire canonical signaling activity when Lrp5 and Fzd4 are present (Mikels and Nusse 2006), has no β cat/TCF trans-activation activity in mammary gland (not surprising because Fzd4 is not expressed) (Table 1). Indeed, as shown for other cell types, Wnt5a not only does not induce Wnt reporter expression, it inhibits Wnt3A/Wnt1 canonical responses. In fact, TGF- β (a well-known mammary gland growth inhibitor) induces Wnt5a expression (directly via Smad binding sites on the promoter), and Wnt5a may be necessary to limit and control mammary gland growth. Thus, when Wnt5a is absent (Wnt5a KO), mammary gland development is hyperproliferative (Roarty and Serra 2007), whereas in the presence of ectopic Wnt5a (produced by slow release beads), ductal extension is inhibited. These phenotypes are the inverse of gain of function of canonical Wnt activity. It is puzzling then that this inhibition is not reproduced in transgenic MMTV-Wnt5a strains (Baxley et al. 2011).

How does a non-canonical ligand inhibit the canonical response? Various ideas have support; the first relies on competition; thus, if Wnt5a competes effectively for Fzd binding (in association with Ror1/2) (Mikels et al. 2009; Grumolato et al. 2011), the amount of the rate-limiting LRP receptor available to other ligands could be reduced. The second is that Wnt5a may generate an intracellular signal that is inhibitory for canonical responses; thus, when Ror1/2 binds Wnt ligands, Rac is activated (leading to JNK activation) and Fzd2 is internalized and inactivated by a clathrin-dependent mechanism (Sato et al. 2010). Witte et al. (2010) suggest that Ror2

generates an inhibitory signal via CK1-mediated dvl phosphorylation and that CK1 constitutes a switch that directs the signaling output (studies of HEK293 and Cos cells) (Foldynova-Trantirkova et al. 2010; Witte et al. 2010). Indeed, the effects of the morphogenetic planar cell polarity pathway, typically mediated by ligands such as Wnt11, are largely unexplored for mammary gland. During development, it is often true that multiple Wnt signals are perceived by cells simultaneously (see the examples drawn from genetic analysis of *Caenorhabditis elegans*, summarized by van Amerongen and Nusse 2009). These signals are key to tube building and the convergent extension processes associated with polarization of epithelial layers (Kouros-Mehr and Werb 2006; Miller and McCrea 2010). In addition, it is likely that there are polarized cell divisions that occur either in the plane of the basal and luminal layers, or that cross between them. Evidence suggests that Notch signaling and p53 are important to determining these division planes, and both pathways are highly cross-regulated by, or interact with, Wnt signaling (Callahan and Egan 2004; Collu and Brennan 2007; Bouras et al. 2008; Cicalese et al. 2009; Yan et al. 2010).

Wnt signaling in other tissue contexts also serves to organize the growth and development of more differentiated cells that emerge from the stem cell compartment. Thus, for intestinal cells, a battery of genes are induced by β cat/TCF signaling (van de Wetering et al. 2002), and these can be divided functionally into at least three programs (maintenance of progenitor phenotype, promotion of differentiation of Paneth cells in the crypt, and compartmentalization of differentiated intestinal cells from progenitors). The latter is regulated by ephrins (EphB2 and EphB3). Thus, in *Apc^{min}* mice, when ephrins are missing and cell sorting is defective, tumor development proceeds even more rapidly (Batlle et al. 2002). For the retinotectum, opposing gradients of ephrin and Wnt signals (via Ryk or Fzd) organize and map the cell function (Schmitt et al. 2006). For mammary gland, the ephrin Robo1 is a suppressor of mammary branching and basal cell division (Macias et al. 2011) and promotes the accumu-

lation of cytoplasmic E-cadherin and β -catenin. Whether ephrin expression is downstream and/or upstream of Wnt signals in this cell type is not fully understood. Other Wnt target genes identified in intestine by van de Wetering et al. (2002) include the orphan GPCR receptors Lgr4 and Lgr5 (indeed, for intestine, an Lgr5-dependent tag serves to identify intestinal stem cells in studies of growth and pathogenesis) (Barker et al. 2007). Lgr4/5 functions to feed forward to amplify the stem cell function in this tissue, by binding RSpO proteins, forming a complex with Lrp/Fzd molecules that potentiates a canonical Wnt (Wnt3a) signal (Carmon et al. 2011; de Lau et al. 2011). Because all of the same components are also expressed in mammary gland, the same principle may apply (Fig. 2).

It is possible that Wnt signaling could act indirectly on mammary morphogenesis and function via its effects on non-epithelial components of the mammary gland, including blood vessels, macrophages, fibroblasts, and adipocytes. For example, endothelial cell maturation is highly Wnt dependent (Parmalee and Kitajewski 2008), including a relatively little characterized pathway that depends on the Norrin ligand and Fzd4/Lrp5 (Ye et al. 2010). Macrophages and other immunomodulatory cells are well known to affect mammary development, involution, and tumor progression (Schwertfeger et al. 2006; Schaale et al. 2011; O'Brien et al. 2012). Fibroblasts are activated by local Wnt signals to initiate a desmoplastic response (Xu et al. 2000) that elicits a mobilization and proliferation response from circulating cells and bone marrow precursors (Kim et al. 2008). These cells are recruited to the tumor, where they participate in the angiogenic and inflammatory response, creating leaky vessels and macrophage activation, neither of which is associated with the tumors that develop in response to the intracellular Wnt effector β -catenin (Kim et al. 2008). Adipocyte differentiation is controlled by Wnt proteins (specifically Wnt10b), and adipocyte differentiation/fat content varies substantially throughout pregnancy and lactation (Prestwich and Macdougald 2007). Indeed, it is likely that the controlled spacing and branching of mammary ducts in the fat pad

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represents an interaction between the host tissue and the expanding ductal epithelial cell population (Sakakura 1987).

Furthermore, responses to Wnt signals can be modulated by cross talk from other effectors and pathways, including metabolic checkpoints such as AMPK, mitochondrial function, autophagy, and lipid effectors (Inoki et al. 2006; Gao et al. 2010; Yoon et al. 2010; Kang et al. 2011). Prostaglandins (Goessling et al. 2009) have been shown to cross-talk to Wnt signaling to maintain hematopoietic stem cells, and there are other pathways that regulate the turnover and metabolism of β -catenin (Incassati et al. 2010). Although the significance of these is largely unknown for normal mammary development and tumor initiation, the interaction of Wnt signaling with EGFR or with nuclear hormone receptors suggests that these could be particularly important for dissecting mammary behaviors (Schlange et al. 2007; Mastroianni et al. 2009; Roarty and Rosen 2010).

WNT4: A WNT PROBLEM CHILD?

Wnt4 knockout glands were reported to show a delay of lobulo-alveolar development (Briskin et al. 2000) during pregnancy, implying that this ligand could be a mammary epithelial cell growth factor with similar properties to Wnt1 (and Wnt10b). Indeed, when mammary epithelial cells were transduced with a Wnt4 expression vector, the outgrowths were hyperplastic (Bradbury et al. 1995). However, unlike Wnt1, Wnt4 does not induce mammary gland hyperplasia when expressed as an MMTV-based transgene. This is despite effective expression levels (equivalent to mid-pregnancy) and evidence of Wnt4-induced gene expression (e.g., Wnt16) (Kim et al. 2009), both in vitro (in HC11 cells) and in vivo in virgin glands. In contrast to Wnt1 (and the other canonical ligands), Wnt4 does not induce axin2 expression or TOP-FLASH activation in mammary epithelial cells or fibroblasts, and does not induce the typical developmental phenotypes associated with canonical Wnt ligand expression in zebrafish embryos.

It may be that Wnt4 is just one arm of a progesterone-induced program, making Wnt4

necessary but not sufficient. Other progesterone-induced components, such as RANKL (Beleut et al. 2010) and/or the differentiation/specification factor bHLH protein Id4 (Fernandez-Valdivia et al. 2005), could also be required for an effective response. Wnt4 may be a prime candidate to explain the regulation of mammary stem cell function by endocrine factors. Thus, recent reports describe a dynamic twofold expansion and retraction of the stem cell activity during the 4-d estrus cycle (Joshi et al. 2010), and an 11-fold difference in activity in estrogen/progesterone positive/negative mammary environments (Asselin-Labat et al. 2010). These hormonal dynamics closely resemble the expression of Wnt4 mRNA and protein expression in luminal mammary epithelial cells (Table 1) (Silberstein et al. 2006).

Information derived from other Wnt4-dependent processes, such as kidney development, could provide a useful precedent for mammary gland. For kidney, several Wnt proteins are expressed by the epithelial ureteric bud, including Wnt6, 7b, 9b, and 11, and two Wnt proteins by the morphogenic mesenchyme, Wnt 2b and Wnt4. Of these, Wnt4, 9b, and 11 are essential, and none show redundant functions, suggesting that the nature of the signal generated by each is unique (Carroll et al. 2005; Pulkkinen et al. 2008). In MDCK and other kidney cell types, Wnt4 can induce a canonical signal, but the receptor mediating this signaling event is still mysterious (although Wnt4 binds Fzd6, this is not the canonical signaling reaction) (Lyons et al. 2004). Interestingly, Bernard et al. (2008) suggest that Wnt4 elicits a distinct response from that induced by Wnt5a, namely, accumulation of β -catenin at the cell membranes (of HEK293T cells). These, or other factors, may be important to understanding the molecular mechanism of Wnt4 activity.

IS WNT SIGNALING A DRIVER FOR BREAST TUMOR GROWTH?

Wnt signaling has been known to be highly oncogenic for mouse mammary glands since 1982, when Nusse and Varmus showed that mouse mammary tumors induced by MMTV

were caused by activation of Wnt ligand gene expression. In fact, not only were Wnt1, Wnt3, Wnt3A, and Wnt10b activated to induce mammary tumors, so indeed were Wnt-pathway coactivators RSp2 and RSp3 (Nusse and Varmus 1982; Nusse 1988; Theodorou et al. 2007; Callahan and Smith 2008). Of these, only Wnt10b and the RSp ligands are usually expressed in significant amounts in mammary gland (Table 1). It may be significant that other Wnt components are not on that list—including Lrp6, even though overexpressed Lrp6 can induce hyperplasia in mouse models (Lindvall et al. 2009; Liu et al. 2010; Zhang et al. 2010a).

However, although it is true that the vast majority of colorectal tumors have a Wnt signaling driver (initiated by mutations in one of three genes in the canonical Wnt signaling pathway, namely, inactivating mutations of the tumor suppressors Apc or axin or activating mutations of the oncogene β -catenin), there is little evidence to suggest that breast tumors share this etiology. Mice with an Apc mutation (Apc^{min}), although they develop intestinal polyps, do not spontaneously develop breast tumors. (They are, however, sensitized to tumor development, revealed by the enhanced rate of tumorigenesis after the administration of carcinogens [Moser et al. 1993].) Indeed, unlike mammary glands expressing Wnt ligands, Apc^{min} mammary glands are not hyperplastic, so this loss of function is insufficient to generate a growth signal. The mechanisms governing inactivation of Wnt signaling components may be different for mammary cells; for example, data derived from combination microarray–CGH suggest that the expression of genes in 5q22.2 is reduced in basaloid breast tumors (Geyer et al. 2011). This interval includes Apc. Perhaps total inactivation of the typical Wnt tumor suppressors by mutation is lethal for mammary epithelial cells.

Preneoplastic lesions with ectopic Wnt signaling often require cooperating mutation(s) to progress. Perhaps the lack of Wnt mutations in breast tumors could be explained if these cooperating mutations did not arise readily during breast cancer development, or were not tolerated. For example, gut tissues in Apc^{min} mice

(after loss of heterozygosity) accumulate β -catenin, and this is sufficient to prevent differentiation and induce morphological changes in tissue architecture. Note that this tissue is remarkable for its high rate of continuous growth, which has no parallel in adult mammary gland. However, nuclear β -catenin does not arise until later in tumor development, after Ras is mutated (Phelps et al. 2009). Ras is rarely mutated in any type of breast tumor, although Ras mutations are relatively common in breast tumor cell lines (Hollestelle et al. 2007). Ectopic Ras activity is often detected by surveillance checkpoints (Zhu et al. 2005), and cells are eliminated in response (typically a p53-dependent process). Thus, Ras activation has to be carefully titrated to contribute effectively to tumor development. Perhaps it is no coincidence that the Fgf signaling pathway is frequently activated during Wnt-induced tumor development (where Ras is activated as a downstream effector in a regulated manner) (Callahan and Smith 2008). In developmental models, when Wnt and Fgf are coordinately expressed, the signal generated is distinct from either alone (ten Berge et al. 2008). This may also explain the observation that human basaloid breast tumor aggression is associated with (and dependent on) hyperactivation of Fgf signaling (Turner et al. 2010a,b; Sharpe et al. 2011). Similarly, activation of the tyrosine kinase Ron has been associated with β -catenin phosphorylation (Y⁶⁵⁴ and Y⁶⁷⁰), together with enhanced nuclear translocation and transactivation activity (Wagh et al. 2011). Furthermore, when a Wnt signal is present, two reports suggest that Akt activation is sufficient to activate the nuclear translocation of β -catenin, in human and mouse breast tumor epithelial cells (Korkaya et al. 2009; Zhang et al. 2010b). Akt has been shown to phosphorylate β -catenin (S-552) to enhance nuclear translocation (He et al. 2007), and an inhibitor of Akt signaling (perifosine) inhibits the accumulation and translocation of β -catenin, Wnt signaling reporters, and stem cell activity, in normal cells and tumor cells deficient in PTEN and/or p53. Up-regulation of Akt signaling in the absence of Wnt signals is unlikely to cross-talk to Wnt end points, despite the fact that they share a

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signaling component (GSK3 β) (Korkaya et al. 2009; Ng et al. 2009); indeed, the data to date suggest that the pools of GSK3 β allocated to these pathways are different and separate (Ding et al. 2000).

It is useful to outline the limitations of assays designed to test whether Wnt signaling is a tumor driver, in the following sections.

Wnt-Dependent Subpopulations Are Obscured by General Wnt Signatures

The tumorigenic effects of ectopic Wnt signaling may be associated with only a subpopulation of (stem/progenitor) cells (especially early in tumor development), whereas the tumor majority shows a different Wnt signaling response. This has been illustrated in a Wnt1–Lrp5-dependent transgenic tumor model system (Gunter et al. 2003; Lindvall et al. 2006). These tumors contain a minority “responder” cell population that depends on a paracrine canonical Wnt ligand (Lrp5-positive cells) from the cell majority (Kim et al. 2011). This cell majority is therefore unable to generate a canonical Wnt signal (Lrp5-negative; visualized by axin2 expression), but is nonetheless recruited to grow in a Wnt1-dependent manner. Thus, the Wnt signature is not activated in the cell majority, and the tumor cell population grows in response to a field effect, with so far unknown mediators.

A “field effect” for growth is common for other tumor drivers, such as the estrogen-ER α axis. Thus, for ER α -positive tumors, the tumorigenic ER α receptor is typically expressed by a minority population (ER α -positive cells comprise 1%–100% of tumor cells, depending on the exact pathological criteria), but the Ki67/mitotic index is raised throughout (Clarke 2003; Schlange et al. 2007; Mastroianni et al. 2009). Extrapolating from this idea, it is clear that transcriptional signatures of whole populations of mixed cell types could be difficult to interpret (Huang et al. 2005). The markers c-myc and cyclinD1 that characterize Wnt-dependent transcription for intestinal cells are not prominent (or required for tumorigenesis) for mammary gland (Huang et al. 2005), and indeed are not specific to Wnt signaling. Data also

suggest that activation of Akt signaling may be a component of Wnt ligand-mediated oncogenesis, and this outcome may make ectopic Wnt signaling difficult to recognize by a genetic signature (Korkaya et al. 2009; Zhang et al. 2010b).

Nuclear β -Catenin

As discussed above, a combination of signaling activities may be required to generate nuclear β -catenin. Nuclear β -catenin has been convincingly observed in some breast tumors (Geyer et al. 2011). However, even when Wnt signaling is known to be the tumor driver, it may not be associated with significant immunostaining of nuclear β -catenin, thus the breast tumors identified with ectopic Wnt signaling by this assay may underrepresent the prevalence of this tumor driver. For example, although the growth of Wnt1-induced breast tumors is known to require the continuous presence of Wnt1/Lrp5, there is little support for the presence of nuclear β -catenin (data not shown), despite high expression of Wnt reporters in responder cells (Kim et al. 2011). Furthermore, basaloid breast tumors (in particular) are demonstrably heterogeneous with respect to basal and luminal cell subpopulations (Rakha et al. 2009; Kim et al. 2012), and the Wnt-dependent subpopulation may be rare.

Loss of E-Cadherin

Loss of function for the tumor suppressor E-cadherin (characteristic of many breast tumors) can lead to redistribution or elimination of β -catenin, an E-cadherin binding partner (van de Wetering et al. 2001; Incassati et al. 2010). However, this is not necessarily accompanied by the functional recruitment of β -catenin to the Wnt signaling pathway (Gumbiner 1998; Geyer et al. 2011).

These issues do not eliminate Wnt signaling as a significant tumor driver. Indeed, there is a large body of literature that describes a disproportionate expression of mRNA for positive effectors of Wnt signaling in tumors, either with respect to normal tissue, sometimes with respect to a larger group of breast tumors (for review, see

Incassati et al. 2010). All of these studies have the caveat that the cell composition of tumors is different from subtype to subtype (e.g., the luminal vs. basaloid subtypes, where the latter are mixtures of both basal and luminal cells), and the samples are seldom microdissected, perhaps including contributions from cells that may express high amounts of Wnt signaling components (such as macrophages). However, the accumulated data suggest that there are significant expression of Wnt ligands (summarized by Benhaj et al. 2006), loss of non-canonical signaling (Jonsson et al. 2002; Serra et al. 2011), and shut-down of expression of extracellular inhibitors. Numerous extracellular inhibitors of Wnt signaling are expressed by mammary gland (including 2/4 dkk species, WIF1, and 3/4 sFRP species) (Fig. 2; Table 1). Thus, Ugolini et al. (2001) originally described the epigenetic inhibition of sFRP1 expression in ER α -positive tumors, and Schlange et al. (2007) showed that autocrine Wnt signaling (measured by dvl phosphorylation) was inhibited by pan siRNA to Dvl species, and by the ectopic administration of sFRP1, for a group of breast tumor cells lines that represent all the major subtypes of breast tumor. This relied partly on transactivation of EGFR (and ERK phosphorylation), mediated by Wnt-induced MMP activation and the release of erbB2 ligands from extracellular matrix. Note that this study (and others) may not implicate canonical Wnt signaling, but Wnt ligand/Fzd-dependent growth and invasion. Alterations in mRNA species for Wnt signaling components require cautious interpretation, because the effects of so-called inhibitors (in particular) are often difficult to predict a priori. Thus, loss of dkk3 (reported in breast tumors) (Suzuki et al. 2008; Veeck et al. 2008) induces apoptosis of lung cancer cells (Jung et al. 2010), and ectopic dkk3 can potentiate Wnt signaling (probably by binding and disabling Kremen) (Nakamura and Hackam 2010).

However, despite all these caveats, Geyer et al. (2011) have recently published clear pictures of the localization and amount of β -catenin (using two different monoclonal antibodies) visualized for a cohort of 245 breast tumors, and showed that nuclear β -catenin is prevalent

in a subpopulation of tumor epithelial cells in approximately one-third of basaloid breast tumors. Basaloid breast tumors comprise about one-sixth of total breast tumors and show high levels of expression of basal cell markers (both protein and mRNA) alongside luminal cell markers. The Wnt receptor, Lrp6, is overexpressed in some of these tumors (by approximately twofold to fivefold) (Lindvall et al. 2009; Liu et al. 2010), and inhibition of Lrp6 in MDA-MB231 basaloid cells in culture inhibits TOPFLASH ($10\times$ – $100\times$), growth ($2\times$), Axin2 expression ($2\times$), colony formation ($5\times$) in vitro, and tumor growth in vivo (Matsuda et al. 2009).

The focus is now on the inhibitors of Wnt signaling for their possible use in tumor treatment, and given the data described above, the most likely target cohort may be patients with basaloid tumors. Drug development of inhibitors of Wnt signaling tend to assay HEK293 cells, and they have been highly successful in producing molecules that inhibit signaling outside and inside of the cell (Barker and Clevers 2006; Dasgupta 2009). It may be possible to exploit unique aspects of Wnt signaling in breast tumor cells to enhance these more generic approaches.

CONCLUDING REMARKS

This is not a comprehensive review, in part because it focuses on the cell surface components of Wnt signaling, and does not detail how that signal is modified by (1) cytoplasmic modifiers; (2) nuclear uptake mechanisms for β -catenin; (3) the nature of the TCF species expressed, together with the associated transcription factors (Archbold et al. 2012); or (4) the epigenetic chromatin status (Hatzis et al. 2008; Gu et al. 2009; Wend et al. 2010). This article mentions these mechanisms only in passing because there is little specific information published to describe the importance of these aspects with respect to mammary gland or mammary cell lines. We have focused instead on some of the outstanding questions likely to be our preoccupation in the near future, such as the evident plasticity of fate induced by Wnt signaling (indicated by transitions between basal and luminal

Table 1. Characterized expression and function of cell surface Wnt signaling components in mammary cells and tissues

Gene	Array	mRNA	Protein	Cell/tissue type	Function	Citation
Wnt1	X					All sources
		X (N)		Normal mouse MG	Tumorigenic in transgenic mice (MMTV-Wnt1) Induces branching and lobuloalveolar hyperplasia	Tsukamoto et al. 1988
		✓ (R)		T-47D cells		Suzuki et al. 2008
					Highly transforming in C57MG cells Transfection into HC11 cells and transplantation into cleared fat pads leads to fibrotic outgrowths	Wong et al. 1994 Humphreys and Rosen 1997
Wnt2 (Wnt2a)	✓	✓ (I)		TEB stroma		Kouros-Mehr and Werb 2006
		✓ (R, I, N)		Basal cells and TEBs	mRNA decreases $10\times - 40\times$ during pregnancy and lactation Transforms C57MG cells	Buhler et al. 1993 Wong et al. 1994
		✓ (RP, I)		Normal human breast fibroblasts and human breast tumor epithelium	Low expression levels in normal tissue and overexpression ($5\times - 8\times$) in some carcinomas Transfection into HC11 cells and transplantation into cleared fat pads leads to fibrotic outgrowths	Dale et al. 1996 Humphreys and Rosen 1997
				Mouse breast cancer cell line bone metastasis model	Significantly down-regulated at tumor–bone interface	Sadanandam et al. 2011
Wnt2b (Wnt13)	X					All sources
		✓ (R)		Normal human mammary tissue and infiltrating ductal carcinoma biopsies	Low expression levels in normal tissue and overexpression ($5\times - 8\times$) in some carcinomas	Bergstein et al. 1995
Wnt3	X					All sources
		✓ (N)		Normal mouse mammary gland	MMTV CIS	Roelink et al. 1990; Theodorou et al. 2007; Callahan and Smith 2008
		✓ (RP)		Normal human breast tissue, MTSV1-7, BT20, MCF7adr (ER [−] , EGFR ⁺)		Huguet et al. 1994

Wnt3a	X					Highly transforming in C57MG cells MMTV CIS	All sources Wong et al. 1994 Roelink et al. 1990; Theodorou et al. 2007; Callahan and Smith 2008
Wnt4	✓					Mature luminal	Lim et al. 2010
	✓					Differentiated luminal	Grigoriadis et al. 2006
	✓					Luminal ER ⁺ cells	Kendrick et al. 2008
		✓				NDE	Kendrick et al. 2008
	✓	✓ (I)				TEBs/mature ducts	Kouros-Mehr and Werb 2006
		✓ (I)				Luminal	Briskin et al. 2000
			X (W)			C3H 10T1/2 cells	Bradbury et al. 1995; Briskin et al. 2000
		✓ (R)				Luminal cells from mammary glands treated with estrogen	Joshi et al. 2010
		✓ (N)				Highest expression in virgin also in cell lines C57MG, C127L, and NMuMG cells	Gavin and McMahon 1992
		✓ (RP)				MDA415	Huguet et al. 1994
Wnt5a	✓	✓				Luminal ER ⁺ cells	Kendrick et al. 2008
	✓					Mature luminal cells	Lim et al. 2010
	✓					Luminal cells	Grigoriadis et al. 2006
	✓	✓ (I)				TEB epithelium	Kouros-Mehr and Werb 2006
		✓ (N)				Weakly expressed in 12-wk-old virgin and induced during pregnancy	Weber-Hall et al. 1994
		✓ (N)				Whole mammary gland	Buhler et al. 1993
						Expression detectable during pregnancy (peak at 10 days), no expression during lactation	
						Required for MG development and TGFβ-mediated inhibition of ductal growth.	Roarty and Serra 2007
						Wnt5a KO mice show accelerated ductal morphogenesis	

Continued

Table 1. Continued

Gene	Array	mRNA	Protein	Cell/tissue type	Function	Citation
	✓ (N)			Virgin, increase during pregnancy; C57MG and C127I cells		Gavin and McMahon 1992
	✓ (N)			Human breast carcinoma	Does not transform C57MG cells	Iozzo et al. 1995
	✓ (RP, I)			Little to no expression in normal breast tissue Increased expression in benign proliferations and invasive breast cancer ISH signal localized to epithelial compartment		Lejeune et al. 1995
					Overexpression results in lactation defect in MMTV-Wnt5a mice	Baxley et al. 2011
Wnt5b	✓			Bipotent CFCs		Raouf et al. 2008
	✓			Luminal		Grigoriadis et al. 2006
	✓	✓ (I)		TEBs/mature ducts		Kouros-Mehr and Werb 2006
		✓ (N)		Whole mammary gland	Expression detectable during pregnancy (d10-18), no expression during lactation Transforms C57MG cells	Buhler et al. 1993 Wong et al. 1994
		✓ (N)		Virgin mammary gland, increases during pregnancy		Gavin and McMahon 1992
Wnt6		✓		Basal cells		Kendrick et al. 2008
	✓	✓ (I)		TEBs/mature ducts		Kouros-Mehr and Werb 2006
		✓ (N)		Expressed in virgin, increase during pregnancy		Gavin and McMahon 1992
Wnt7a	X					All sources
					Highly transforming in C57MG cells	Wong et al. 1994
		✓ (N)		NMuMG cell line		Gavin and McMahon 1992
Wnt7b	✓	✓		Luminal ER+ cells		Kendrick et al. 2008
		✓		Luminal ER- cells		Kendrick et al. 2008
	✓			Mature luminal cells		Lim et al. 2010
	✓	✓ (I)		TEB epithelium		Kouros-Mehr and Werb 2006
					Does not alter MG development Transforms C57MG cells	Wong et al. 1994; Naylor et al. 2000

				Transfection into HC11 cells and transplantation into cleared fat pads leads to fibrotic outgrowths and palpable adenocarcinomas	Humphreys and Rosen 1997
	✓ (N)				
	✓				Gavin and McMahon 1992
	(RP)				Huguet et al. 1994
Wnt9a	✓				Grigoriadis et al. 2006
(Wnt14)		✓			Kirikoshi et al. 2001
Wnt9b		✓			Qian et al. 2003
(Wnt14b					
Wnt15)		✓			Kirikoshi et al. 2001
Wnt10a	✓	✓			Kendrick et al. 2008
Wnt10b					
(Wnt12)					
		✓			
	(RPR)				Bui et al. 1997
		✓ (N)			Lee et al. 1995
		✓			Christiansen et al. 1995
Wnt11	X				All sources
					Christiansen et al. 1996
					Dwyer et al. 2010
Wnt16	✓				Kim et al. 2009

Continued

Table 1. *Continued*

Gene	Array	mRNA	Protein	Cell/tissue type	Function	Citation
RSpo1	✓	✓ (I)		Luminal ER- Mammary mesenchyme (E15.5)		Kendrick et al. 2008 Nam et al. 2006, 2007 Chadi et al. 2009
					KD might result in absence of ductal side-branching and alveolar formation	
RSpo2	X	✓ (R)		Detected at very low levels early in MG development but not detectable in MG of day 15 pregnant mice		Not in adult tissues Lowther et al. 2005
		X (R)	X (W, ICC)	HC11, C57MG mouse cell lines		Klauzinska et al. 2011
		X (R)		HC11 cells		SA Fakhraldeen and CM Alexander, unpubl.
RSpo3	X				CIS for MMTV	Not in adult tissues Theodorou et al. 2007; Callahan and Smith 2008
Fzd1	✓	✓		Basal/myoepithelial		Kendrick et al. 2008
	✓			Down-regulated in mature luminal cells		Lim et al. 2010
	✓			Mature myoepithelial cells		Raouf et al. 2008
	✓	✓ (I)		TEB stroma		Kouros-Mehr and Werb 2006
		✓ (R)		HMEC, MDA-MB-468, MDA-MB-453, MCF- 7, T-47D, BT-20, and BT-474 cells		Benhaj et al. 2006
Fzd2	✓	✓		Basal/myoepithelium		Kendrick et al. 2008
	✓	✓ (I)		TEB		Kouros-Mehr and Werb 2006
		✓ (R)		MDA-MB-468, MDA-MB-453, MCF-7, T47D, BT-20, and BT-474 cells		Benhaj et al. 2006
Fzd3	✓			Basal/myo		Kendrick et al. 2008
	✓			Myo		Grigoriadis et al. 2006
		✓ (R)		MDA-MB-231 and SUM-159 cells	Functions in MDA-MB-231 cell motility	Valastyan et al. 2009
			✓ (W)	MDA-MB-231 cells		
		✓ (R)		HMEC, MDA-MB-468, MCF-7, T-47D, BT-20, and BT-474 cells		Benhaj et al. 2006

Fzd4	X					All sources
Fzd5	✓				Luminal	Grigoriadis et al. 2006
		✓ (R)			MDA-MB-468 cells	Benhaj et al. 2006
Fzd6	✓				Basal/myoepithelium	Jones et al. 2004
	✓				Basal/myoepithelium	Grigoriadis et al. 2006
		✓ (R)			HMEC, MDA-MB-468, MDA-MB-453, MCF-7, T-47D, BT-20, and BT-474 cells	Benhaj et al. 2006
Fzd7	✓	✓			Basal/myoepithelium	Kendrick et al. 2008
	✓				Luminal-restricted CFCs	Raouf et al. 2008
		✓ (R)			HMEC, MDA-MB-468, MDA-MB-453, T-47D, and BT-20 cells	Benhaj et al. 2006
					Plays a role in proliferation and invasiveness of TNBC cell lines	Yang et al. 2011
Fzd8	✓	✓			Basal/myoepithelium	Kendrick et al. 2008
	✓				MaSC-enriched fraction	Lim et al. 2010
		✓ (R)			HMEC and MDA-MB-453 cells	Benhaj et al. 2006
		X (R)			MDA-MB-468	
Fzd9	✓				Differentiated luminal cells	Raouf et al. 2008
Fzd10	X					
Lrp5	✓					
		✓ (E, IHC)			Basal cells	Badders et al. 2009
		✓			Translation induced in Wnt1-induced luminal cells	Kim et al. 2011
		✓ (R)			HMEC, MDA-MB-468, MDA-MB-453, MCF-7, T-47D, BT-20, and BT-474 cells	Benhaj et al. 2006
		✓ (R)			Breast cancer specimens and normal breast tissue specimens	Bjorklund et al. 2009
					Mammary stem cell maintenance	Lindvall et al. 2006
					Maintenance of basal cell population	Badders et al. 2009

Continued

Table 1. *Continued*

Gene	Array	mRNA	Protein	Cell/tissue type	Function	Citation
Lrp6	✓			Luminal		Grigoriadis et al. 2006
			✓ (W)	SUM1315 cells		DiMeo et al. 2009
		✓ (R)		HMEC, MDA-MB-468, MDA-MB-453, MCF-7, T-47D, BT-20, and BT-474 cells		Benhaj et al. 2006
		✓ (R)		Breast tumor, MDA-MB-231 and MDA-MB-468 cells		Li et al. 2004
					OE in mice (MMTV-Lrp6) induces hyper-lobular development and increased TEB number	Zhang et al. 2010a
			✓ (E, IHC)	Basal in adult Basal and luminal in newborns	Loss interferes with mammary placode, fat pad, and branching development during embryogenesis	Badders et al. 2009; Lindvall et al. 2009
					Heterozygosity for inactivating mutation leads to reduced TEBs and branches	
Ror1	✓			Luminal CFCs		Raouf et al. 2008
	✓			Myoepithelium/basal cells		Kendrick et al. 2008
Ror2		✓ (R)	✓ (IHC)	Expressed in basal/myoepithelial cells and luminal cells of mouse mammary gland		K Roarty and JM Rosen, pers. comm.
		✓ (R)		Expressed in breast cancer cell lines (MCF7 and MDA-MB231) and brain metastases of brCA in vivo		Klemm et al. 2011
Lgr4	✓			Basal cells		Kendrick et al. 2008
	X			Normal luminal epithelium		Grigoriadis et al. 2006
Lgr5	✓			Basal cells		Kendrick et al. 2008
Dkk1		✓		Basal cells		Kendrick et al. 2008
	✓			NDE		Kendrick et al. 2008
	✓			Luminal cells		Grigoriadis et al. 2006
			✓ (W)	SUM1315 cells		DiMeo et al. 2009
					OE prevents mammary placode development	Chu et al. 2004
		X (R)		HC11 mouse cells		SA Fakhraldeen and CM Alexander, unpubl.

Dkk2	X						All sources
		X (R)		HC11 mouse cells			SA Fakhraldeen and CM Alexander, unpubl.
Dkk3	✓	✓		Bipotent CFCs			Raouf et al. 2008
			✓	Differentiated basal cells			Raouf et al. 2008
	✓			MaSC-enriched			Lim et al. 2010
	✓			Myoepithelium/basal cells			Jones et al. 2004
	✓			Myoepithelium/basal cells			Grigoriadis et al. 2006
	✓			Myoepithelium/basal cells			Kendrick et al. 2008
		X (R)		HC11 cells			SA Fakhraldeen and CM Alexander, unpubl.
Dkk4	X						All sources
		X (R)					SA Fakhraldeen and CM Alexander, unpubl.
WIF1	✓	✓		Myoepithelium/basal cells			Kendrick et al. 2008
	✓			MaSC-enriched			Lim et al. 2010
		✓ (R)		T47D, normal human breast tissue	Targeted for epigenetic silencing in human breast cancer		Ai et al. 2006
			✓	Normal breast tissue	Down-regulated in invasive ductal breast carcinoma		Wissmann et al. 2003
	✓		(IHC)	Mouse breast cancer cell line bone metastasis model	Increased expression at tumor-bone interface		Sadanandam et al. 2011
SFRP1	✓	✓		Myoepithelium/basal cells			Kendrick et al. 2008
	✓			Down-regulated in mature luminal			Lim et al. 2010
					Maintains/reduces canonical signaling levels		Cowling et al. 2007
					mRNA expression lost in >80% of invasive breast carcinomas (medullary)		Ugolini et al. 2001
			✓	Mammary epithelial cells	Tumor suppressive function		Klopocki et al. 2004
			(IHC)		Lost in tumors		
					Loss associated with poor prognosis		

Continued

Table 1. Continued

Gene	Array	mRNA	Protein	Cell/tissue type	Function	Citation
SFRP2	✓		X (W)	SUM1315 cells		DiMeo et al. 2009
				Mature luminal		Grigoriadis et al. 2006
		✓ (R)		Normal breast tissue		Suzuki et al. 2008
	X (R)			MDA-MB-435S, MDA-MB-436, MDA-MB-468, MDA-MB-157, MDA-MB-361, and ZR-75-1 cells		
				MCF-7, MDA-MB-231, T47D, and SK-BR-3 cells		
SFRP4	✓			Myoepithelium/basal cells		Grigoriadis et al. 2006
		✓ (R)		Normal breast tissue and MDA-MB-157 cells		Suzuki et al. 2008
		X (R)		MCF-7, MDA-MB-231, MDA-MB-435S, MDA-MB-468, T-47D, SK-BR-3, MDA-MB-453, and ZR-75-1 cells		
SFRP5		✓		Luminal		Grigoriadis et al. 2006
	✓			Mouse breast cancer cell line bone metastasis model	Increased expression at tumor-bone interface	Sadanandam et al. 2011
WISP1	X					All sources
WISP2	✓			Myoepithelium/basal cells		Grigoriadis et al. 2006
	✓			Myoepithelium/basal cells		Kendrick et al. 2008
	✓			Luminal		Grigoriadis et al. 2006
		✓ (R)	✓ (W)	MCF7, T47D, ZR-75.1, SKBr3	Knockdown promotes proliferation (including E-independent) of MCF7 cells	Fritah et al. 2008
		X (R)	X (W)	HMEC, MDA-MB-231	Inducing expression inhibits proliferation of MCF7 and MDA-MB-231 cells

WISP3	✓	Bipotent CFCs	Grigoriadis et al. 2006
Naked1	✓	Myoepithelium/basal cells	Kendrick et al. 2008
Naked2	✓	Bipotent CFCs	Raouf et al. 2008
	✓	Myoepithelium/basal cells	Kendrick et al. 2008

The expression patterns of Wnt-receptor signaling components are summarized, deduced from significant expression by mRNA species by microarray analysis (Array), or more specific analysis by qPCR/RT-PCR (R), in situ hybridization (I), RNase protection (RP), or Northern blotting (N), and of cognate proteins, shown by flow cytometry (F), immunohistochemical/cytochemical localization (IHC/ICC), or Western blotting (W). Functional information for each component is summarized.

Specificity of expression in mammary gland cell types: The array results reported are from several separate studies, and the cell fractions and gene chips are as follows:

1. (Raouf et al. 2008) Normal human mammary epithelial cells were sorted into four fractions. Both two stem/progenitor-enriched fractions (a bipotent basal stem cell and a luminal-restricted luminal progenitor; both scored as colony-forming cells in vitro, CFCs) showed high EpCAM expression compared with their mature myoepithelial and luminal counterparts. Two mature cell types extracted from the low-EpCAM fraction were luminal cells (high Muc1/CD133, low Thy1/CD10) and myoepithelial cells (high Thy1 and low Muc1). mRNA was analyzed using Affymetrix human X3P GeneChip arrays or PCR-LongSAGE libraries.

2. (Lim et al. 2010) Normal mouse mammary glands were sorted into three epithelial cell fractions, and one stromal. A basal stem cell-enriched fraction (MaSC) was separated as $\beta 1$ integrin (CD29)-high, CD24-low, and CD61-positive. Luminal cells were isolated as CD29-low (CD24-positive), and the progenitors were CD61-negative, whereas the mature cells were CD61-positive. For normal human mammary gland cells, the sorting parameters were different, but the cell populations were broadly similar. Three fractions described were a basal, mammary stem cell-enriched (MaSC) fraction— $\alpha 6$ integrin (CD49f)-high, EpCAM-negative—and two luminal fractions—EpCAM-positive, the progenitor fraction CD49f-positive, and the mature fraction CD49f-negative. mRNA was analyzed using Illumina MouseWG-6 v 2.0 BeadChips.

3. (Kendrick et al. 2008) Normal mouse mammary glands were sorted into three epithelial cell fractions, broadly similar to those described by Lim et al. (2010), although the cell surface markers used are different, as is the preparation and exclusion of other non-epithelial cell types. A basal cell fraction (stem/myoepithelial) was collected as CD24-low and Sca1-negative. Two luminal cell fractions both expressed CD24; the ER α -negative, luminal progenitor cell fraction was isolated as Sca1-low; and the mature, ER α -positive cells were Sca1-positive. mRNA analysis was performed using Mouse Affymetrix Mouse Expression MOE430 2.0 arrays.

4. (Grigoriadis et al. 2006) This is the only study to use cultured primary cells. Cells from normal human breast and from primary tumors were separated by double immunomagnetic sorting, either as EMA⁺/integrin $\beta 4$ -negative luminal cells, or CD10⁺/BerEP4-negative myoepithelial cells. The mRNA was analyzed using MPSS and Affymetrix Human Genome U133 Plus 2.0 GeneChip, CodeLink Human Whole Genome Bioarray, Agilent Whole Human Genome Oligo Microarray 44K cDNA array, and 20K cDNA microarray (constructed at the Breakthrough Breast Cancer Research Center, UK).

(CFC) Colony-forming cell; (NDE) not differentially expressed; (TNBC) triple negative breast cancer; (OE) overexpression; (KO) knockout; (ISH) in situ hybridization; (IHC) immunohistochemistry; (CIS) common integration site (applied to MMTV retroviral tagging studies). Wnt ligand and Fzd receptor expression in embryonic mammary tissues has been previously described (Chu et al. 2004).

(✓) Detectable expression.

(X) No detectable expression.

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mammary epithelial cell fates), the molecules that regulate the mammary stem cell niche, the combinatorial outputs that are offered by the multiplicity of Wnt signals perceived by mammary epithelial cells, the difficulties of conclusively implicating Wnt signaling in tumor growth or tumor cell survival, and the potential Wnt responses of the Lrp-deficient luminal cell population. Key Wnt signaling components that discriminate luminal and basal cell function, such as Lrp5, are regulated at the posttranscriptional level (Joshi et al. 2010; Kim et al. 2011), and this requires more investigation. Much of the information available to describe the regulation of Wnt signaling involves ectopic expression of various components. At many levels, this does not reflect the obvious competition for binding partners that regulates the transcriptional response and the response to Wnt ligands at the cell surface. The next generation of data will focus more heavily on studying the function of Wnt signaling components when expressed at endogenous levels. Given the new generation of Wnt signaling inhibitors, there are new horizons for investigations of mechanism and therapeutic potential.

ACKNOWLEDGMENTS

This work is supported by the BCRP/DOD Era of Hope Scholars Award W81XWH (to C.M.A., S.G., S.K., and S.F.).

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