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From the ranks of mammary progesterone mediators, RANKL takes the spotlight

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Abstract

Whether during the diestrus phase of the estrous cycle or with pregnancy onset, the mitogenic effects of progesterone are well-established in the murine mammary epithelium. Importantly, progesterone-induced mitogenicity is critical for mammary tumor promotion, providing one explanation for the increase in breast cancer-risk observed with prolonged progestin-based hormone therapy. At the cellular level, progesterone projects its mitogenic influence through an evolutionary conserved paracrine mechanism of action. In this regard, recent studies provide compelling support for receptor activator of NF- κ B ligand (RANKL) as a key paracrine mediator of the progesterone mitogenic signal. Induction of RANKL is sufficient to elicit mammary ductal side-branching and alveologenesis, the very morphogenetic responses elicited by progesterone during pregnancy and at diestrus. Significantly, the proliferative and pro-survival signals triggered by RANKL are also required for progestin-promotion of mammary tumorigenesis, underscoring a dual role for RANKL in progesterone-dependent mammary morphogenesis and tumorigenesis. Recently, RANKL has been shown to be critical for progesterone-induced expansion of the mammary stem cell population (and its lineal descendents), thereby advancing our conceptual understanding not only of RANKL's involvement in normal mammary morphogenesis but also in breast cancer risk associated with sustained hormone exposure. Finally, these studies together suggest that chemotherapeutic intervention of RANKL signaling represents a feasible approach for the effective prevention and/or treatment of hormone-responsive breast cancers.

Keywords

Progesterone; Mammary mitogenesis; Receptor Activator of NF- κ B Ligand; Paracrine signaling; Tumorigenesis; Stem cells

I. Progesterone: a mammary mitogen

1.1. Introduction

Toward defining a causal relationship between “internal secretions” of sex glands and the development of secondary sexual characteristics, the young Vienna-trained gynecologist,

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Josef Halban (1870–1937), demonstrated by transplantation of guinea pig tissues that the ovary is an essential sex gland for mammary development (Halban, 1905). Halban's British contemporary, the distinguished surgeon Sir George Thomas Beatson (1848–1933), made the seminal discovery that breast cancer progression can be markedly curtailed by bilateral oophorectomy (Beatson, 1896), thereby linking ovarian secretions to both mammary development and malignancy. Following the identification of estrogen and progesterone from these secretions (Allen and Doisy, 1923; Corner and Allen, 1929), endocrine ablation and/hormone “add-back” studies would underscore the collective importance of both ovarian steroids along with pituitary factors in the control of mammary morphogenesis and function (Nandi, 1958).

For decades since, however, ovarian estradiol was considered the primary steroidal mitogen that is preferentially required for normal mammary morphogenesis as well as for early establishment and expansion of mammary tumors. Influenced in large part by progesterone's recognized role in suppressing estrogen-induced mitogenesis in the endometrial epithelium (the rationale for the inclusion of progestins in postmenopausal hormone therapies), a similar anti-mitogenic role for progesterone was assumed in the mammary gland; reviewed in (Lange, 2008). However, this simple extrapolation is undercut by findings which show that mammary epithelial mitogenesis is highest during the progesterone-dominant luteal (or secretory) phase of the human menstrual cycle (Goebelsmann and Mishell, 1979); that progestin-inclusion in postmenopausal hormone therapy expands the mammary epithelium beyond that detected with estrogen treatment alone (Hofseth et al., 1999); that progestin-inclusion in combined oral contraceptives reduces estradiol-dependent endometrial carcinomas but not breast cancers (Pike and Spicer, 2000); and that a statistically significant increase in breast cancer risk is associated with progestin-based postmenopausal hormone therapies but not with estrogen alone therapies (Writing group for women's health initiative investigators, 2002; LaCroix et al. 2011). The uncertainty in the field is based in part on the obvious experimental limits to which the human can serve as a model for further in-depth investigational studies as well as on the inherent difficulties in separating estrogen responses from those of progesterone. Therefore, the mouse—with its genetic malleability—has proven to be an indispensable surrogate for the human in disclosing the cellular and molecular mechanism(s) specifically attributable to progesterone in the mammary epithelium.

1.2. A mitogenic role confirmed

Most of the physiological effects of systemic progesterone are mediated by its nuclear receptor (the progesterone receptor (PR)), a class I member of the nuclear receptor superfamily of transcription factors (Mangelsdorf et al., 1995), which also includes the cognate nuclear receptor for estrogen (the estrogen receptor (ER); the alpha form is considered in this review). Upon binding progesterone or progestin ligand, activated ligand-bound PR is thought to directly or indirectly interface with specific response elements in the genome—during which coregulators (*i.e.* coactivators or corepressors) are recruited to the nucleating transcriptional complex—to turn on or off discrete subsets of target genes. At the molecular level, the transcriptional induction and/or silencing of these target genes manifests the physiological response of the target cell (*i.e.* the mammary epithelial cell) to progesterone. In many physiological contexts, PR expression is induced by agonist-bound ER thereby confounding the assignment of specific physiological responses to each of these nuclear receptors *in vivo*.

Because of the decades-long controversies surrounding progesterone's role in a number of target tissues, of the necessity to clearly distinguish progesterone responses from those of estrogen, and of the need to define progesterone's genomic responses from its non-genomic effects (Sen and Hammes, 2010), a PR knockout (PRKO) mouse was generated by gene-

targeting methods (Lydon et al., 1995). While the spectrum of phenotypes displayed by the PRKO mouse underscored the functional pleiotropy of PR-mediated signaling in female reproductive biology, studies on the PRKO mammary gland in particular would reinforce and greatly extend our understanding of progesterone as a mammary mitogen.

As with all mammals, postnatal mammary development in the mouse broadly comprises two separate allometric growth phases (Daniel and Silberstein, 1987). The first manifests with puberty onset, the second in response to pregnancy. With puberty, cap cells of terminal end-buds proliferate to elongate epithelial ducts and generate simple dichotomous branching to the periphery of the mammary fat pad. In the juvenile, the mammary gland is relatively growth quiescent except for some nascent side-branching and limited alveolar budding as a consequence of recurrent ovarian cyclicity. During early pregnancy, however, the mammary epithelium undertakes an expansive and accelerated proliferative program which results in numerous ductal side-branches and alveoli that progressively in-fill the interductal spaces as pregnancy advances to term. The net result of these overt proliferative changes is to generate sufficient numbers of alveolar epithelial cells that undergo prolactin-dependent differentiation during mid-late pregnancy. Removal of the suckling stimulus at weaning elicits breakdown of the alveolar-ductal network *via* proteinase and apoptotic mediated reductive remodeling events, termed involution. Following involution, the return of the mammary gland to a ductal morphology akin to that of the pre-pregnant mouse completes the cycle of development. Importantly, it's the ability of the mammary gland to reenter this cycle with each successive pregnancy that previously suggested the existence of a resident mammary stem cell (MaSC) population (DeOme et al, 1959; Daniel et al, 1975; Smith et al., 1988; Kordon et al, 1998) and reviewed in (Wagner and Smith, 2005).

In contrast to the ER knockout (ERKO) mouse (Bocchinfuso and Korach, 1997), in which abrogation of estrogen signaling blocks mammary ductal outgrowth at puberty, PRKO mammary development progresses normally through the first allometric growth stage (Lydon et al., 1995). These observations underscore the importance of ovarian estrogen over progesterone during immediate post-pubertal ductal morphogenesis. However, applying the hormone-treated ovariectomized mouse model to the PRKO, morphological and cellular studies disclosed the indispensable role of progesterone signaling for mammary ductal side-branching and alveologenesi (Lydon et al., 1995), hallmarks of the second allometric growth phase. At the cellular level, the underlying cause of the PRKO mammary defect is an inability of the PRKO mammary epithelium to initiate a mitogenic response to hormone exposure (Lydon et al., 1999). Put simply, these results

confirmed progesterone as a mammary mitogen which signals its proliferative effects through its nuclear receptor. As further testament to the critical importance of mammary PR during pregnancy-induced ductal side-branching and alveologenesi, mammary epithelial cell transplantation and tissue reconstitution studies revealed that transplants containing PRKO mammary epithelial cells within wild-type (WT) hosts fail to elicit these structural changes despite exposure to the full-spectrum of pregnancy hormones (Briskin et al., 1998). While these studies unequivocally defined the necessity of PR in mammary morphogenetic changes that occur with pregnancy-onset, these findings immediately raised the critical question: what are the cellular and molecular mechanisms that transduce the progesterone signal to a mitogenic response?

2. A paracrine mechanism of action revealed

2.1. At the cellular level

In common with the human breast (Clarke, 2003), ER and PR expression colocalize (ER+/PR+) to a subset of luminal epithelial cells in the murine mammary gland (Aupperlee and

Haslam, 2007). Remarkably, co-labeling experiments demonstrated that ER+/PR+ cells are a separate cell entity from nearby ER-/PR- cells that proliferate in response to steroid hormone (Seagroves et al., 2000). Importantly, this segregation pattern between steroid receptor positive and proliferating cells is observed in human breast tissue (Clarke et al., 1997) and more recently in three dimensional cultures of human breast epithelial cells (Graham et al., 2009), suggesting an evolutionary conserved mechanism of action for steroid hormone signaling in the mammary epithelium. Although counterintuitive, these findings suggest that progesterone (along with estrogen) projects its proliferative signal through an indirect or paracrine signaling mechanism in which ER+/PR+ cells ("transmitter" cells) release progesterone-induced paracrine factors that instruct neighboring ER-/PR- cells ("responder" cells) to proliferate. Indeed early functional support for this proposal comes from mammary epithelial cell transplant studies which showed that PRKO mammary epithelial cells (when mixed with WT cells) contribute to alveoli and ductal side-branches in a reconstituted gland of a pregnant mouse (Briskin et al., 1998). These results suggest that responder cells (most likely alveolar and ductal progenitor cells) within the PRKO mammary epithelial cell population receive paracrine signals from transmitter cells derived from co-mingled WT cells which enable PRKO cells to contribute to alveoli formation. Interestingly, recent pulse-labeling experiments suggest that paracrine signaling develops from an earlier autocrine mode which rapidly (but transiently) manifests within the first 24-hours of hormone exposure (Beleut et al., 2010).

2.2. At the molecular level

To formulate a molecular model to explain progesterone's paracrine signaling mechanism at the cellular level, identification of the key molecular mediators of the mammary progesterone signal is essential. Not unexpectedly, microarray data predict a multitude of genes, pathways, and networks most likely mediate (directly or indirectly) mammary PR action (Fernandez-Valdivia et al., 2008). Despite this complexity, however, recent investigations provide compelling support for the unique importance of receptor activator of NF- κ B ligand (RANKL) as a pivotal paracrine mediator of progesterone-dependent mammary morphogenesis and tumorigenesis.

3. RANKL is a potent mediator of mammary progesterone action

3.1. Mammary morphogenesis requires the RANKL signaling axis

A cytokine member of the tumor necrosis factor (TNF) superfamily, RANKL signals through its cognate receptor, RANK (Blair et al., 2006; Dougall and Chaisson, 2006). Both RANKL and RANK engage as transmembrane homotrimers; however, depending on signaling context, RANKL also acts (*via* RANK) through its cleaved ectodomain (Ikeda et al., 2001). Following RANKL binding, RANK initiates signaling cascades that are essential for a multitude of physiological processes which include osteoclastogenesis and bone-remodeling, T-cell and dendritic cell survival and communication, lymph-node organogenesis, thymic epithelial development and central whole-body thermoregulation (Dougall et al., 1999; Hanada et al., 2009; Kong et al., 1999; Rossi et al., 2007).

Over a decade-ago, Penninger's group revealed that mice deficient in RANKL (or RANK) exhibit a significant decrease in parity-induced mammary alveologenesis which results in a lactational defect (Fata et al., 2000). Initial cell and molecular studies demonstrated that the mammary defect is underpinned by an absence of RANKL-induced proliferative and pro-survival signaling programs, both of which are required for expansion and maintenance of the epithelial compartment during pregnancy. As its moniker indicates, RANKL signals through I κ B kinase- α (IKK- α) to activate the pleiotropic transcription factor: NF- κ B (Cao and Karin, 2003). One of the targets of this activation is cyclin D1 which is thought to

license mammary cell-cycle progression in response to RANKL (Cao et al., 2001). Signaling through the activated phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Fata et al., 2000), RANKL also enables the mammary epithelium to evade premature apoptosis thereby maintaining the increases in epithelial cell content gained during pregnancy. Additional studies reveal that mammary RANKL signals through Id2 and CAAT/enhancer binding protein- β (C/EBP- β) (Kim et al., 2002; Kim et al., 2006), highlighting a wider spectrum of mammary RANKL targets than previously suspected.

3.2. How does RANKL "rank" as a mammary PR mediator?

Obviously, the striking impairment in alveolar development resulting from the absence of mammary RANKL (or RANK) drew immediate parallels with the PRKO mammary phenotype (Lydon et al., 1995), implicating a regulatory link between progesterone and RANKL. Indeed, the original Penninger study demonstrated that mammary RANKL is induced by exogenous progesterone (Fata et al., 2000), providing the first tangible support for a possible mediator role for RANKL in progesterone-induced mammary morphogenesis. This support was further strengthened by the finding that: (a) RANKL is markedly induced in the mammary gland during early-to-mid pregnancy (Fernandez-Valdivia et al., 2009; Gonzalez-Suarez et al., 2007; Srivastava et al., 2003), the proliferative-phase of pregnancy in which progesterone signaling drives mammary epithelial expansion and morphogenesis; (b) mammary RANKL expression is rapidly induced following acute progesterone exposure in the WT virgin (Fernandez-Valdivia et al., 2008), suggesting that RANKL is an early paracrine mediator of mammary progesterone action. Indeed, recent studies indicate that RANKL represents one of the earliest progesterone targets to emerge following the autocrine-to-paracrine switch in mammary PR signaling (Beleut et al., 2010); (c) progesterone-induced RANKL in an ovariectomized mouse (Mulac-Jericevic et al., 2003) — or RANKL expression in the mammary epithelium of a pregnant mouse (Fernandez-Valdivia et al., 2009)—is confined to ER+/PR+ transmitter cells that are located near responder cells which express cyclin D1 and proliferate. This observation suggests that mammary RANKL acts as a direct molecular link between transmitter and responder cells and does so, in part, by mediating progesterone-induced cyclin D1 expression; and finally (d) transgenic overexpression of mammary RANKL or RANK alone elicits ductal side-branching and alveologenesis (Fernandez-Valdivia et al., 2009; Gonzalez-Suarez et al., 2007), the very morphogenetic responses that are dependent on progesterone signaling (Lydon et al., 1995).

As described above, previous transplant studies demonstrated that the PRKO mammary epithelium retains the responder cell population that receives paracrine signals from WT transmitter cells to enable rescue of the PRKO phenotype (Briskin et al., 1998). Therefore, these results raised the question: Apart from WT cells, can introduction of the RANKL signal alone similarly rescue the PRKO mammary phenotype? To address this question, the Briskin group employed both retroviral and systemic delivery systems to globally target RANKL expression to PRKO mammary epithelial cells which were subsequently transplanted into the cleared mammary fat-pad of WT host mice (Beleut et al., 2010). Remarkably, RANKL triggered mammary ductal side-branching and alveolar budding in the PRKO transplant within a pregnant WT host, providing much needed functional support for a crucial mediator role for RANKL in mammary progesterone signaling. However, a key question remained unanswered: Can RANKL rescue the PRKO mammary phenotype *via* a paracrine mechanism of action as observed in the WT?

Apart from harboring responder cells, the PRKO mammary epithelium also retains the transmitter cell which normally co-expresses ER and PR in the WT mammary epithelium (Mukherjee et al., 2010). In other words, abrogation of PR does not remove its cell of origin (the transmitter cell) in the PRKO mammary epithelium but rather molecularly disengages

this cell-type from its neighboring responder cells. Based on the foregoing, the transmitter (ER+/PR+) cell in the WT mammary epithelium is equivalent to the transmitter (ER+/PR-) mammary cell in the PRKO. With this insight, an inventive PRKO bigenic mouse was generated to induce (in response to doxycycline administration) transgene-derived RANKL expression specifically in the transmitter cell within the PRKO mammary epithelium (Mukherjee et al., 2010) (Fig. 1A). This genetic strategy essentially recapitulates in the PRKO mammary epithelium the observed restricted spatial expression of mammary RANKL in transmitter cells (ER+/PR+) in the WT.

In response to acute doxycycline exposure, ordered RANKL-driven ductal side-branching and alveologenesis was observed throughout the mammary gland of the PRKO bigenic mouse (Fig. 1B). These morphogenetic responses were shown to be primarily dependent on RANKL-induced proliferation of local responder cells. As in the WT mammary gland, cyclin D1 was also shown to be indirectly induced by RANKL in this PRKO bigenic mouse.

Interestingly, removal of RANKL expression (through doxycycline withdrawal) caused a rapid reversal of the above morphological and proliferative changes (Mukherjee et al., 2010), indicating that other (as yet unidentified) signaling pathways are likely required to maintain and advance these cellular responses in the case of the WT mammary gland as pregnancy progresses to term. Importantly, the speed with which the mammary epithelial proliferative response reverts to base-line following removal of the RANKL signal emphasizes the growing interest in this signaling pathway as a possible target for chemotherapeutic intervention in a number of pathophysiological contexts such as bone metastases from prostate and breast cancers (Fizazi et al., 2009; Holland et al., 2010; Miller et al., 2008).

So how does RANKL “rank” as a paracrine signal for mammary progesterone action? Exceptionally well, if one considers that conditional overexpression of Wnt-4 alone in the murine mammary gland fails to elicit a morphogenic response as witnessed with RANKL induction (Kim et al., 2009). Early expression studies first implicated Wnt-4 as a paracrine factor involved in progesterone-induced mammary morphogenesis (Briskin et al., 2000; Weber-Hall et al., 1994). Mammary transplant studies using Wnt-4 knockout mice demonstrated that absence of Wnt-4 reduces ductal side-branching and alveologenesis during early pregnancy but this phenotype is rescued as pregnancy progresses to term (Briskin et al., 2000), suggesting other Wnt ligands compensate for Wnt-4's absence. Though necessary, it appears that Wnt-4 alone is not sufficient to trigger *de novo* ductal side-branching and alveologenesis to the level that is observed with RANKL expression. Therefore, recognizing the singular importance of RANKL as a pivotal mediator of PR action during normal mammary morphogenesis, the following question is inevitable: Does RANKL participate in progesterone-dependent mammary tumorigenesis?

3.3. RANKL's dual role in progesterone-dependent mammary morphogenesis and tumorigenesis

Compared to WT, PRKO mammary epithelial cells are significantly less susceptible to neoplastic transformation due to their inability to proliferate in response to progesterone (or progestins) (Chatterton et al., 2002; Lydon et al., 1999; Medina et al., 2003). These findings provide strong support for an important role for progesterone-driven mitogenicity not only in normal mammary epithelial biology but also in the promotion of mammary epithelial neoplastic progression. This proposal broadly agrees with conclusions drawn from independent rodent investigations (Poole et al., 2006), human observational studies (Colditz et al., 1993; Ross et al., 2000; Schairer et al., 2000), much publicized clinical trials (Beral, 2003; Writing group for women's health initiative investigators, 2002; Rossouw et al., 2002) and recent correlations made between the decline (since 2002) in hormone therapy use in

some populations in the United States and the concurrent reduction in newly diagnosed breast cancer cases (Clarke et al., 2006).

However, an obvious question arising from the murine mammary tumor studies is: Does progesterone (or progestins) use the same molecular mediators in both mammary morphogenesis and tumorigenesis? In the case of RANKL, the question is particularly pertinent when considering this cytokine's unique importance in progesterone-dependent mammary morphogenesis and its role outside the mammary gland in such clinicopathologies as malignant bone disease and metastasis, humoral hypercalcemia of malignancy, and prostate cancer (Chen et al., 2006; Farrugia et al., 2003; Hansen et al., 1999; Jones et al., 2006).

Therefore, using an established carcinogen-induced murine mammary tumor model, the Dougall and Penninger teams recently demonstrated that the RANKL transduction axis is essential for progestin-promotion of mammary tumorigenesis (Gonzalez-Suarez et al., 2010; Schramek et al., 2010). Both groups revealed that abrogation or accentuation of RANKL signaling renders the mammary epithelium markedly resistant or susceptible to mammary tumorigenesis respectively. The progestin, medroxyprogesterone acetate (MPA), significantly increased RANKL expression in the ER+/PR+ “transmitter” cell population in the WT mammary epithelium as well as in pre-malignant and early neoplastic mammary tissue. These findings indicate that MPA (like progesterone in normal mammary tissue) is reliant on RANKL as a paracrine mediator of its proliferative effects which in-turn facilitate carcinogen-induced epithelial transformation and subsequent neoplastic progression. Indeed, signature signaling pathways (*i.e.* IKK α →NF- κ B→cyclin D1) induced by RANKL in the normal mammary epithelium are markedly elevated during progestin-driven mammary tumorigenesis. These observations support the proposal that unfettered mammary epithelial proliferation due to prolonged overexpression of the RANKL paracrine signal (and its downstream effector pathways) accounts for the majority of progestin-driven pre-neoplasias from which develop primary mammary tumors. This proposal therefore provides one mechanistic explanation for why sustained progesterone/progestin exposure represents a well-established risk-factor for breast cancer (Fig. 2).

Dougall's group showed that RANKL expression was detected in only ~11% of human invasive breast carcinomas under study (Gonzalez-Suarez et al., 2010). However, its not clear whether these advanced tumors express ER and PR and most importantly are hormone responsive (Cui et al., 2005). Clearly, future investigations will need to examine RANKL expression in pre-neoplastic human breast tissue that are positive for ER and PR and responsive to progestins. Such a study design will be better able to determine whether RANKL represents a common signal shared by human and rodent during early hormone-dependent primary tumor formation. Intriguingly, however, most of the RANKL immunoreactivity in these human invasive tumors is concentrated in infiltrating immune cells, suggesting this alternative source of RANKL exerts some role during the later stages of the neoplastic progression program.

3.4. RANKL is a pro-metastatic factor

Recently, the Dougall and Karin groups revealed that pharmacological inhibition or genetic suppression of the RANKL signaling axis markedly reduces the incidence of mammary cancer pulmonary metastases in the MMTV-HER2/*Neu* transgenic mouse (Gonzalez-Suarez et al., 2010; Tan et al., 2011). Amplified and/or overexpressed in ~30% of human breast cancers (Slamon et al., 1989), HER2/*Neu* (also known as ErbB-2, a transmembrane tyrosine kinase receptor and a member of the epidermal growth factor receptor family) endows human breast cancers not only with an aggressive growth phenotype but with a heightened metastatic potential (Tiwari et al., 1992). Remarkably, tumor-infiltrating regulatory T (Treg)

cells rather than epithelial cells within invasive mammary tumors were shown to express the majority of RANKL protein (Tan et al., 2011). In both human and mouse, the appearance of tumoral Treg cells is invariably linked with aggressive tumor invasion and metastasis (Bohling and Allison, 2008). These observations suggest that RANKL derived from infiltrating Treg cells is essential for driving RANK expressing breast cancer cells toward an invasive and metastatic phenotype. To directly address this proposal, Karin's group employed an orthotopic tumor model in which MMTV-HER2/*Neu* primary mammary cancer cells were injected into the thoracic mammary gland of WT mice (Tan et al., 2011). With this approach, Karin demonstrated that systemic administration of recombinant RANKL (after tumor cell injection) significantly increased the incidence of pulmonary metastases, providing compelling support for the proposal that RANKL derived from Treg cells is a driver of breast cancer metastasis to the lung. Importantly, injected RANK+ human breast cancer cells also exhibited increased metastatic potency in response to RANKL administration, furnishing strong translational support for RANKL's role in breast cancer metastases. While we know that RANKL-positive Treg cells are attracted to the tumor microenvironment by chemokines emanating from cancer-associated myofibroblasts (CAFs) (Liao et al., 2009), we are still unclear as to the signaling cue(s) which induce RANKL expression within these cell-types.

Together, the mammary tumor and metastasis studies posit that intraepithelial paracrine signaling underpins RANKL's role in progestin-dependent primary tumor formation whereas paracrine signaling between infiltrating T cells expressing RANKL and advanced cancer cells expressing RANK underscores RANKL's involvement in cancer cell metastasis (Fig 2). Because of RANKL's pro-metastatic effects, blocking RANKL signaling is now gaining significant interest not only in the prevention and treatment of hormone-dependent primary breast cancers but also as an anti-metastatic therapeutic approach.

3.5. Progesterone, RANKL, and the stem cell connection

The paracrine signaling model of progesterone action in the mammary epithelium has been further refined by recent mouse studies which reveal progesterone as a critical factor in the transient expansion and regenerative activity of the MaSC population (Asselin-Labat et al., 2010; Joshi et al., 2010). Previous cell sorting approaches followed by transplant assays identified an enriched MaSC pool which is capable of regenerating all cell-lineages that comprise the murine mammary gland (Shackleton et al., 2006; Stingl et al., 2006). In addition to MaSCs, the identification of downstream committed progenitors (alveolar and ductal) as well as mature luminal cells (ER+/PR+ and ER-/PR-) provided strong support for a hierarchical organization for mammary epithelial cells (Shackleton et al., 2006; Stingl et al., 2006) as similarly inferred from human studies (Dontu et al., 2003).

Given both progesterone paracrine-signaling and MaSC function are indispensable for mammary morphogenesis, the question was raised as to whether these processes are linked. The finding that the MaSC is devoid of ER and PR expression (Asselin-Labat et al., 2006) and resides in the myoepithelial/basal compartment rather than in the luminal epithelium (Asselin-Labat et al., 2010; Joshi et al., 2010) frames this question in more specific terms: Does the MaSC represent a distinct responder cell for steroid hormone paracrine signaling?

This question has recently been addressed by insightful studies conducted by the Khokha and Visvader groups which demonstrated that increased progesterone levels during the diestrus phase of the estrous cycle or with pregnancy-onset drives transient expansion (*via* symmetrical cell division) of the MaSC population (Asselin-Labat et al., 2010; Joshi et al., 2010). It's thought through asymmetrical cell division, the amplified MaSC pool then generates ductal and alveolar progenitors which rapidly give rise to luminal and basal cells in sufficient numbers to account for the numerous ductal side-branches and alveoli observed

during the proliferative-phase of pregnancy and to a lesser extent with ovarian cyclicity (Asselin-Labat et al., 2010; Joshi et al., 2010) and (Fig. 3). Importantly, recent studies on cultured human breast epithelial cells revealed that progesterone also signals in a paracrine manner to elicit proliferation of bipotent progenitor cells (Graham et al., 2009), underscoring the evolutionary conserved nature of this signaling paradigm between ER+/PR+ transmitter cells and the stem/progenitor responder cell population.

Apart from normal mammary morphogenesis, progesterone-induced expansion of the MaSC pool also provides a cellular basis for the well-established risk factor for breast cancer, namely sustained steroid hormone exposure (which occurs with repeated menstrual cycles (Pike et al., 1993), postmenopausal hormone therapy (Chlebowski et al., 2010), or as a short-term risk with pregnancy (Lambe et al. 1994)). The idea being that progesterone-driven increases in MaSC number translates to increases in cell targets for carcinogen or genetic-induced malignant transformation (Visvader, 2011). Conversely, retraction of the MaSC pool—through removal of progesterone or suppression of its paracrine signals—is predicted to decrease breast cancer risk.

Intriguingly, the Khokha and Visvader groups—through expression studies and signaling blockade approaches—furnish compelling support for RANKL as one such important paracrine signal which mediates progesterone-induced expansion of the MaSC pool (Asselin-Labat et al., 2010; Joshi et al., 2010). Furthermore, Penninger's group recently revealed that MPA also increases the MaSC population prior to carcinogenic exposure when using the carcinogen-induced mammary tumor model (Schramek et al., 2010), and that this stem cell expansion is suppressed by blocking RANKL signaling. Collectively, these findings not only provide a novel stem-cell perspective for RANKL's role in progesterone-dependent mammary morphogenesis and tumorigenesis but also offer a convincing rationale (from a cellular perspective) for targeting RANKL action in the prevention and/or treatment of breast cancer (Fig. 3). It should be noted, however, that because of the wide-spread distribution of its signaling receptor (RANK) within the mammary epithelium (Fernandez-Valdivia et al., 2009; Gonzalez-Suarez et al., 2007), RANKL likely signals to responder cell-types other than MaSCs. In this cellular context, RANKL not only drives division of the stem cell but propels proliferation of its lineal descendents (Fig. 3), all of which serves to rapidly amplify epithelial cellularity within a short-time frame.

Future Directions—In just three years, we have witnessed stunning advances in our understanding of RANKL as a paracrine mediator of progesterone-dependent mammary morphogenesis and tumorigenesis. These advances notwithstanding, important questions remain: (1) Does ligand-bound PR induce mammary RANKL transcription through direct binding to response elements within control regions of the RANKL gene? Now recognizing that sustained progesterone-induction of RANKL expression underpins prolonged progesterone exposure as a risk factor for breast cancer, understanding the mechanisms by which mammary PR controls RANKL transcription is an imperative if we are to gain meaningful insight into how this cytokine contributes to hormone-dependent mammary tumor promotion; (2) Does progesterone coordinate with prolactin hormone in the induction of mammary RANKL transcription? Required for mammary alveolar formation and differentiation (Ormandy et al., 1997; Horseman et al., 1997; Brisken et al., 1999), prolactin also has been shown to induce mammary RANKL expression (Srivastava et al., 2003). Since progesterone and prolactin are both essential for completion of the second allometric growth phase that occurs with pregnancy, whether RANKL represents a convergence point for these important hormonal cues remains an open question; (3) Is RANKL's function regulated by post-translational control mechanisms in the mammary gland? Recent studies indicate that the soluble ectodomain of RANKL—which is generated or shed by proteolytic cleavage—is a mechanism by which RANKL extends its influence beyond local responder cells

(Schramek et al., 2010). If so, identifying the key proteinases involved in local RANKL shedding and the factors which induce their expression and activity will be essential to gaining a better understanding of this complex post-translational regulatory control mechanism; (4) Does RANKL signaling cross-talk with other signaling pathways which also are implicated to act as paracrine factors (*i.e.* Wnt-4) of mammary progesterone action? Cyclin D1 is a known molecular effector for both RANKL and Wnt/catenin signaling (Cao et al., 2001; Shtutman et al., 1999), suggesting that cyclin D1 may represent at least one point of intersection for these important signaling pathways; (5) What regulates the signaling receptor, RANK? In contrast to its ligand, RANK displays a more general spatiotemporal expression pattern in the mammary epithelium (Fernandez-Valdivia et al., 2009; Gonzalez-Suarez et al., 2007), providing little clues as to the hormonal signals (if any) that regulate its expression. Interestingly, however, recent *in vivo* and *in vitro* studies reveal that RANK expression is downregulated by increased levels of RANKL (Fernandez-Valdivia et al., 2009; Gonzalez-Suarez et al., 2007). As with progesterone-downregulation of PR expression (Ismail et al., 2002), RANKL's control of its signaling receptor may represent a mechanism by which this transduction pathway is kept in check in response to changing levels of extracellular signaling cues. If true, questions as to whether this ligand-receptor control paradigm occurs at the transcriptional or post-translational level and whether deregulation of this control mechanism contributes to RANKL's role in mammary tumorigenesis are critical questions that await answers. Noteworthy, transgenic expression of RANK—which is free of the constraints imposed by RANKL control—leads to RANKL-dependent tumorigenesis (Gonzalez-Suarez et al., 2007), a finding which in part addresses the latter question; (6) Does the transcriptional read-out from RANK-mediated signaling differ between stem cells, lineage-committed progenitors, and mature epithelial cells? RANK is expressed throughout the epithelial hierarchy of the mammary gland therefore disclosing the transcriptional signature of MaSCs and their lineal descendents when exposed to RANKL will be essential in further resolving our mechanistic understanding as to whether this cytokine differentially influences the proliferative status of each epithelial cell category of the mammary gland; (8) Is it the MaSC and/or an adjacent cell within the stem-cell niche that is directly responsive to the RANKL signal? So far, we can only conclude that a MaSC enriched fraction (albeit heterogeneous) responds to RANKL. Only through the development of more specific antibodies to the MaSC will this question be addressed; and finally (9) Is mammary RANKL signaling of clinical significance? While RANKL's role in breast cancer metastasis to distant anatomic sites is currently being considered for therapeutic intervention (Stopeck et al., 2010), the involvement of this cytokine in hormone-dependent primary breast cancer formation and progression has yet to be clearly established. As mentioned previously, nothing short of examining early progestin-responsive human (or the very least non-human primate) breast cancers will answer whether recent mouse findings (Gonzalez-Suarez et al., 2010; Schramek et al., 2010) ultimately translate to the clinic.

Undoubtedly, answers to the above questions will further expand and accelerate the extraordinary progress already achieved in our understanding of mammary RANKL signaling and importantly will furnish invaluable insights which will aid in the design of more effective chemotherapeutics in the prevention and/or treatment of progestin-dependent breast tumor formation and progression.

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Abbreviations

C/EBP-β	CAAT/enhancer binding protein- β
CAF	cancer associated myofibroblasts
ER	estrogen receptor- α
ERKO	estrogen receptor- α knockout
HER-2	human epidermal growth factor receptor-2
I$\kappa$$\kappa$-$\alpha$	I κ B kinase- α
MaSC	mammary stem cell
MPA	medroxyprogesterone acetate
MMTV	mouse mammary tumor virus
PR	progesterone receptor
PRKO	progesterone receptor knockout
RANKL	receptor activator of NF- κ B ligand
TNF	tumor necrosis factor
Treg	regulatory T cells
WT	wild type

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Fig. 1.

The PRKO mammary phenotype is rescued by RANKL. (A) Using a state-of-the-art PRKO bigenic mouse (Mukherjee et al., 2010), transgene-derived RANKL expression is conditionally expressed in PRKO transmitter cells (ER+/PR-) within the luminal epithelium of the mammary gland and only in the presence of the inducer, doxycycline. Location of basal cells and the extracellular matrix (ECM) are also shown in this schematic. (B) An example of the mammary gland of the PRKO bigenic prior to doxycycline administration. As previously reported for the PRKO mouse (Lydon et al., 1995), note the simple ductal structure (black arrow) and absence of alveoli. (C) Typical mammary response in the PRKO bigenic mouse following one-month of doxycycline intake. Note the clear evidence of alveologensis (black arrowhead) and extensive side-branching. Scale bar in panel B applies to panel C. The data represent a condensed version of those previously reported ((Mukherjee et al., 2010).

Fig. 2.

Dual role of RANKL in progesterone/progestin dependent mammary morphogenesis and tumorigenesis. (A) Schematic showing RANKL acting as a paracrine mediator of progesterone-induced mammary side-branching and alveologenesis during pregnancy progression. Increased levels of progesterone bind PR in (PR+) transmitter cells in the luminal epithelium. Ligand-bound PR induces the expression of RANKL which in-turn binds its cell-surface signaling receptor (RANK) on nearby responder cells in the luminal epithelium (LE) and in the basal cell (BC) compartment (see: section 3.5) to elicit mitogenesis (ECM denotes the extracellular matrix). (B) A conceptual depiction of the promotional effect of sustained progestin-induced RANKL signaling on carcinogen (or genetic lesion) induced breast cancer development. In the case of more advanced invasive tumors, RANK+ cancer cells no longer express RANKL due to loss of PR. However, these cancer cells respond in a paracrine manner to an alternative source of RANKL from infiltrating Treg cells; these cells are attracted to the tumor microenvironment by CAF-derived chemokines (Liao et al., 2009). As a consequence of the paracrine signaling between cancer cells and immune cells, cancer cells gain access to the invasion-metastasis cascade which allows for breast cancer cell colonization to distant anatomic sites such as lung and bone. Note: CAF and Treg denote: cancer-associated myofibroblast and regulatory T cell respectively.

Fig. 3.

Progesterone-regulates the size and regenerative activity of the MaSC pool through paracrine RANKL signaling. In response to increased levels of progesterone during pregnancy, the PR+ transmitter cell in the luminal epithelium relies on the RANKL paracrine signal to instruct the MaSC population to undergo symmetric cell division. Asymmetric cell division of MaSCs produces lineal-restricted progenitor cells which in-turn generate mature luminal and myoepithelial cells. The rapid increase in mature epithelial cells accounts for most of the increased cellularity observed during the second allometric growth phase of mammary development. Because RANK is expressed in epithelial cells other than the MaSC (Fernandez-Valdivia et al., 2009; Gonzalez-Suarez et al., 2007), RANKL likely influences the mitogenicity of lineal descendants of the MaSC also (dashed line). While this schematic depicts progesterone control of MaSC homeostasis during pregnancy, this signaling paradigm also applies during the progesterone-dominant diestrus stage of the estrous cycle (Joshi et al., 2010).