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The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse

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ABSTRACT

Receptor of Activated NF-κB Ligand (RANKL) is implicated as one of a number of effector molecules that mediate progesterone and prolactin signaling in the murine mammary epithelium. Using a mouse transgenic approach, we demonstrate that installation of the RANKL signaling axis into the mammary epithelium results in precocious ductal side-branching and alveologenesis in the virgin animal. These morphological changes occur due to RANKL-induced mammary epithelial proliferation, which is accompanied by increases in expression of activated NF-kB and cyclin D1. With age, prolonged RANKL exposure elicits limited mammary epithelial hyperplasia. While these transgenics exhibit RANKL-induced salivary gland adenocarcinomas, palpable mammary tumors are not observed due to RANKL-suppression of its own signaling receptor (RANK) in the mammary epithelium. Together, these studies reveal not only that the RANKL signaling axis can program many of the normal epithelial changes attributed to progesterone and prolactin action in the normal mammary gland during early pregnancy, but underscore the necessity for tight control of this signaling molecule to avoid unwarranted developmental changes that could lead to mammary hyperplasia in later life.

Introduction

Receptor of Activated Nuclear Factor-κB Ligand (RANKL (also known as OPGL, TRANCE, and ODF)), which is a member of the tumor necrosis factor (TNF) superfamily, is a transmembrane trimeric signaling ligand that mediates its downstream effects by engaging its cognate receptor, RANK (Blair et al., 2006; Dougall and Chaisson, 2006). A member of the TNF receptor family, RANK also acts as a homotrimer transmembrane protein which upon binding RANKL triggers signaling cascades required for a wide variety of physiological processes, ranging from osteoclastogenesis and bone-remodeling to T-cell and dendritic cell survival and communication (Theill et al., 2002; Walsh and Choi, 2003). RANKL also binds the soluble decoy receptor, osteoprotegrin (OPG), which blocks the RANKL/RANK interaction (Khosla, 2001).

Knockout studies in mice demonstrated an unexpected role for RANKL and its receptor RANK in parity-induced mammary morphogenesis and function (Fata et al., 2000). Absence of RANKL or RANK resulted in a marked attenuation in mammary alveologenesis resulting in a lactational defect at parturition (Fata et al., 2000). This mammary phenotype shares many similarities with the previously described progesterone receptor knockout (PRKO), prolactin knockout (PRLKO) and prolactin receptor knockout (PRLRKO) mammary phenotypes (Brisken et al., 1999; Horseman et al., 1997; Lydon et al., 1995; Oakes et al., 2008), suggesting a connection between RANKL, progesterone, and prolactin signaling during parity-induced mammary morphogenesis. In support of this proposal, molecular studies demonstrate that RANKL can be induced in the murine mammary epithelium by progesterone or prolactin exposure (Fata et al., 2000; Mulac-Jericevic et al., 2003; Srivastava et al., 2003). Moreover, in the case of progesterone, recent microarray studies reveal mammary RANKL transcription can be rapidly induced by short-term progesterone treatment (Fernandez-Valdivia et al., 2008), suggesting that RANKL may be required for early progesterone-dependent proliferative changes which occur in the mammary epithelium of the parous animal. Further support for this supposition is the observation in the hormone-treated ovariectomized mouse model that mammary RANKL induction is restricted to PR positive luminal epithelial cells which are segregated from (but in close apposition to) a subset of PR negative cells that express cyclin D1 and proliferate in response to progesterone (Mulac-Jericevic et al., 2003).

Notwithstanding the growing evidence supporting RANKL as a mediator of progesterone and prolactin-induced mammary morphogenesis, we are still uncertain as to the extent to which RANKL functionally contributes to these morphogenetic events at the gross

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morphological, cellular, and molecular level. This uncertainty is further exacerbated by an increasing number of seemingly unrelated mediators that have also been ascribed to PR and/or PRLR action in the mammary epithelium (Brisken et al., 2002, 2000; Fernandez-Valdivia et al., 2008; Harris et al., 2006; Naylor et al., 2005; Satoh et al., 2007).

To address the above, we have used a transgenic approach to evaluate the response of the mammary epithelium of the virgin mouse to RANKL exposure. It's important to note that although the parous RANKL (and RANK) knockout mouse demonstrated that absence of RANKL signaling results in impairments in alveologenesis, the direct effects of RANKL on mammary epithelial cell biology were not fully explored. Furthermore, our transgenic approach was used to determine whether continuous (or inappropriate) RANKL expression in the virgin mammary gland leads to adverse developmental consequences such as hyperplasia and neoplastic transformation. The latter question is important since in other physiological contexts RANKL signaling has been shown to underlie a number of aberrant cellular processes, which include proliferation and survival, programmed cell-fate, intercellular communication as well as cell-migration. The perturbation of such cellular functions has been linked to the etiology and/or progression of such clinicopathological disorders 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).

Here, our transgenic studies reveal that the RANKL signaling axis alone can trigger both precocious ductal side-branching and alveolar budding within the simple ductal structure of the pubescent and adult virgin mammary gland. These epithelial changes are the very processes that are abrogated in the PRKO, PRLKO, and PRLRKO mammary gland (Brisken et al., 1999; Horseman et al., 1997; Lydon et al., 1995; Oakes et al., 2008). The underlying cause of these morphological changes results from accelerated RANKL-induced mammary epithelial proliferation, which is paralleled by an upregulation in the pleiotropic transcription factor Nuclear Factor-κB (NF-κB) as well as cyclin D1. Despite this proliferation, however, RANKL transgenics do not exhibit mammary tumors. One plausible explanation for the lack of mammary tumor formation is the observed suppression by RANKL of mammary RANK expression, the net result of which is to curtail RANKL action. Interestingly, in other developmental systems in which RANKL-induced RANK downregulation is not observed (i.e. in the salivary epithelium), RANKL initiated epithelial hyperplasia and neoplastic transformation is free to progress. Collectively, our findings support the proposal that, despite a growing number of effectors assigned to mammary PR and PRLR action, the RANKL signaling cascade may represent a critical mediator of the normal mammary epithelial response to progesterone and prolactin signaling during early pregnancy and that tight control of this effector molecule is required to avoid unwarranted hormone-induced mammary epithelial expansion.

Materials and methods

Generation of MMTV RANKL transgenic mouse lines

The full-length (2.221 kb) murine RANKL cDNA (GenBank accession number: AF013170) was directionally inserted (with the aid of an Xba1 and Not1 linker) into a unique EcoR1 site within exon 3 of the rabbit β-globin gene which comprises part of the MMTV-KCR transgene construct (Ma et al., 1999). In addition to exon 3, the MMTV-KCR construct also contains a short segment of exon 2, full-length intron 2 as well as the polyadenylation signal of the rabbit β-globin gene. Prior to microinjection into male pronuclei of single-cell (FVB/N) embryos and subsequent transfer to pseudopregnant ICR recipients (Harlan Sprague Dawley Inc., Indianapolis, IN), the Xho1 excised MMTV-RANKL transgene (4.9 kb) was isolated from the pBluescript KS ll cloning vector (Stratagene, La Jolla, CA). Founders (and progeny positive for the transgene) were identified by PCR before further characterization by Southern blot analysis. As indicated in Fig. 1A, the PCR primers (1: 5′-GAAAATGTACTATAGTTTATCAGCC-3′; and 2: 5′-GTCAAGGCTTTTCTATGGAATAAGG-3′) flank the junction between the 3′ untranslated region (UTR) of the RANKL cDNA and exon 3 of the rabbit β-globin gene to generate an amplicon of 489 bp in size; this amplicon was also used as a transgene specific probe for Southern analysis (Figs. 1B, C). For these studies, mice were maintained as hemizygotes for the transgene in a FVB/N genetic background using stock mice from The Jackson Laboratory, Bar Harbor, ME. In the case of salivary tumor studies, palpable salivary tumors were allowed to reach 1.0 cm in diameter before transgenic animals were euthanized for histological and molecular analysis.

All mice were maintained in a temperature controlled (22±2 °C) environment, 12-h light, 12-h dark photocycle, and fed rodent chow meal (Purina Mills Inc., St. Louis, MO) and fresh water, *ad libitum*. Surgical procedures as well as experimental and euthanasia protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and were in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85–23).

Histological analysis

For immunohistochemical analyses, tissues were fixed overnight in Bouin's fixative or 4% paraformaldehyde; for immunofluorescence detection, tissues were fixed in 4% paraformaldehyde for 2 h. Immunohistochemistry and dual-immunofluorescence were performed as described previously (Mukherjee et al., 2006). The following primary antibodies were used in these studies: rabbit anti-human PR (DAKO Corporation, Carpinteria, CA, (A0098; 1:100)); goat anti-mouse RANKL (R&D Systems Inc., Minneapolis, MN, (AF462; 1:200)); goat anti-mouse RANK (R&D Systems Inc., (AF692; 1:100)); mouse anti-BrdU (Amersham, Piscataway, NJ, (1:50 dilution)); mouse anti-human alpha-actin (Sigma-Aldrich, St. Louis, MO, (A2547; 1:100)); mouse anti-human cytokeratin 8 (Fitzgerald Industries International, Inc., Concord, MA, (RDI1038; 1:40)); rabbit anti-human cyclin D1 (Lab Vision Corporation, Fremont, CA, (RB-9041-P0; 1:40)); and rat anti-mouse Mac-3 (BD Biosciences, San Jose, CA, (550292; 1:200)). For dual-immunofluorescence analysis, tetramethyl-rhodamine isothiocyanate -conjugated streptavidin (red color) and fluorescein isothiocyanate (green color) were used to fluorescently simultaneously detect two proteins *in situ*; full instructions are detailed in the Tyramide Signal Amplification (TMA) Fluorescence Kit (NEL701; Perkin Elmer Life Sciences, Boston, MA) as well as in (Ismail et al., 2002; Mukherjee et al., 2006). Slides were washed and mounted in Vectashield mounting medium with 4′, 6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA). Sections were viewed with an Axioplan 2 microscope equipped for epifluorescence detection with appropriate filters for tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate detection (Carl Zeiss, Jena, Germany). For Supplemental Fig. 2, immunopositive cells (single or double immunopositive) were quantitated from 1000 DAPI stained luminal epithelial cells in three separate histological sections per mouse; five mice per genotype were analyzed. Results are expressed as percentage mean±SEM; differences were considered significant (at $P<0.05$) by Student's *t* test (Fernandez-Valdivia et al., 2008; Lydon et al., 1995). Digital images were captured with Metavue Software 4.6r9 (Universal Imaging Inc., Downingtown, PA); final image composites were constructed with Photoshop CS3 (Adobe Systems, Inc., San Jose, CA).

To quantitate 5-bromo-2-deoxyuridine (BrdU) incorporation (as detected by immunohistochemistry (Fig. 3J)), mice were injected (intraperitoneally) with BrdU (Amersham Biosciences, NJ) at 0.1 ml/10 g of body weight, 2 h prior to sacrifice. Tissues were fixed, processed, paraffin-embedded, and sectioned as described (Lydon et