

Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland

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The mouse mammary gland develops postnatally under the control of female reproductive hormones. Estrogens and progesterone trigger morphogenesis by poorly understood mechanisms acting on a subset of mammary epithelial cells (MECs) that express their cognate receptors, estrogen receptor α (ER α) and progesterone receptor (PR). Here, we show that in the adult female, progesterone drives proliferation of MECs in two waves. The first, small wave, encompasses PR(+) cells and requires cyclin D1, the second, large wave, comprises mostly PR(–) cells and relies on the tumor necrosis factor (TNF) family member, receptor activator of NF- κ B-ligand (RANKL). RANKL elicits proliferation by a paracrine mechanism. Ablation of RANKL in the mammary epithelium blocks progesterone-induced morphogenesis, and ectopic expression of RANKL in MECs completely rescues the PR^{–/–} phenotype. Systemic administration of RANKL triggers proliferation in the absence of PR signaling, and injection of a RANK signaling inhibitor interferes with progesterone-induced proliferation. Thus, progesterone elicits proliferation by a cell-intrinsic and a, more important, paracrine mechanism.

cell proliferation | cyclin D1 | mammary epithelium | progesterone | RANKL

Lifetime exposure to reproductive hormones, in particular estrogens and progesterone, affects the risk of breast cancer, a complex disease that is under hormonal control (1). The same hormones also control development of the mouse mammary gland, most of which occurs after birth. In response to ovarian estrogen secretion at puberty, the rudimentary ductal system extends from the nipple area into the fat pad through dichotomous branching. The complexity of the milk duct system increases through the formation of side branches triggered by cyclic changes in estrogen and progesterone secretion during adulthood. Side branching is enhanced during the first half of pregnancy. Subsequently, saccular outpouchings, alveoli, bud off the ducts and differentiate to become sites of milk production during lactation. Once pups are weaned, the mammary gland returns to a pre-pregnancy state through involution.

Tissue recombination experiments with mammary epithelium from mice with germ-line deletion of the estrogen receptor (ER) α (2), the progesterone receptor (PR) (3), or the prolactin receptor (PrIR) (4) revealed that epithelial intrinsic ER α is required for ductal elongation (5), PR for ductal side branching (6), and PrIR for alveologenesis and differentiation into milk-producing cells (7). PRs are composed of two proteins that are expressed from a single gene as a result of transcription from two alternative promoters (8), both of which are expressed in the mouse mammary gland (9). Deletion of either PR-A or PR-B from the mouse germ line revealed that PR-B is specifically required for mammary gland development (3, 10, 11).

The mechanisms by which hormones induce proliferation *in vivo* are poorly understood. The mammary epithelium consists of an inner layer of luminal cells that are surrounded by basal cells juxtaposed to the basal lamina. Some of these are in suprabasal position and thought to comprise progenitor cells others are spindle shaped and called myoepithelial cells because they exhibit

features of smooth muscle cells. The receptors for both steroid hormones are expressed in about 30% of the luminal cells and have been shown to colocalize in the human breast (12). Colabeling studies in humans and rodents revealed that most of the proliferating cells are hormone receptor negative (12–14). Chimeric epithelia in which ER α - or PR-deficient cells were mingled with WT cells showed that the mutant cells participated actively in ductal growth in the context of surrounding WT MEC (5, 6).

The tumor necrosis family (TNF) member, receptor activator of NF- κ B-ligand (RANKL), is important for osteoclast differentiation and lymph-node organogenesis (15) and was shown to be required for pregnancy-induced lactational mammary gland development (16) and implicated upstream of cyclin D1 in mammary epithelial cells (MECs) acting via the IKK α subunit of I κ B kinase (17). Ectopic expression of RANKL in the mammary epithelium was shown to elicit ductal side branching and alveologenesis (18); similarly, overexpression of the cognate receptor RANK resulted in increased proliferation (19). Here, we show that progesterone induces proliferation of a subset of PR-positive (+) MECs by a cell-autonomous, cyclin D1-dependent mechanism and a larger wave of proliferation by a paracrine, RANKL-dependent mechanism.

Results

Two Waves of Progesterone-Induced Proliferation. To examine cell proliferation induced by ovarian steroids, we administered 17- β -estradiol and progesterone to adult mice that had been ovariectomized 10 days earlier to deplete their endogenous steroids, and pretreated with 17- β -estradiol for 24 h to restore PR expression (20). Neither vehicle, nor 17- β -estradiol alone (Fig. 1*A* and *B*) elicited proliferation considerably above background levels as assessed by 5'-bromo-2'-deoxyuridine (BrdU) labeling, but extensive proliferation occurred when progesterone was added (Fig. 1*A* and *B*), highlighting that 17- β -estradiol, a major mitogen in the pubertal gland (21), is merely permissive for the proliferative effects of progesterone in the adult mammary gland.

Based on previous reports that few proliferating MECs express ER α and PR in humans and rodents (12–14), and genetic evidence that progesterone can act by paracrine mechanisms (6), we hypothesized that progesterone may be directly mitogenic for some PR(+) MECs and elicit proliferation of PR(–) MECs by a paracrine mechanism. As cell divisions elicited by direct mitogenic stimulation should occur earlier than those following paracrine stimulation, we assessed proliferation at distinct time points. To identify all early proliferation events, we administered BrdU

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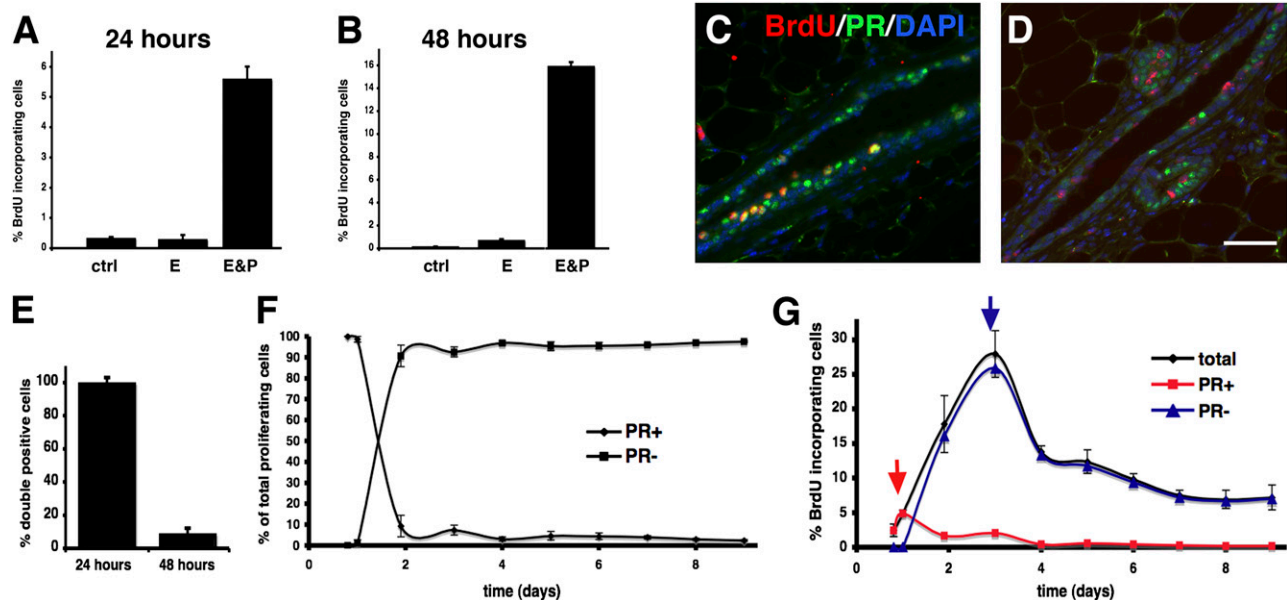


Fig. 1. Progesterone induces two waves of proliferation. (A–E) Ten-week-old female mice were ovariectomized, pretreated with 17- β -estradiol 10 days later, and then injected with vehicle, 17- β -estradiol, or 17- β -estradiol and progesterone. BrdU was administered repeatedly for analysis at 24 h (A and C) or as a single bolus at 46 h (B and D). Bars show BrdU incorporating MECs \pm SEM in different treatment groups 24 h (A) and 48 h (B) after injection ($n = 3$). Double immunofluorescence after 24 h (C) and 48 h (D) of stimulation. Green, PR; red, BrdU; blue, DAPI. (Scale bar: 40 μ m.) (E) Percentage of PR(+) and PR(-) cells \pm SEM among BrdU incorporating MECs 24 h and 48 h after progesterone stimulation. (F and G) Ten-week-old female mice were ovariectomized and treated every 24 h with 17- β -estradiol and progesterone. Percentage of PR(+) and PR(-) BrdU incorporating MECs determined by double immunofluorescence; 40–400 BrdU(+) MECs counted per mouse ($n = 3$ –4) (F). BrdU incorporation indices were determined by counting 3,000 cells per mouse ($n = 3$ –4), and plotted over time. Percentage of PR(+) (red) and PR(-) cells (blue) incorporating BrdU was calculated based on F (G).

continuously for 24 h following hormone stimulation. Anti-BrdU staining revealed that 5% of MECs incorporated the label (Fig. 1A); double immunohistochemistry showed that most of these were PR(+) (Fig. 1C and E), and found in the luminal compartment (Fig. 1C). Then, we pulsed mice with BrdU 46–48 h after progesterone stimulation. Under these conditions, about 15% of MECs incorporated the label (Fig. 1B); out of these less than 10% were PR(+) (Fig. 1D and E). A time-course analysis revealed that BrdU incorporation was first above background level at 18 h after stimulation, with 3.2% of the MECs staining positive for the labeled nucleotide. Colabeling for BrdU and PR confirmed that up to 24 h, almost all of the proliferating MECs were PR(+); on day 2, the PR(+) cell fraction represented 9.3%, and by day 5 less than 5% of the proliferating MECs (Fig. 1F). From day 2 on, proliferating cells were found in luminal and subluminal locations as well as in the myoepithelium. Proliferation peaked on day 3 with 27% of MECs incorporating BrdU (Fig. 1G). Analysis of PR(+) and PR(-) MEC populations revealed two distinct peaks of proliferation (Fig. 1G, red and blue line): a small peak of PR(+) cell proliferation at 24 h and a large peak of PR(-) cell proliferation at 72 h. We note that the incorporation index at 48 h is higher under repeated hormone stimulation (Fig. 1F and G) than following a single bolus (Fig. 1A–E). Taken together, progesterone stimulation elicits two waves of proliferation in the mammary epithelium, a first small wave of PR(+) cells and a subsequent large wave of PR(-) MECs.

Cyclin D1 and Progesterone-Induced Proliferation. Cyclin D1 is amplified specifically in ER α (+) breast cancers and is a progesterone target gene in the PR(+) breast cancer cell line T47D (22). To assess whether cyclin D1 is required in vivo for progesterone-induced proliferation, we generated mice with cyclin D1 $^{-/-}$ and WT mammary epithelia in contralateral glands by tissue recombination. Briefly, in 3-week-old mice, the inguinal glands can be cleared of endogenous epithelium by surgically removing the nipple-near half

that contains the rudimentary ductal system. MECs that are introduced into the remaining “cleared” fat pad will give rise to a new ductal system. They can grow out from a piece of mammary tissue that is implanted (23), or from single-cell suspensions injected into the fat pad (24). To distinguish the graft from endogenous epithelium inadvertently left behind, we crossed the mutant cyclin D1 allele into a transgenic strain that ubiquitously expresses green fluorescent protein (GFP) (25) and grafted the GFP(+) donor tissue into GFP(-) hosts. Eight weeks later, recipients were analyzed 24 or 48 h after progesterone stimulation. As expected, within 24 h of progesterone stimulation, 6–16% of WT MECs had incorporated BrdU (Fig. 2B and E). The contralateral cyclin D1 $^{-/-}$ epithelial grafts, however, incorporated only background levels (Fig. 2A and E). At 48 h, BrdU incorporation indices in both WT (Fig. 2D and F) and cyclin D1 $^{-/-}$ epithelia were in the range of 10–24% (Fig. 2C and F). Double immunofluorescence revealed that among both WT and mutant MECs more than 90% of the BrdU-incorporating cells were PR(-) (Fig. S1). Thus, cyclin D1 function is required for cell-autonomous division of PR(+) cells induced by progesterone, but the second wave of proliferation is cyclin D1 independent. We note that grafted epithelia in general have higher proliferative indices than endogenous ones.

Role of RANKL in the Mammary Epithelium. Next, we sought to identify the mediator of progesterone’s paracrine mitogenic effects. Several factors had been implicated downstream of progesterone signaling in the mammary gland, including Wnt-4 (26), RANKL (10, 27), Calcitonin (28), and Id4 (29). RANKL had been proposed as a candidate paracrine mediator based on the observation that RANKL protein was shown to be expressed in PR(+) cells (10). Yet, analysis of RANKL-deficient mice indicated that the cytokine is required late in pregnancy for alveologenesis and lactogenic differentiation (16). As analysis of the mammary gland phenotype in RANKL $^{-/-}$ mice may have been confounded by systemic effects of the deletion (15), we performed mammary

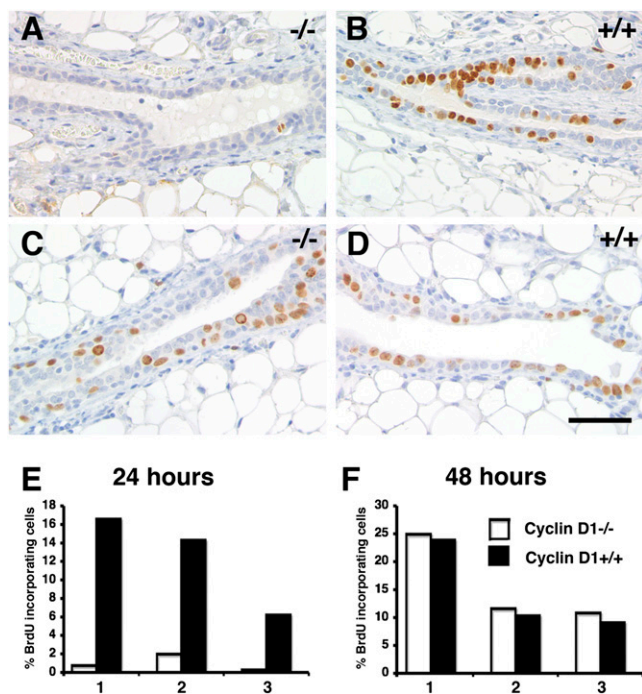


Fig. 2. Cyclin D1- and progesterone-induced proliferation. (A and D) Mice engrafted with cyclin D1^{-/-} and WT epithelia were stimulated with progesterone for 24 h (A, B, and E) or 48 h (C, D, and F). Histological sections of contralateral mammary glands engrafted with cyclin D1^{-/-} (A and C) or WT (B and D) epithelia and stained with an anti-BrdU antibody. (Scale bar: 40 μ m.) (E and F) Bar graphs showing BrdU incorporation in cyclin D1^{-/-} and WT MECs \pm SEM ($n = 3$), 1,000 cells counted per mouse.

gland recombination experiments to discern the epithelial intrinsic effects of the deletion.

Fluorescent stereomicroscopy of contralateral glands engrafted with GFP⁺.RANKL^{-/-} versus GFP⁺.RANKL^{+/+} MECs (Fig. S2 A and C) revealed that pubertal ductal outgrowth (9 weeks) was

comparable between WT and mutant grafts. At 13 weeks of age, WT grafts gained complexity due to side branching, whereas the RANKL^{-/-} epithelium did not. By day 14.5 of pregnancy, WT grafts had developed side branches and alveolar buds, whereas RANKL^{-/-} grafts had only bifurcated. At the end of pregnancy, WT epithelia showed extensive alveogenesis, whereas RANKL^{-/-} epithelia had few side branches and alveoli (Fig. S2A–C). Quantification of side branches at 13 weeks confirmed that WT grafts were consistently more complex than the RANKL^{-/-} counterparts (Fig. S2D).

Histological analysis of the engrafted glands revealed normal tissue structure at all stages (Fig. S2B) with immunohistochemistry for the myoepithelial markers p63 (Fig. S2E) and smooth muscle actin (Fig. S2F) showing normal organization of the two epithelial layers. Morphological hallmarks of secretion, such as lipid droplets, characteristic of the WT lactating gland, were sparse in the RANKL^{-/-} epithelium (Fig. S2B and G). Immunostainings for β -casein revealed the presence of this milk protein (Fig. S2G), suggesting differentiation occurs in the absence of RANKL.

Thus, epithelial RANKL is required for mammary gland side branching before and during pregnancy, consistent with this cytokine mediating progesterone function.

RANKL and Progesterone-Induced Proliferation. To address whether RANKL is required for progesterone-induced proliferation, mice engrafted with RANKL^{-/-} and WT epithelia were stimulated with progesterone. During the first 24 h, on average 16% of the cells in both epithelia incorporated BrdU (Fig. 3A), most of which expressed PR (Fig. 3B and C). Forty-eight hours after progesterone injection, an average of 17% of WT but less than 3% of RANKL^{-/-} MECs incorporated BrdU (Fig. 3D–F), suggesting that RANKL is not required for cell-autonomous proliferation, but rather paracrine proliferation induced by progesterone.

Analysis of apoptosis in contralateral glands engrafted with RANKL^{-/-} and WT MECs by caspase 3 staining (Table S1) and TUNEL assays (Fig. S3) revealed no difference.

It was conceivable that RANKL deletion affected PR signaling; however, expression of PR (Fig. S4A and B) and the PR target genes *wnt-4* and *calcitonin* was comparable (Fig. S4B). Similarly, cyclin D1 protein levels were independent of RANKL status (Fig.

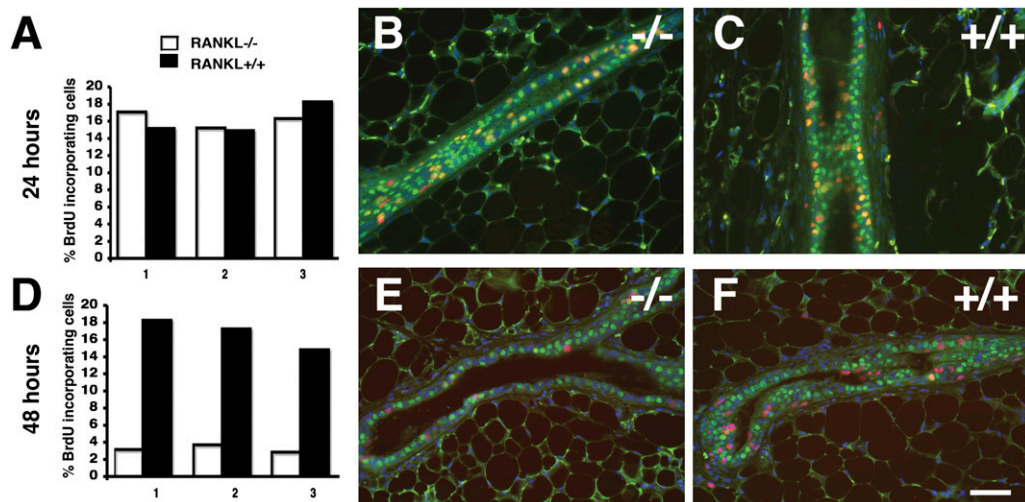


Fig. 3. Response to progesterone in RANKL^{-/-} MECs. (A–F) Mice engrafted with RANKL^{-/-} or WT MECs stimulated with progesterone for 24 h (A, B, and C) or 48 h (D, E, and F). The percentage of BrdU-incorporating cells \pm SEM in contralateral mammary epithelia of three mice after 24 h (A) and 48 h (D) of stimulation. Open bars, RANKL^{-/-}; black bars, WT MECs; total of 1,000 cells counted in three different sections from each mouse. (B, C, E, and F) Double-immunofluorescence at 24 h (B and C) and 48 h (E and F) of stimulation. Green, PR; red, BrdU; blue, DAPI. (Scale bar: 40 μ m.) Note: at 24 h, RANKL^{-/-} (B) and WT (C) MECs incorporate BrdU similarly and are PR(+). At 48 h, fewer mutant MECs incorporate BrdU (E) vs. WT (F), and most BrdU-incorporating cells are hormone receptor negative.

S4C), and RANKL expression levels independent of cyclin D1 status. (Fig. S4 D-H). We concluded that the first wave of progesterone-induced proliferation is cyclin D1 dependent and RANKL independent, whereas the second wave is cyclin D1 independent and RANKL dependent.

Ectopic Expression of RANKL in PR^{-/-} Epithelium. To test whether the paracrine, RANKL-dependent mechanism is sufficient to mediate progesterone function, we ectopically expressed RANKL in PR^{-/-} epithelium using retroviral vectors and used the infected cells to reconstitute cleared fat pads. Two months later, the recipients were mated. At the end of pregnancy, the glands were analyzed by whole-mount microscopy. As expected, endogenous control glands showed full alveolar development (Fig. 4A) and control virus-infected PR^{-/-} cells formed simple ductal systems (Fig. 4B). In glands reconstituted with RANKL-infected cells, some sectors had highly branched ductal systems decorated by alveoli (Fig. 4C and D), and some of these were undistinguishable from WT control glands (Fig. 4A), indicating that ectopic RANKL expression rescued the PR^{-/-} phenotype. At the cellular level, histological sections containing rescued areas showed fat droplets, a morphological hallmark of milk secretion (Fig. 4E and F) and expression of the milk protein β -casein (Fig. 4E and F). Thus, RANKL expression rescued the PR^{-/-} phenotype with regard to morphogenesis and differentiation.

In view of the 10–30% efficiency of retroviral infection of primary MECs, which is usually reflected by a similar extent of transgene expression in vivo (27), the large extent of rescue in the grafts with 74% of the grafts showing more than 40% rescue (Table S2) was surprising. To test whether RANKL is diffusible, we generated a retrovirus expressing both RANKL and GFP. In glands reconstituted with PR^{-/-} MECs infected with this virus, GFP expression overlapped with areas of rescue ($n = 10$) (Fig. 4G–I), indicating that RANKL confers a growth advantage to the MECs expressing it and their immediate neighbors.

Immunohistochemistry on PR^{-/-} MECs infected with the retrovirus expressing both RANKL and GFP revealed that BrdU-incorporating cells are frequently found next to ectopic RANKL-expressing cells, indicating that RANKL indeed elicits proliferation by a paracrine mechanism (Fig. 4K). Similarly, colabeling of mammary glands from WT females at day 12.5 of pregnancy revealed dissociation of BrdU incorporation and RANKL expression (Fig. 4L), with RANKL expressing cells frequently as direct neighbors of BrdU-incorporating cells (Fig. 4L Right). Thus, RANKL elicits proliferation by a paracrine mechanism.

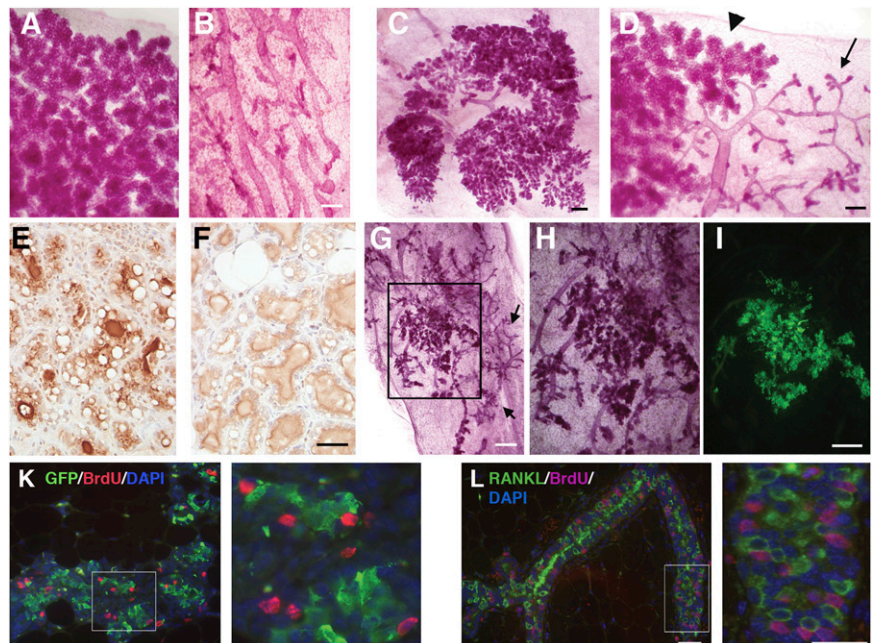
Systemic Modulation of Proliferation in the Mammary Gland. To assess whether proliferation of MECs can be induced by systemic RANKL administration, we injected PR^{-/-} females with recombinant RANKL (rRANKL). In rRANKL-treated mice, 14% of the MECs incorporated BrdU versus 4% in control-injected mice (Fig. 5A). Next, we sought to block the interaction between RANKL and its receptor by administering the decoy receptor OPG. Injection of rOPG resulted in 54% inhibition of the progesterone-induced proliferation (Fig. 5B). Double immunohistochemistry revealed that most of the cells that proliferate in the presence of OPG are PR(+) (Fig. 5C). Thus, systemic interference with RANKL signaling affects proliferation of MECs in vivo.

Discussion

The present study shows that progesterone elicits proliferation in the mammary epithelium by two distinct mechanisms. First, a subset of PR(+) cells proliferates by a cyclin D1-dependent mechanism. Second, a large number of PR(-) cells proliferate by a RANKL-dependent mechanism. These findings, with experimental hormone stimulation, are consistent with studies under physiologic conditions in different species showing that most proliferation occurs in hormone receptor-negative cells (12–14).

Approximately 20% of all PR(+) MECs proliferated during the first 24 h of hormone stimulation, prompting the question whether proliferation is a stochastic event or whether distinct

Fig. 4. RANKL is sufficient to mediate PR function in the mammary epithelium. (A–D) Whole-mount micrographs of mammary glands from WT hosts postpartum. (A) Endogenous gland showing highly branched ductal system with dilated alveoli. (B) Gland engrafted with PR^{-/-} epithelium infected with control virus. Note the simple ductal system. (Scale bar: 300 μ m.) (C and D) Two representative mammary glands engrafted with PR^{-/-} MECs infected with a retrovirus expressing RANKL showing extensive alveologensis (C) or PR^{-/-} phenotype in one area (arrow) next to a fully developed sector (arrowhead) (D). (E and F) Histological sections of postpartum mammary glands stained with an anti- β -casein antibody, gland engrafted with PR^{-/-} MECs infected with a retrovirus expressing RANKL (E), and endogenous control gland (F). Note the presence of fat droplets and β -casein expression in both WT epithelium and PR^{-/-} MECs expressing ectopic RANKL. (Scale bar: 40 μ m.) (G–I) Stereomicrographs of a mammary gland reconstituted with PR^{-/-} MECs expressing ectopic RANKL and GFP. The sector highlighted in G is magnified in H; same sector shown with a fluorescent image taken before tissue processing (I). Note: area of rescue coincides with GFP expression. Areas showing the PR^{-/-} phenotype (arrows; G) do not express detectable levels of GFP (G). (Scale bar: 1 mm.) (H and I) 400 μ m. (K) Double immunofluorescence for GFP (green) and BrdU (red) on mammary gland reconstituted with PR^{-/-} MECs expressing ectopic RANKL and GFP. Blue, DAPI. Note: BrdU incorporation in MECs adjacent to cells expressing viral GFP. (L) Anti-RANKL (green) and BrdU (red) immunofluorescence of histological sections of mammary glands from WT female at day 12.5 of pregnancy. Note: BrdU incorporation in MECs adjacent to cells expressing RANKL. (K and L Right) Zoom of marked area. (Scale bar: 40 μ m.)



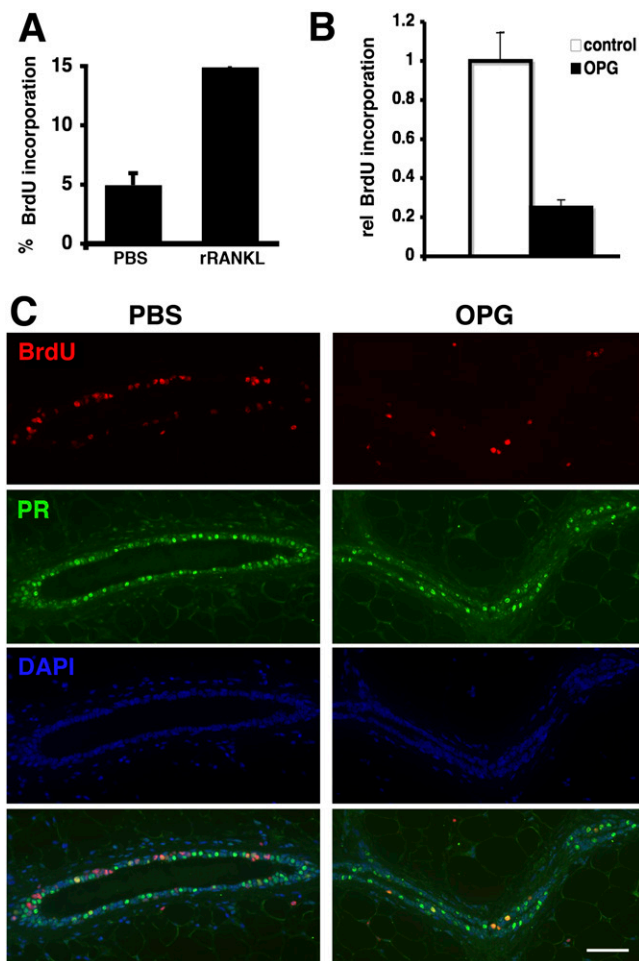


Fig. 5. Systemic manipulation of RANKL signaling affects the mammary epithelium. (A) Twelve-week-old $PR^{-/-}$ females were injected i.v. with either 8 μ g of Fc-RANKL or PBS. BrdU was administered continuously for 72 h. BrdU-incorporating cells \pm SEM; 1,200 cells were counted per mouse ($n = 6$), representing two independent experiments. (B and C) WT females were stimulated with progesterone and treated either with PBS or OPG. (B) BrdU incorporation in MECs is plotted \pm SEM. Open bars, PBS treated ($n = 9$); filled bars, OPG treated ($n = 12$); three independent experiments were performed. (C) Double immunofluorescence of histological sections from mammary glands stimulated with progesterone and treated with PBS (Left) or OPG (Right). Green, PR; red, BrdU; blue, DAPI; bottom, overlay. Note: most BrdU (+) MECs in OPG-treated animals are PR(+).

populations of PR(+) cells exist, some with a higher proliferation potential and some more differentiated. It is tempting to speculate that the BrdU incorporating PR(+) cells represent hormone receptor-positive stem cells, identified as label retaining epithelial cells (LREC) that incorporate label during pubertal ductal elongation and proliferate again upon hormonal stimulation during adulthood (30, 31), and that these cells may be expressing integrin $\beta 3$ (CD61), a marker for luminal progenitor cells, some of which express $ER\alpha$ (32).

Our finding that cyclin D1 expression is not affected by deletion of RANKL in the mammary epithelium of virgin mice is in line with observations on pregnant $RANKL^{-/-}$ mice (33). Yet, cross-talk between progesterone and RANKL signaling with cyclin D1 may occur at the level of posttranslational modification. Cyclin D1 function was shown to be controlled by phosphorylation on threonine-286 by GSK3- β (34), and I κ B kinase phosphorylates the steroid receptor coactivator (SRC)-3 (35), which is an important coactivator of PR (36).

Cyclin D1 has been shown to be required in MECs for alveolar development (37) downstream of PrIR signaling and IGF-2 (27). Whether the block in alveologensis is related to the failure of at least some PR(+) cells to divide in response to progesterone or whether it reflects a distinct function of cyclin D1 in MECs remains to be addressed.

Previous overexpression studies of both RANKL (18) and its receptor (19) indicated that RANK signaling is strongly mitogenic in MECs both in vivo and in vitro. Observations in 3D cultures pointed to a synergism between RANKL and hepatocyte growth factor (HGF) in eliciting proliferation of myoepithelial cells (38). Here, we provide genetic evidence that RANKL is required for the proliferation of PR(-) MECs and sufficient to rescue the phenotype of $PR^{-/-}$ MECs, consistent with the finding that ectopic expression of RANKL elicits pregnancy-related morphogenetic changes in virgin mice (18). We reported previously that *wnt-4* is required for progesterone-induced side branching (26). How RANKL and *wnt-4* interact, and whether they are coexpressed in the same PR-positive cells, remains to be addressed.

RANKL signaling may be directly or indirectly mitogenic. BAFF/BLYS and CD40L/CD144 also belong to the TNF family—the former induces direct proliferation of B cells, possibly through TRAF2-mediated activation of MEKK1, JNK, AP1, and up-regulation of cyclin D2 (39), whereas the latter indirectly promotes mitogenic signals originating from the B-cell receptor by promoting survival (40). RANKL was shown to trigger nuclear translocation of Id2, which in turn resulted in the down-regulation of the cell-cycle inhibitor p21 and proliferation in primary MECs (33).

Breast cancer risk correlates with the number of menstrual cycles a woman experiences, and cell proliferation occurs during the luteal phase of the menstrual cycle when progesterone levels are high, suggesting a link between progesterone exposure and the disease. Similarly, studies on women on hormone replacement therapy showed that concomitant administration of estrogens and progesterone results in increased breast cancer incidence, whereas administration of estrogen only did not have significant effect (41). Our finding that progesterone-induced proliferation can be inhibited by administering OPG may have important implications if RANKL signaling should have a similar role in the human breast. Clinical trials to test the utility of anti-RANKL agents in the therapy of bone erosion diseases such as osteoporosis, rheumatoid arthritis, and multiple myeloma are ongoing (42); might it turn out that these agents interfere with proliferation, in the breast epithelium possibly in a subset of patients, and protect against breast cancer?

Materials and Methods

Mice. GFP transgenic, RANKL, PR, and cyclin D1 mutant mice (3, 25, 43, 44) were bred in C57Bl6 or 129SV/C57Bl6 genetic background. Transplantation and mammary gland whole mounts were performed as described (5).

Hormone and BrdU Treatments. Ten-week-old female mice were ovariectomized and injected 10 days later s.c. with 17 β -estradiol (Sigma; 4 ng/g body weight) in tocopherol-stripped corn oil (MP Biomedicals). Twenty-four hours later, vehicle, 17 β -estradiol, or 17 β -estradiol and progesterone (Sigma; 100 μ g/g body weight) were injected. For continuous stimulation, both steroids were injected every 24 h. BrdU (Sigma; 30 μ g/g body weight) was injected either every 3 h for 24 h, or as bolus 2 h before sacrifice.

Injection of recombinant RANKL and OPG. Human OPG (amino acids 1–202) was fused at the N terminus of hlgG-Fc, expressed, and purified as described (45). The receptor-binding domain of mRANKL was fused at the C terminus to hlgG-Fc and purified as described (46). Eight micrograms of Fc-RANKL in PBS were administered intravenously, and BrdU was injected every 6 h for 72 h. Twelve-week-old female mice were ovariectomized and injected 10 days later s.c. with 17 β -estradiol and 24 h later with progesterone (100 μ g/g body weight). Two hours and 24 h later, mice were injected intraperitoneally with OPG (2.4 μ g/g of body weight) in PBS. BrdU was injected every 6 h for 48 h.

Histological Examination and Immunohistochemistry. Glands fixed with 4% paraformaldehyde were paraffin embedded. We stained 4- μ m sections with anti-BrdU (1:300) (Oxford Biotechnology; OBT0030), anti-GFP (1:4,000) (Molecular Probes; A6444), anti-PR (1:400) (Neomarkers; SP2), and anti-RANKL (1:200) (R&D Systems) overnight at 4 °C after antigen retrieval in citrate buffer and revealed with Vectastain Elite kit (Vector Laboratories). RANKL was detected using a TSA Signal Amplification System (PerkinElmer). Pictures were acquired with a Leica DM2000 microscope and Pixelink PL-A622C camera, and Zeiss Axioplan 2-imaging fluorescence microscope with Axiocam MRm camera.

Retroviral Production and Cell Infection. The *mRANKL* coding region (GenBank accession no. AB022039) was extracted from pCR3-RANKL subcloned via

Bluescript into MSCV (47) or PINCO (48). High-titer retrovirus was produced as described (49).

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