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The Osteoclast Differentiation Factor Osteoprotegerin-Ligand Is Essential for Mammary Gland Development

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Summary

Osteoprotegerin-ligand (OPGL) is a key osteoclast differentiation/activation factor essential for bone remodeling. We report that mice lacking OPGL or its receptor RANK fail to form lobulo-alveolar mammary structures during pregnancy, resulting in death of newborns. Transplantation and OPGL-rescue experiments in *opgl*$^{-/-}$ and *rank*$^{-/-}$ pregnant females showed that OPGL acts directly on RANK-expressing mammary epithelial cells. The effects of OPGL are autonomous to epithelial cells. The mammary gland defect in female *opgl*$^{-/-}$ mice is characterized by enhanced apoptosis and failures in proliferation and PKB activation in lobulo-alveolar buds that can be reversed by recombinant OPGL treatment. These data provide a novel paradigm in mammary gland development and an evolutionary rationale for hormonal regulation and gender bias of osteoporosis in females.

Introduction

The morphogenesis and remodeling of bone requires the synthesis of bone matrix by osteoblasts and its coordinated resorption by osteoclasts. The TNF family molecule Osteoprotegerin-ligand (OPGL; also known as ODF, TRANCE, and RANKL) (Anderson et al., 1997; Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998) is a key factor stimulating the differentiation and activation of osteoclasts, and is therefore essential for bone remodeling and calcium mobilization from the bones. Inactivation of the *opgl* gene in mice results in a complete block in osteoclast development that leads to severe osteopetrosis and a failure in tooth eruption (Kong et al., 1999a). Treatment of *opgl*$^{-/-}$ mice in vivo with recombinant OPGL increases serum calcium levels by activating osteoclasts (Lacey et al., 1998). OPGL exerts its effects on osteoclasts by binding to the TNFR family receptor RANK (receptor activator of NFκB) (Hsu et al., 1999). Osteoprotegerin (OPG) acts as a soluble decoy receptor and competes with RANK for binding to OPGL (Lacey et al., 1998). The balance between OPGL and OPG levels determines osteoclast activation, skeletal calcium release, and bone remodeling (Karsenty, 1999). Abnormalities in the OPGL/RANK/OPG system lead to the increased bone resorption that underlies the bone damage of postmenopausal osteoporosis (Simonet et al., 1997), Paget's disease (Kurihara et al., 2000), bone loss in metastatic cancers (Honore et al., 2000), and crippling rheumatoid arthritis (Kong et al., 1999b).

The expression of OPGL and OPG is regulated by multiple hormones and cytokines shown to affect the development and activation of osteoclasts, including 25-dihydroxyvitamin D3, IL-1, IL-11, PGE_2, calcitonin, and TNFα (Ross, 2000). Intriguingly, expression of OPGL and OPG is also strongly influenced by the female sex hormones progesterone and estrogen, and by hormones involved in reproduction and lactation such as prolactin and parathyroid hormone-related peptide (PTHrP) (Karsenty, 1999). Reduction of ovarian function following menopause in women, and ovariectomy in animal models, result in osteoporosis and fractures, conditions that can be completely reversed, at least in animals, by treatment with OPG (Simonet et al., 1997). The evolutionary and functional rationale for OPGL/OPG regulation by reproductive hormones and the prevalence of hormonally regulated and gender biased osteoporosis in older females is not known.

In mammals, sex and pregnancy hormones control mammary gland morphogenesis and formation of a lactating mammary gland. Mammary gland morphogenesis proceeds in distinct steps, beginning with a fetal mammary anlage that undergoes ductal elongation and branching (Robinson et al., 1999). Mammary branching commences with puberty and leads to infiltration of the epithelial ductal tree into the mammary fat pad. During pregnancy, increased ductal side branching and development of lobulo-alveolar structures result from the expansion and proliferation of ductal and alveolar epithelium (Neville, 1999; Robinson et al., 2000). Formation of a lactating mammary gland provides essential nourishment to mammalian newborns in the form of calcium-rich milk. Calcium transport from the mother to the fetus and neonates is a vital process to preserve the species.

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Mothers meet the increased requirements for calcium during pregnancy and lactation by doubling their intestinal calcium absorption and demineralizing their skeletons via activation of bone-resorbing osteoclasts (Kovacs and Kronenberg, 1997).

We report that pregnancy hormones induce OPGL expression in mammary epithelial cells. OPGL expression is required for cellular survival/proliferation. Mutations of the *opgl* or *rank* genes result in the total inhibition of lobulo-alveolar epithelial development and a complete block in the formation of a lactating mammary gland, leading to the death of newborn pups. Local reconstitution of OPGL in *opgl*$^{-/-}$ mammary tissue, but not in *rank*$^{-/-}$ mammary glands, restores epithelial proliferation, lobulo-alveolar development, and mammary gland formation during pregnancy. Thus, OPGL and RANK, the master regulators of skeletal calcium release, are essential for the formation of the lactating mammary gland, the organ required for transmission of maternal calcium to neonates in mammalian species.

Results

Essential Role of OPGL in the Formation of Lobulo-Alveolar Mammary Structures in Pregnancy

Since OPGL expression was known to be regulated by sex and reproductive hormones (Karsenty, 1999; Roodman, 1999; Ross, 2000), we investigated whether OPGL function was required during pregnancy and lactation. Although *opgl*$^{-/-}$ mothers are osteopetrotic and display a failure in tooth eruption, they are fertile and give birth to litters of morphologically normal pups whose numbers were comparable to those of *opgl*$^{+/-}$ and *opgl*$^{+/+}$ mothers. However, despite normal nursing and mothering characteristics displayed by *opgl*$^{-/-}$ females and suckling attempts made by their pups, newborns of *opgl*$^{-/-}$ mothers failed to thrive and all pups died within 48 hr of birth. Lethality of the newborns was independent of their genotype (*opgl*$^{+/-}$ or *opgl*$^{-/-}$). Examination of one-day-old pups revealed that they lacked milk in their stomachs. Fostering by surrogate wild-type females rescued the survival of both *opgl*$^{+/-}$ and *opgl*$^{-/-}$ pups born to *opgl*$^{-/-}$ females. These data suggested that *opgl*$^{-/-}$ mothers might have a defect in the production of breast milk.

Defective milk production can be caused by various developmental and/or functional abnormalities in the mammary glands. Mammary gland morphogenesis proceeds in distinct steps, beginning with a fetal mammary anlage that undergoes ductal elongation and branching (Robinson et al., 1999). Mammary branching commences with puberty and leads to infiltration of the epithelial ductal tree into the mammary fat pad. In age-matched nulliparous *opgl*$^{-/-}$, *opgl*$^{+/-}$, and *opgl*$^{+/+}$ females, formation of the mammary gland anlage, elongation and extension of the ductal tree, as well as ductal side branching were comparable (Figures 1A and 1B). Histological analyses of nulliparous mammary tissues at different time points after birth showed that the epithelial ductal structures of *opgl*$^{-/-}$ mice were of the usual size and surrounded by normal stromal matrix and fibroblasts (Figures 2A and 2B). Importantly, *opgl*$^{-/-}$ mice contained normal numbers of myoepithelial cells, as assessed by histological examination and immunohistochemical determination of smooth muscle actin expression. As in the wild type, these cells were localized to the basal surfaces of the epithelial ducts (inset in Figure 2B). These results indicate that the absence of OPGL expression has no apparent effect on the development of the parenchymal breast tissue during postnatal life, puberty, or in nulliparous adult female mice. The rate of ductal side branching during puberty needs to be further analyzed in *opgl*$^{-/-}$ mice.

Since morphogenesis of the ductal tree appeared normal in nulliparous *opgl*$^{-/-}$ females, we hypothesized that loss of OPGL might affect mammary gland development during pregnancy. In wild-type females, pregnancy hormones induce proliferation of ductal epithelium and sprouting of alveolar buds in mid-pregnancy (Figures 1C and 2C). Proliferation and terminal differentiation of the alveolar buds follows, resulting in fully developed lobulo-alveolar structures and dilated primary ducts at one day lactation (L1) (Figures 1E and 2E). In the mammary glands of pregnant *opgl*$^{-/-}$ females, increased ductal side branching and formation of the initial alveolar buds occurred normally (Figures 1D and 2D), indicating that OPGL is not involved in these processes. However, differentiation and expansion of the alveolar buds into mature lobulo-alveolar mammary structures was completely arrested in *opgl*$^{-/-}$ females. The earliest defects in alveolar development in pregnant female *opgl*$^{-/-}$ mice were observed on day 14.5 of pregnancy (P14.5) (Figure 1D), and these defects remained evident until lactation (Figure 1F). Nevertheless, at lactation, the mammary glands of *opgl*$^{-/-}$ mice displayed the usual dilation of primary ducts (Figure 2F; compare to ducts at P14.5 in Figure 2D) indicating that luminal expansion of epithelial ducts is not affected by the absence of OPGL. In addition to the defect in lobulo-alveolar development, expression of mRNA for the major milk protein β-casein was significantly impaired starting at P14.5 and continuing until at least day one of lactation (L1; Figure 2G). Therefore, the death of pups born to *opgl*$^{-/-}$ females appears to be the consequence of inappropriate mammary glandular growth and a failure in maternal milk production. These data show that OPGL expression is essential for the development of the initial mammary alveolar buds into mature lobulo-alveolar epithelial structures, and the formation and differentiation of a functional, milk-producing mammary gland.

OPGL Is Necessary for Mammary Gland Development during Pregnancy

The defect in lobulo-alveolar development in pregnant *opgl*$^{-/-}$ females could point to a primary role for OPGL in mammary tissue development, or might be secondary to systemic endocrine abnormalities. We therefore determined whether local reconstitution of *opgl*$^{-/-}$ mammary tissue with recombinant mouse OPGL (rOPGL) could rescue lobulo-alveolar development. Slow-release pellets containing soluble rOPGL were implanted into the mammary glands of nonpregnant and pregnant females. To first determine if soluble rOPGL could induce ductal and/or alveolar differentiation in the absence of pregnancy, we implanted rOPGL-containing pellets into one mammary fat pad of nulliparous, nonpregnant wild-type females, and control pellets containing the vehicle

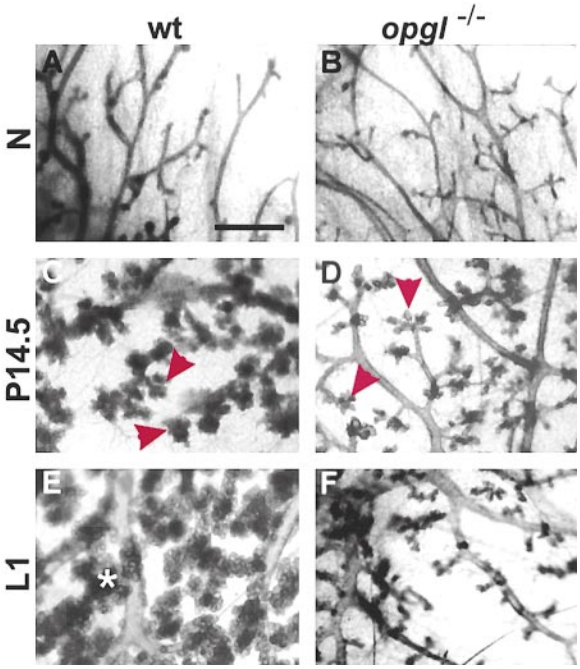

Figure 1. Essential Role of OPGL in the Formation of Lobulo-Alveolar Mammary Structures in Pregnancy

Whole-mount analyses of mammary tissue of (A) nulliparous wild-type females; (B) nulliparous *opgl*$^{-/-}$ females; (C) wild-type females at P14.5; (D) *opgl*$^{-/-}$ females at P14.5; (E) wild-type females at L1; and (F) *opgl*$^{-/-}$ females at L1. Mammary gland ductal morphogenesis appears normal in nulliparous *opgl*$^{-/-}$ females (A and B). Alveoli in gestating wild-type females (arrowhead in C) have progressed to form lobular structures, whereas development is arrested at a rudimentary alveolar bud in *opgl*$^{-/-}$ females (arrowheads in D). Lobulo-alveolar structures (asterisks in E) in wild-type females at L1 encompass the whole mammary fat-pad. In contrast, *opgl*$^{-/-}$ mammary tissue is devoid of lobulo-alveolar structures at L1 (F) and resembles that at P14.5 (D). Scale bars: (A)–(F) 0.5 mm.

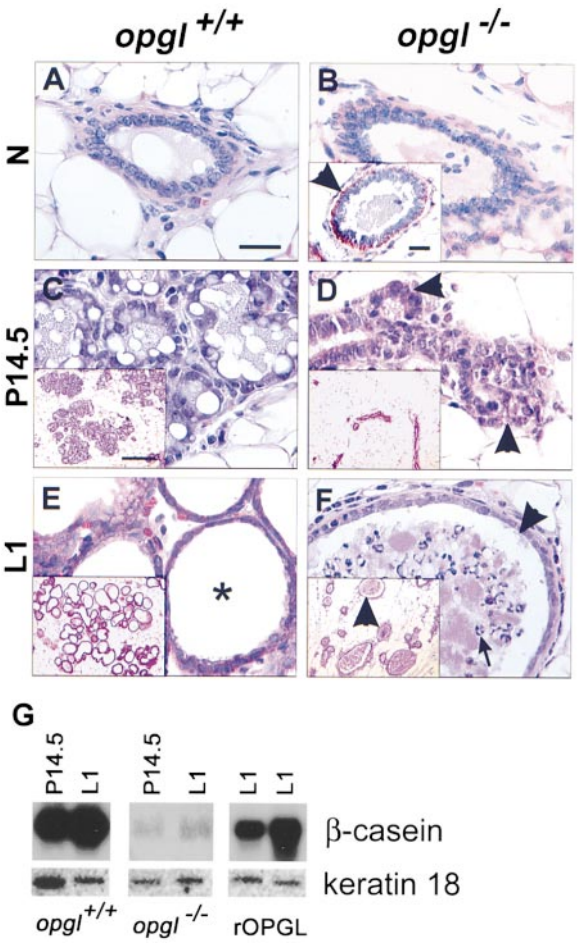

Figure 2. Impaired Development of Mammary Lobulo-Alveolar Structures in *opgl*$^{-/-}$ Females

(A–F) Histological appearance of mammary tissue of (A) nulliparous wild-type females; (B) nulliparous *opgl*$^{-/-}$ females; (C) wild-type females at P14.5; (D) *opgl*$^{-/-}$ females at P14.5; (E) wild-type females at L1; and (F) *opgl*$^{-/-}$ females at L1. Arrowhead in inset of (B) shows the normal appearance of myoepithelial cells surrounding the epithelial ducts in *opgl*$^{-/-}$ mammary tissue as assessed by immunoperoxidase staining for smooth muscle actin. Alveolar development is arrested at a rudimentary alveolar bud in *opgl*$^{-/-}$ females (arrowheads in D). Note that primary ducts (arrowheads in F) are enlarged in *opgl*$^{-/-}$ females at L1 as compared to P14.5 (D). These enlarged ducts at L1 contain lipid droplets and often contain CD45^{+} leukocytes (arrow in F). The distribution and number of CD45^{+} leukocytes adjacent to the myoepithelial mammary tissue was comparable between wild-type and *opgl*$^{-/-}$ nulliparous females and females at P14.5. H&E stainings. Scale bars: (A)–(F) 25 μm; (B) insert, 25 μm; inserts in (C)–(F) 0.1 mm.

(G) Expression of the milk protein β-casein. Total RNA was isolated at P14.5 and L1 from mammary tissues of wild type (*opgl*$^{+/+}$) females, *opgl*$^{-/-}$ females, and two different *opgl*$^{-/-}$ females implanted with pellets containing rOPGL [10 μg/pellet] (rOPGL). A representative Northern blot of β-casein and keratin 18 mRNA expression is shown.

alone into the contralateral mammary fat pad of the same mice. Local administration of rOPGL did not induce proliferation, elongation, ductal side-branching, or alveolar bud formation in nonpregnant animals (not shown).

However, when rOPGL pellets were implanted into the mammary tissues of pregnant *opgl*$^{-/-}$ females, lobulo-alveolar development was rescued (Figures 3A–3D), in that the morphological appearance of lobulo-alveolar structures was comparable to that of pregnant wild-type females (Figure 3B to Figure 1E). Moreover, local implantation of rOPGL restored the expression of β-casein mRNA (Figure 2G), indicating a recovery of milk production. In contrast, implantation of control pellets into the contralateral mammary tissue of the same pregnant *ogpl*$^{-/-}$ females did not restore lobulo-alveolar development, and mammary gland formation remained blocked at the alveolar bud stage (Figures 3A and 3C). These results indicate that the developmental arrest of *opgl*$^{-/-}$ mammary glands is directly due to a lack of OPGL. It should be noted that ovulation and subsequent luteal growth in the ovaries, development and differentiation of uteri, as well as trophoblastic implantation are comparable among *opgl*$^{-/-}$, *opgl*$^{+/-}$, and *opgl*$^{+/+}$ females, indicating that OPGL has no effect on these tissues. Thus, local administration of rOPGL restores lobulo-alveolar development in the mammary gland.

The Effect of OPGL Is Autonomous to Epithelial Cells

Mammary gland development is controlled by an interplay between epithelial and stromal cells (Robinson et

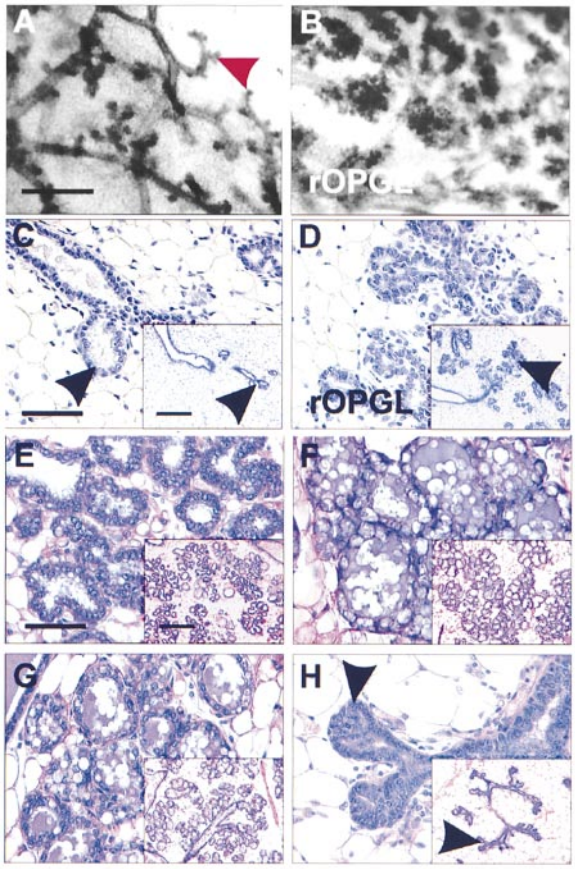

Figure 3. OPGL Protein Restores Mammary Gland Development in Pregnant *opgl*$^{-/-}$ Females

(A–D) Rescue of lobulo-alveolar development in pregnant *opgl*$^{-/-}$ females by rOPGL. (A,C) Control pellets and (B and D) pellets containing rOPGL [10 μg/pellet] were implanted into contralateral mammary glands of *opgl*$^{-/-}$ females at P14.5. Mammary glands were analyzed at L1. Lobulo-alveolar development (arrowhead in D) proceeds normally in pregnant *opgl*$^{-/-}$ mice implanted with rOPGL, whereas the development of these structures is not rescued in the control contralateral mammary glands of the same mouse (arrowheads in A and C).

(E-H) *opgl*$^{+/+}$ and *opgl*$^{-/-}$ mammary epithelial tissues were transplanted into cleared fat-pads of *rag1*$^{-/-}$ females prior to pregnancy. (E and G) Normal development of lobulo-alveolar structures in nontransplanted mammary glands of *rag1*$^{-/-}$ females. (F) *rag1*$^{-/-}$ stroma supports the development into normal lobulo-alveolar structures of *opgl*$^{+/+}$ mammary epithelial cells transplanted in the contralateral mammary gland of the same mouse as in (E). (H) *rag1*$^{-/-}$ stroma fails to rescue lobulo-alveolar development (arrowheads) of *opgl*$^{-/-}$ mammary epithelial cells transplanted into the contralateral mammary gland of the same mouse as in (G). Data from analyses at L1 are shown. Scale bars: (A) and (B), 0.5 mm; (C)–(H), 50 μm; inserts in (C)–(H), 0.1 mm.

al., 1999). Since the slow-release rOPGL pellets could possibly override and mask essential epithelial-stromal interactions, the impaired mammary gland development in *opgl*$^{-/-}$ mice could be due to defects in stromal cells and/or a cell-autonomous defect localized to the epithelial cells. Thus, we evaluated whether *opgl*$^{+/+}$ stromal tissue could induce *opgl*$^{-/-}$ epithelial cells to form lobulo-alveolar structures during pregnancy. To separate stromal from epithelial signals, *opgl*$^{+/+}$ or *opgl*$^{-/-}$ nulliparous mammary epithelial tissues were transplanted into cleared mammary fat pads of nulliparous *rag1*$^{-/-}$ females (Robinson et al., 2000). *Rag1*$^{-/-}$ mice lack T and B cells and do not reject the epithelial transplants. The contralateral fat pad of the transplanted animals was left untreated. *Rag1*$^{-/-}$ animals that carried transplanted *opgl*$^{+/+}$ or *opgl*$^{-/-}$ mammary epithelium were mated and mammary gland development was examined (Figures 3E–3H). At lactation, the development of *opgl*$^{+/+}$ tissue transplanted into *rag1*$^{-/-}$ females (Figure 3F) paralleled that of the nontransplanted control gland (Figure 3E), and the fat-pads of both glands were filled with lobulo-alveolar structures exhibiting secretory activity. However, mammary gland development was arrested at the alveolar bud stage when *opgl*$^{-/-}$ epithelium was transplanted into *rag1*$^{-/-}$ mammary stroma (Figure 3H). It should be noted that although *rag1*$^{-/-}$ stroma did not rescue lobulo-alveolar development of *opgl*$^{-/-}$ epithelium, it was able to support elongation and side-branching of *opgl*$^{-/-}$ ductal epithelial cells (Figure 3H). These transplantation studies show that the lobulo-alveolar differentiation defect in pregnant *opgl*$^{-/-}$ females is inherent and cell autonomous to mammary epithelial cells and cannot be overcome by the presence of wild-type stroma. Thus, local OPGL is essential for the development of a mammary gland during pregnancy.

Loss of RANK Phenocopies *opgl* Deficiency in Mammary Tissue

RANK is a member of the TNFR family that acts as a receptor for OPGL on osteoclasts and dendritic cells (Anderson et al., 1997; Hsu et al., 1999). Mice with mutations in *rank* have defects in osteoclastogenesis and lymph node formation (Dougall et al., 1999; Li et al., 2000) that are identical to those we have previously reported for *opgl*$^{-/-}$ mice (Kong et al., 1999a). However, *opgl*$^{-/-}$ mice also show defects in T cell development that are not found in *rank*$^{-/-}$ mice, suggesting that OPGL may associate with another receptor. To test whether OPGL's role in mammary gland formation is mediated by RANK, or by another as yet unidentified receptor, we analyzed mammary gland development in *rank*$^{-/-}$ female mice that are osteopetrotic and display a failure in tooth eruption (Li et al., 2000). Like *opgl*$^{-/-}$ mothers, *rank*$^{-/-}$ mothers are fertile and give birth to litters of morphologically normal pups whose numbers were comparable to those of *opgl*$^{+/-}$ and *opgl*$^{+/+}$ mothers. However, after giving birth, *rank*$^{-/-}$ females failed to lactate and their litters did not survive, despite normal nursing and suckling behaviors displayed by mothers and pups.

Formation of the mammary gland anlage, elongation and extension of the ductal tree, and ductal side branching were comparable between age-matched nulliparous *rank*$^{-/-}$, *rank*$^{+/-}$, and *rank*$^{+/+}$ mice (Figures 4A–4C, and data not shown). Thus, neither OPGL nor RANK is required for mammary gland development before pregnancy. In pregnant *rank*$^{-/-}$ females, the initial alveolar buds sprouted but differentiation and expansion of these buds into mature lobulo-alveolar mammary structures was completely abolished (Figures 4D, 4E, 4G, and 4H). Importantly, whereas implantation of rOPGL into the mammary tissues of pregnant *opgl*$^{-/-}$ females restored lobulo-alveolar differentiation (Figures 3A–3D) and milk

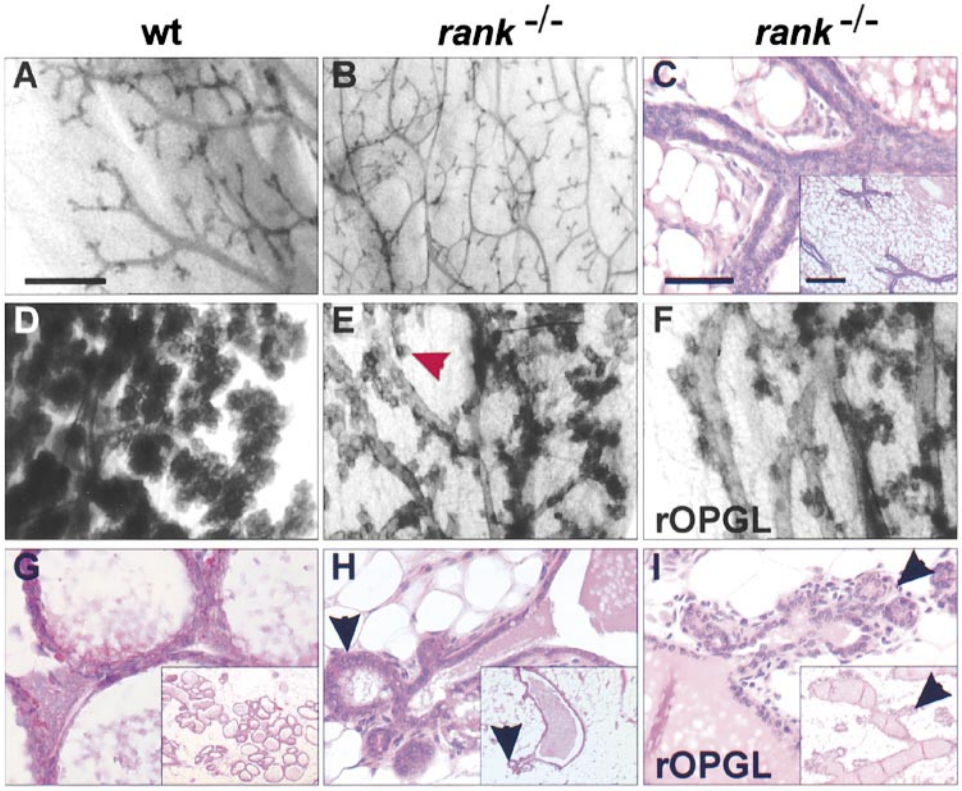

Figure 4. *rank*$^{-/-}$ Mammary Tissue Phenocopies Defects in *opgl*$^{-/-}$ Mammary Tissue

(A–C) Normal mammary ductal morphogenesis in nulliparous wild-type (A) and *rank*$^{-/-}$ (B,C) female mice. Inset in (C) is a low power magnification showing normal branching morphogenesis.

(D,G) Typical development of lobulo-alveolar structures at lactation in wild-type tissue. (E,H) Absence of lobulo-alveolar development in pregnant *rank*$^{-/-}$ females. Development is arrested at the initial alveolar bud stage (arrowheads), as occurs in *opgl*$^{-/-}$ females. (F and I) Resistance of the lobulo-alveolar defect to rescue by implantation of rOPGL-containing pellets [10 μg/pellet] into pregnant *rank*$^{-/-}$ females. Only alveolar buds (arrowheads) are detectable. Representative whole mount stainings and H&E stained sections are shown at L1. Scale bars: (A), (B), (D), and (F), 0.5 mm; (C), and (G–I), 50 μm; inserts in (C) and (G–I), 0.1 mm.

production (Figure 2G), implantation of rOPGL into the mammary tissues of pregnant *rank*$^{-/-}$ mice did not rescue the mammary defects (Figure 4F and 4I). Thus, pregnant *rank*$^{-/-}$ females display defects in mammary gland development that are phenocopies of those in pregnant *opgl*$^{-/-}$ mice. These results show that expression of both OPGL and RANK is essential for differentiation of mammary alveolar cells during pregnancy, and that OPGL functions through RANK alone to mediate lobulo-alveolar morphogenesis in preparation for lactation.

Hormone-Induced Expression and In Situ Localization of OPGL and RANK

OPGL is expressed in lymph nodes, spleen, thymus, intestinal lymphoid patches, and in areas of active bone remodeling in the skeleton, primitive mesenchymal cells surrounding the cartilaginous anlagen, hypertrophying chondrocytes, and activated T cells (Wong et al., 1997; Lacey et al., 1998). RANK is expressed on hematopoietic osteoclast progenitors, mature osteoclasts, chondrocytes, endothelial cells of large arterial blood vessels, and dendritic cells of the immune system (Anderson et al., 1997; Hsu et al., 1999). It has also been reported that RANK is expressed on some human breast cancer cell lines (Thomas et al., 1999). In the absence of published reports on the expression of OPGL and RANK in normal human mammary tissue, we determined the levels and kinetics of OPGL and RANK expression, as well as the cellular localization of these molecules, in mammary tissues of nulliparous wild-type females and at different stages of pregnancy. Expression of OPGL protein in mammary tissue was evident as early as P12.5 and gradually increased to maximal expression at L1 (Figure 5A). OPGL protein could not be detected in nulliparous mammary tissue undergoing active ductal development in a 5-week-old mouse (Figure 5A) or in mammary tissue of adult nonpregnant females (not shown).

Because OPGL is not expressed in the mammary tissue of nonpregnant females but is strongly induced during pregnancy, we hypothesized that OPGL expression might be regulated by pregnancy hormones. Progesterone, estrogen, prolactin, and PTHrP are systemically upregulated during pregnancy and are essential for both pregnancy and lactation (Ormandy et al., 1997; Couse and Korach, 1998; Dunbar and Wysolmerski, 1999; Horseman, 1999). These hormones were individually injected into nonpregnant, nulliparous female mice and OPGL and RANK expression in the mammary tissues was examined by RT-PCR and Western blotting. Expression of OPGL mRNA (not shown) and protein (Figure

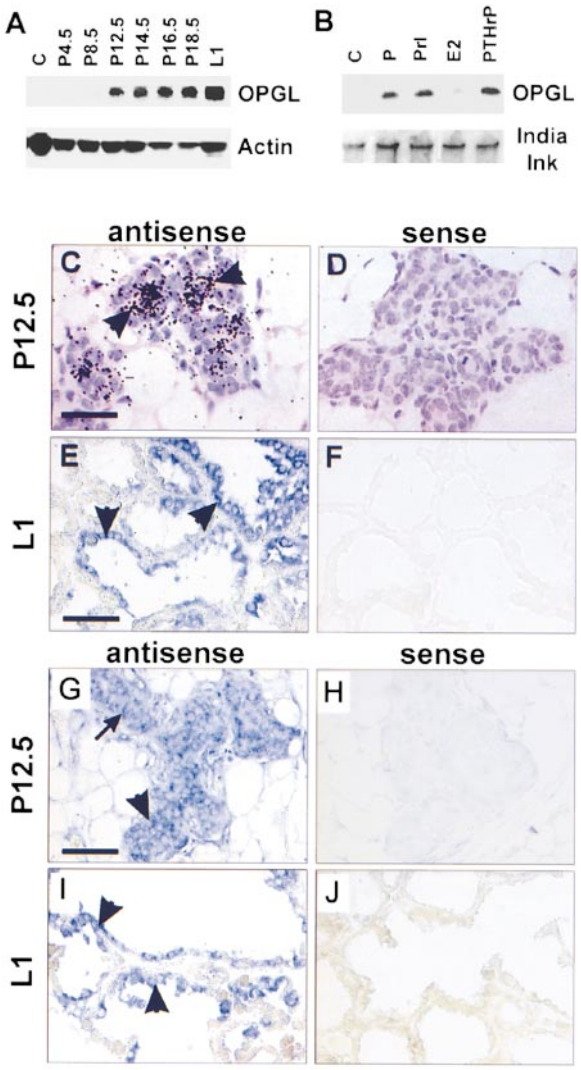

Figure 5. OPGL and RANK Expression in Mammary Tissues

(A) OPGL protein expression in mammary glands of a 5-week-old nulliparous control female (C), at different days of pregnancy (P4.5-P18.5) and L1. OPGL expression was detected by Western blotting. One result representative of three experiments is shown.

(B) Induction of OPGL in mammary glands in response to hormones. Nulliparous wild-type females were ovarectomized and injected with PBS (control, C), progesterone (P), prolactin (Prl), 17-β-estradiol (E2), or PTHrP. OPGL expression in mammary tissues was determined by Western blotting 24 hr after the initial injection. A representative India ink control is shown.

(C–J) In situ hybridization to localize (C–F) OPGL mRNA and (G–J) RANK mRNA expression at P12.5 and L1. Expression of both OPGL and RANK mRNA is restricted to epithelial cells. (C and D) Expression of OPGL in the alveolar bud epithelium at P12.5 (arrowheads). At day P12.5, RANK mRNA is abundantly expressed in ductal (arrow in H) and alveolar bud epithelium (arrowhead in H). (E and I) At L1, both OPGL and RANK are highly expressed in lobulo-alveolar epithelial cells (arrowheads). (C), (E), (G), and (I) were hybridized to antisense and (D), (F), (H), and (J) to sense (control) riboprobes. (C) and (D) are ^{35}S -UTP and (E)–(J) are DIG-UTP in situ hybridizations. Scale bars: 50 μm.

5B) was strongly induced by progesterone, prolactin, or PTHrP, but not by 17-β-estradiol. Furthermore, both prolactin and PTHrP were able to induce OPGL expression in ovariectomized female mice that do not produce ovarian-derived estrogen or progesterone. In contrast to the inducible expression of OPGL, RANK mRNA was found to be constitutively expressed in the mammary tissue of nonpregnant females and at all stages of pregnancy and lactation analyzed (not shown).

In situ mRNA hybridization studies showed that expression of OPGL was restricted to mammary epithelial cells both at P12.5 (Figures 5C and 5D) and L1 (Figures 5E and 5F). Interestingly, at P12.5, OPGL mRNA was most strongly expressed in the developing alveolar buds but OPGL mRNA was not detected in ductal epithelial cells. Similarly to OPGL expression, RANK mRNA expression was localized to epithelial cells at P12.5 (Figures 5G and 5H) and L1 (Figures 5I and 5J). At day 12.5 of pregnancy, RANK mRNA is abundantly expressed in ductal and alveolar bud epithelium (Figure 5G). At L1, both OPGL and RANK are highly expressed in lobulo-alveolar epithelial cells (Figures 5E and 5I). These data show that pregnancy hormones induce OPGL in mammary epithelial cells whereas RANK is constitutively expressed on the same or a neighboring epithelial cell.

OPGL Is Required for Proliferation and Survival of Mammary Epithelial Cells

What is the mechanism of impaired lobulo-alveolar differentiation in *opgl*$^{-/-}$ and *rank*$^{-/-}$ mice? During pregnancy in wild-type females, increased ductal side branching and development of lobulo-alveolar structures result from the expansion and proliferation of ductal and alveolar epithelium (Neville, 1999). *opgl*$^{-/-}$ and *rank*$^{-/-}$ mice show rudimentary alveolar development and form the initial alveolar buds, suggesting that OPGL does not initiate mammary differentiation. Rather, impaired alveolar epithelial cell proliferation and/or excessive cell death occurring as the gland tries to form lobular-alveolar structures might result in the observed defects.

To address this issue, the status of epithelial proliferation was assessed by in situ immunostaining for proliferating cell nuclear antigen (PCNA). The proliferation index (PI) was defined as the number of PCNA-positive nuclei of alveolar epithelial cells/total nuclei. The proliferation of alveolar bud epithelium was significantly reduced in *opgl*$^{-/-}$ females compared to the wild-type. Implantation of control pellets (Figures 6A and 6C) did not rescue epithelial proliferation and the number of PCNA-positive cells remained similar to that in nonmanipulated pregnant *opgl*$^{-/-}$ mice (PI = 16 ± 4.9 versus 21 ± 6.1, respectively). Implantation of rOPGL-containing pellets restored PCNA expression in alveolar epithelial cells of pregnant *opgl*$^{-/-}$ mice (PI = 62 ± 6.7; Figures 6B and 6D) to the level observed in pregnant wild-type mice (PI = 57 ± 4.5). Induction of alveolar epithelial proliferation by rOPGL in *opgl* $^{-/-}$ females is clearly evident in alveolar buds (Figure 6D) when contrasted to impaired PCNA in the rudimentary alveolar buds of the contralateral control mammary tissue (Figure 6C). In contrast to alveolar epithelium, the proliferation of ductal epithelial cells was comparable between *opgl*$^{+/+}$ and *opgl*$^{-/-}$ nontransplanted females both at P14.5 (PI = 18 ± 2.8 versus 19 ± 4.2, respectively) and at L1 (PI = 20 ± 6.3 versus 17 ± 3.9). Implantation of rOPGL had no apparent influ-

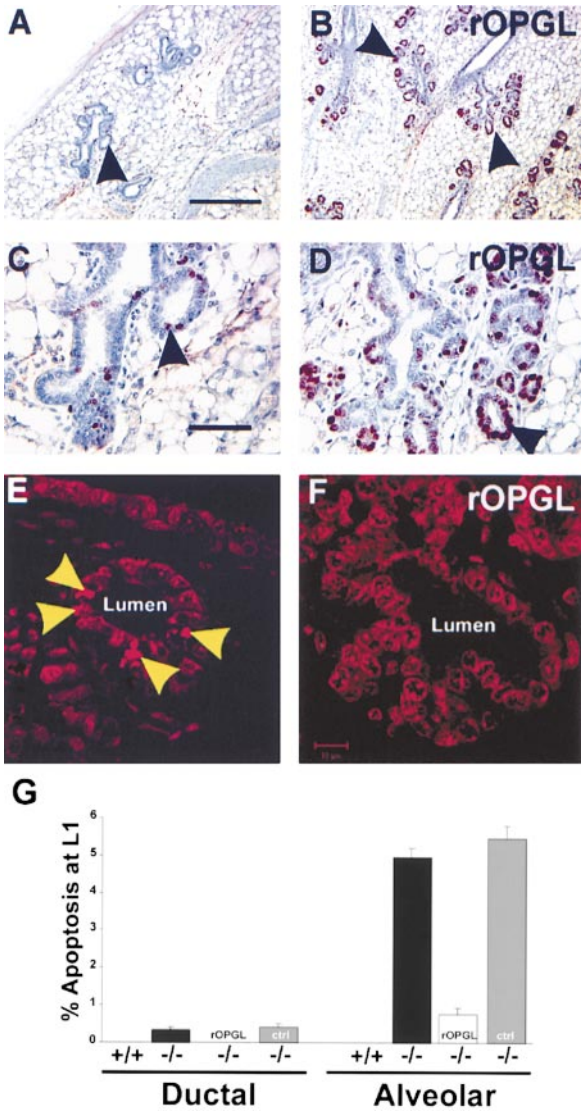

Figure 6. OPGL Promotes Alveolar Epithelial Proliferation and Survival

(A–D) Impaired proliferation of alveolar epithelium in *opgl*$^{-/-}$ mammary glands as detected by in situ PCNA immunostaining. (A and C) *opgl*$^{-/-}$ mammary tissue implanted with a control pellet at P14.5 exhibits few proliferating epithelial cells in alveolar buds (arrowheads) at L1. (B and D) Contralateral tissue of the same mouse as in (A) and (C) implanted with rOPGL [10μg/pellet] at P14.5 displays induction of lobulo-alveolar structures with rescued proliferation of alveolar epithelial cells (arrowheads) at L1. (C) and (D) are magnifications of (A) and (B), respectively.

(E–G) Increased mammary epithelial cell apoptosis in the absence of OPGL, as detected by confocal microscopy of Hoechst stained cells. (E) Apoptotic nuclei (arrows) are apparent at L1 in epithelial cells of developing alveoli in mammary tissue isolated from *opgl*$^{-/-}$ females implanted with a control pellet. (F) Contralateral tissue of the same mouse as in (E) implanted with rOPGL [10 μg/pellet]. No apoptotic nuclei are detectable in the developing lobulo-alveolar structure at L1. (G) Quantification of ductal and alveolar epithelial apoptosis at L1. A minimum of 1000 nuclei of ductal and alveolar epithelial cells was counted for each sample. Results shown are of individual mammary tissues. For (E)–(G), apoptotic nuclei were detected by confocal microscopy and cell death confirmed by in situ DNA end-labeling. Scale bars: (A) and (B), 0.1 mm; (C) and (D), 50 μm. Magnification in (E) and (F) 400×.

ence on the proliferation of ductal epithelial cells in pregnant *opgl*$^{-/-}$ mice. These data indicate that OPGL is a critical regulator of alveolar epithelial cell proliferation.

Because OPGL has been shown to provide survival signals to osteoclasts and dendritic cells (Anderson et al., 1997; Wong et al., 1999), we analyzed the apoptosis of mammary epithelial cells in the presence and absence of OPGL (Figures 6E–6G). No apoptotic epithelial cells were detected in ductal and alveolar tissues of wild-type mammary glands throughout pregnancy. In contrast, apoptotic cells were readily detected among mammary epithelial cells from *opgl*$^{-/-}$ females both at P14.5 (not shown) and L1 (Figure 6G). The increased apoptosis occurred primarily in the epithelium of alveolar buds (Figure 6E), although minimal (<0.5%) cell death was also observed in *opgl*$^{-/-}$ ductal epithelium (Figure 6G). Implantation of rOPGL pellets markedly reduced apoptosis in *opgl*$^{-/-}$ alveolar epithelium, whereas contralateral implantation of control pellets (ctrl) did not (Figures 6E–6G). These results indicate that loss of OPGL expression results in increased apoptosis of mammary alveolar epithelial cells during pregnancy, but can be rescued by rOPGL. Thus, OPGL is required for proliferation and survival of mammary alveolar epithelial cells during pregnancy.

Impaired PKB/Akt Activation in *opgl*$^{-/-}$ Mammary Epithelial Cells

The above data suggested that OPGL is a crucial survival factor for alveolar epithelium, prompting us to examine the expression and activation status of the anti-apoptotic molecule PKB/Akt in *opgl*$^{-/-}$ mammary tissue. In situ immuno-localization studies showed that phosphorylated serine 473 of PKB/Akt (indicative of active PKB/Akt) was not detectable in nonpregnant tissues of wild type mice (not shown). However, PKB/Akt phosphorylation did occur in wild type mammary epithelial cells during pregnancy (not shown) and at lactation (Figures 7A and 7B). Phosphorylation of PKB/Akt was most apparent in alveolar epithelial cells, with markedly less activation in ductal epithelium (not shown). Western blot analyses confirmed that phospho-PKB/Akt was present in wild type mammary glands during pregnancy and at L1, but undetectable in nonpregnant females (not shown). In contrast, although total PKB/Akt protein expression was normal in mammary tissues from pregnant *opgl*$^{-/-}$ females (Figure 7C), active phospho-PKB/Akt was completely absent both at P14.5 (not shown) and L1 (Figure 7D). In contrast to defective PKB/Akt activation, in vivo activation of ERK1/ERK2, STAT3, and STAT5A/B (as detected by Abs reactive to phosphorylation-specific epitopes indicative of activation) was preserved in mammary epithelial cells of *opgl*$^{-/-}$ females at P14.5 and L1 (not shown). Thus, the loss of OPGL leads to impaired PKB/Akt activation in alveolar mammary epithelial cells.

We next determined whether the reintroduction of OPGL expression could restore PKB/Akt activation in *opgl*$^{-/-}$ mammary epithelium. Whereas implantation of control pellets did not rescue PKB/Akt phosphorylation (Figure 7E), implantation of rOPGL was able to induce PKB/Akt activation in alveolar epithelium (Figure 7F). Total PKB/Akt levels were not affected by implantation

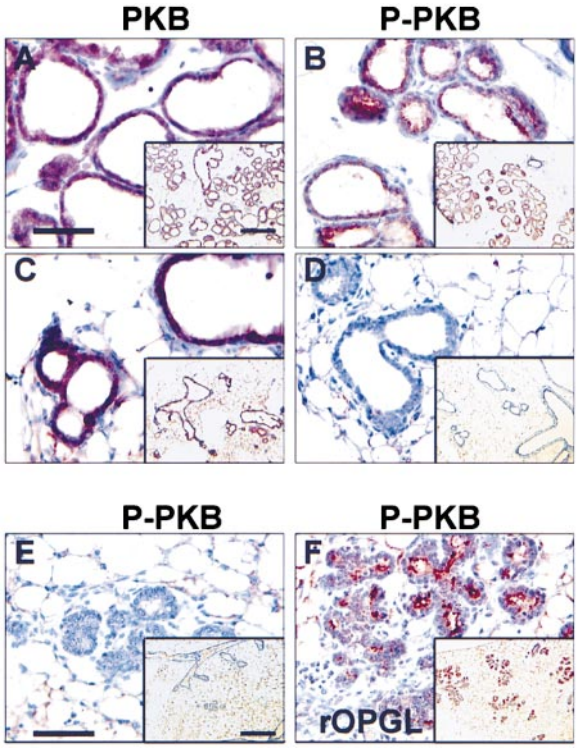

Figure 7. Impaired PKB/Akt Phosphorylation in *opgl*$^{-/-}$ Mammary Tissue

(A–D) Expression of (A and C) total PKB/Akt and (B and D) activated PKB/Akt (phosphorylated Ser473, P-PKB) in mammary tissue of (A and B) wild-type and (C and D) *opgl*$^{-/-}$ females at L1. Total and activated PKB were visualized by in situ immunohistochemistry. Note the complete absence of activated PKB in *opgl*$^{-/-}$ epithelium (D). Similar results were obtained with tissue at different stages of pregnancy.

(E and F) Restoration of PKB/Akt activation in developing lobulo-alveolar epithelium of pregnant *opgl*$^{-/-}$ females by rOPGL administration. Control pellets (E) and pellets containing rOPGL [10 μg/pellet] (F) were implanted into contralateral mammary fat pads of *opgl*$^{-/-}$ females at P14.5. Sections were analyzed by immunohistochemistry at L1. Data are representative of six mice in three experiments. Inserts in (A)–(F) show lower magnification. Scale bars: (A)–(F), 50 μm; inserts in (A)–(F), 0.1 mm.

of rOPGL (not shown). These data indicate that in vivo activation of PKB/Akt, but not activation of ERK, STAT3, and STAT5A/B, in mammary epithelial cells appears to be dependent on the OPGL/RANK pathway.

Discussion

Our results show that OPGL and its receptor RANK, the two critical molecules for osteoclast development/activation and skeletal calcium release, are also essential for the formation of a lactating mammary gland, the organ required to provide essential nourishment and calcium to offspring in mammalian species. Osteoclast-mediated skeletal demineralization is the principal mechanism by which lactating mammals meet the calcium demands of newborns, and calcium transport from mothers to the fetus and neonates is a vital process to preserving the species (Kovacs and Kronenberg, 1997). In phylogenetic evolution, the formation of lactating mammary glands is a relatively recent event, occurring when the first mammals appeared about 200 million years ago. Thus, mammals took a gene product that is the master regulator of bone metabolism and calcium turnover in the whole organism and subverted it to stimulation of mammary gland development during pregnancy.

Our results indicate that pregnancy hormones induce OPGL expression in mammary epithelial cells, and that OPGL acts through RANK to deliver proliferative and survival signals that promote the development of lobulo-alveolar structures and the formation of a lactating mammary gland. Various factors have been implicated at distinct stages in the differentiation and involution of mammary epithelial structures, including estrogen, prolactin, progesterone, Wnt4, Stat5a, cyclin D1, or Csf-1 (Pollard and Hennighausen, 1994; Liu et al., 1997; Neville, 1999; Gouon-Evans et al., 2000; Robinson et al., 2000). Hormonal regulation of OPGL expression suggests that at least some of these molecules might mediate their effects on mammary gland development in pregnancy via OPGL. During pregnancy, progesterone and the progesterone receptor are required for the development of the ductal tree and subsequent ductal branching (Couse and Korach, 1998). These early effects of progesterone have been genetically linked to Wnt4 signaling (Brisken et al., 2000). However, in later stages of pregnancy, *wnt4*$^{-/-}$ mammary glands resemble wild-type glands and form normal lobulo-alveolar structures. Since OPGL expression is strongly induced by progesterone, it is possible that the later effects of progesterone on alveolar differentiation could be mediated by OPGL-RANK. Consistent with this scenario, female *opgl*$^{-/-}$ and *rank*$^{-/-}$ mice display normal ductal elongation and ductal outgrowth during puberty and normal side branching and ductal responses to hormones during early pregnancy. In addition, *opgl*$^{-/-}$ and *rank*$^{-/-}$ mice display normal luteinization and ovulation, unlike progesterone receptor- or prolactin receptor-deficient mice (Lydon et al., 1995; Ormandy et al., 1997). During pregnancy, *csf-1*$^{-/-}$ (*op/op*) mice show impaired ductal sprouting, but increased lobulo-alveolar development of mammary epithelium and expression of milk proteins (Pollard and Hennighausen, 1994). On the other hand, OPGL has no apparent role in ductal sprouting but OPGL is essential for lobulo-alveolar development during gestation. Similar to the synergy between CSF-1 and OPGL in osteoclastogenesis, it appears that CSF-1 regulates ductal sprouting which is followed by OPGL-dependent lobulo-alveolar development. Thus, OPGL and RANK appear to be involved only in the terminal differentiation of alveolar cells in late pregnancy.

Using *rank* knockout animals, it has been shown that essentially all factors that inhibit or enhance bone resorption and calcium mobilization via osteoclasts in vitro and in vivo, including IL-1β, dexamethasone, or PTHrP, act via regulation of OPGL-RANK (Li et al., 2000). CSF-1 and OPGL cooperate to stimulate the differentiation of hematopoietic progenitors into mature multinucleated osteoclasts (Lacey et al., 1998). It is thought that CSF-1 provides the initial survival signal for myeloid/osteoclast progenitors that is followed by the action of OPGL, the critical factor for osteoclast lineage determination (La-

gasse and Weissman, 1997). Thus, the complex system of osteoclast-regulated bone remodeling is ultimately controlled by OPGL-RANK. We now report the surprising finding that OPGL and RANK, the master regulators of skeletal calcium release and bone remodeling, are essential for the formation of the lactating mammary gland, the organ required for transmission of maternal calcium to neonates in mammalian species. Similar to the regulation of osteoclasts, OPGL-RANK could be the essential mediators downstream of multiple factors known to affect lobulo-alveolar differentiation. Importantly, our data provide a novel paradigm in mammary gland biology and define a novel functional signaling pathway involved in lobulo-alveolar development.

The balance between OPGL, RANK, and the decoy receptor OPG is critical for the regulation of bone loss in osteoporosis, arthritis, and lytic bone metastases (Karsenty, 1999). Osteoporosis affects hundreds of millions of people, particularly postmenopausal women. Intriguingly, genetic and functional models have shown that osteoclast-regulated bone remodeling is under the control of powerful sex and pregnancy hormones (Ross, 2000). When estrogen production falls, such as occurs in ovarectomy models in animals or in postmenopausal women, induction of OPG is decreased, allowing uncontrolled demineralization of bone by OPGL-stimulated osteoclasts (Simonet et al., 1997). Osteoporosis is strongly associated with increased morbidity and premature death in older women. The evolutionary question then arises, why has the OPGL/RANK/OPG system, a key regulator of a structural organ such as the skeleton, come under the regulatory umbrella of reproductive hormones? Our results provide a molecular and evolutionary explanation for gender bias and high incidence of osteoporosis in females. The strong bias toward bone loss in postmenopausal women may be due to the fact that the OPG/RANK/OPG system is essential for reproduction and the survival of mammalian offspring.

Conclusions

OPGL is the key regulator of skeletal calcium resorption and osteoclasts development/activation. We report the surprising finding that OPGL is essential for the formation of a lactating mammary gland. Female *opgl* gene-deficient mice have a complete block in the formation of lobulo-alveolar mammary structures during pregnancy which leads to neonatal death of newborns. The TNF-Receptor family protein RANK was identified as the critical receptor of this OPGL action. OPGL expression in mammary epithelial cells is induced by pregnancy hormones whereas RANK is constitutively expressed on mammary epithelial cells. Transplantation and OPGL-rescue experiments in $opgl^{-/-}$ and $rank^{-/-}$ pregnant females, showed that OPGL acts directly on RANK-expressing mammary epithelial cells. The mammary gland defect in female $opgl^{-/-}$ mice is characterized by enhanced cell death, lack of proliferation, and defective PKB/Akt activation in alveolar buds. All of these functional and biochemical defects can be reversed by local rescue with OPGL. Our findings show that the master regulator of osteoclast activation and calcium release from the skeleton is also essential for the morphogenesis of a lactating mammary gland, the vital organ required for transmission of maternal calcium to neonates in mammalian species.

Experimental Procedures

Mice

Mice genetically deficient for *opgl*, *rank,* or *rag1* have been described previously (Kong et al., 1999a; Li et al., 2000). For timed pregnancies, male and female mice were mated overnight and female mice were scored for vaginal plaques the next morning. The presence of vaginal plaques was taken to represent pregnancy day 0.5 (P0.5). All mouse strains used were of the C57BL/6 background ($H\text{-}2^{b/b}$). It should be noted that in all experiments $opgl^{+/-}$ and $rank^{+/-}$ mice displayed a mammary gland phenotype that was indistinguishable to that of wild-type mice. All mice were maintained at the animal facilities of the Ontario Cancer Institute and Amgen.

Histology, Whole-Mount, and Immunohistochemistry

For histological analysis, 5 μm sections were cut and stained with hematoxylin and eosin (H&E). Whole-mount staining of mammary glands was performed as described (Fata et al., 1999). For immunoperoxidase staining, paraffin-embedded sections were dehydrated and antigenic epitopes exposed using a 10 mM citrate buffer and microwaving. Sections were incubated with rabbit polyclonal Abs to PKB/Akt, phospho-PKB/Akt (Ser473), phospho-ERK1/ERK2 (Thr202/Tyr204), phospho-Stat3 (Y705), phospho-Stat5A/B (Y694) (New England Biolabs), and mouse monoclonal anti-PCNA (Santa Cruz Biotech), and visualized using peroxidase-conjugated secondary antibodies. Apoptotic cells were determined either by in situ DNA end-labeling or Hoechst staining and confocal microscopy. Histomorphometric indices (proliferation and apoptosis) were calculated as the number of positive epithelial cells divided by the total number of epithelial cells, with no fewer than 1000 nuclei counted per section.

In Situ Hybridizations and RNA Analysis

In situ hybridizations were performed on 4% formalin fixed mammary sections. Strand-specific riboprobes for *opgl* and *rank* were generated by incorporating digoxygenin (DIG)-labeled UTP or [^{35}S]-UTP. In situ hybridization using DIG-UTP or ^{35}S-UTP labeled sense and antisense *opgl* and *rank* probes have been described previously (Lacey et al., 1998; Hsu et al., 1999). Northern analysis for β-casein and keratin 18 mRNA was performed on 20 μg total mammary RNA.

Mammary Tissue Manipulations and Transplants

Elvax-40 pellets (Dupont) containing rOPGL were prepared as described (Fata et al., 1999). All OPGL and control pellets were surgically implanted into the fourth inguinal mammary fat pad distal to the lymph node. For transplantation studies, mammary epithelial tissue was isolated from nulliparous 3-week-old donors and implanted into cleared mammary fat pads (devoid of endogenous epithelium) of 3-week-old host $rag1^{-/-}$ mice. Three weeks after surgery, hosts were mated and mammary tissue was isolated for analysis. Bilateral ovarectomies (OVX) were performed on anesthesized 4-week-old female mice. Mammary tissue from female mice injected with 17-β-estradiol (Sigma; 10 μg/ml; 50 μl s.c. at 0 and 8 hr post-OVX), progesterone (Sigma; 10 mg/ml; 50 μl s.c. at 0, 8 hr post-OVX), prolactin (Sigma; 20 μg/ml; 50 μl i.p. at 0, 8, 16 hr post-OVX), and PTHrP (Sigma; 400 μg/ml; 30 μl s.c. at 0, 8, 16 hr post-OVX) were collected at 24 hr post-OVX.

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References

Anderson, D.M., Maraskovsky, E., Billingsley, W.L., Dougall, W.C., Tometsko, M.E., Roux, E.R., Teepe, M.C., DuBose, R.F., Cosman, D., and Galibert, L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature *390*, 175–179.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S.K., McMahon, J.A., McMahon, A.P., and Weinberg, R.A. (2000). Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. Genes Dev. *14*, 650–654.

Couse, J.F., and Korach, K.S. (1998). Exploring the role of sex steroids through studies of receptor deficient mice. J. Mol. Med. *76*, 497–511.

Dougall, W.C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M.E., Maliszewski, C.R., et al. (1999). RANK is essential for osteoclast and lymph node development. Genes Dev. *13*, 2412–2424.

Dunbar, M.E., and Wysolmerski, J.J. (1999). Parathyroid hormone-related protein: a developmental regulatory molecule necessary for mammary gland development. J. Mamm. Gland. Biol. Neoplasia. *4*, 21–34.

Fata, J.E., Leco, K.J., Moorehead, R.A., Martin, D.C., and Khokha, R. (1999). Timp-1 is important for epithelial proliferation and branching morphogenesis during mouse mammary development. Dev. Biol. *211*, 238–254.

Gouon-Evans, V., Rothenberg, M.E., and Pollard, J.W. (2000). Postnatal mammary gland development requires macrophages and eosinophils. Development *127*, 2269–2282.

Honore, P, Luger, N.M., Sabino, M.A., Schwei, M.J., Rogers, S.D., Mach, D.B., O'keefe, P.F., Ramnaraine, M.L., Clohisy, D.R., and Mantyh, P.W. (2000). Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. Nat. Med. *6*, 521–528.

Horseman, N.D. (1999). Prolactin and mammary gland development. J. Mamm. Gland. Biol. Neoplasia. *4*, 79–88.

Hsu, H., Lacey, D.L., Dunstan, C.R., Solovyev, I., Colombero, A., Timms, E., Tan, H.L., Elliott, G., Kelley, M.J., Sarosi, I., et al. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc. Natl. Acad. Sci. USA *96*, 3540–3545.

Karsenty, G. (1999). The genetic transformation of bone biology. Genes Dev. *13*, 3037–3051.

Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A., et al. (1999a). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature *397*, 315–323.

Kong, Y.Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., Capparelli, C., Li, J., Elliott, R., McCabe, S., et al. (1999b). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature *402*, 304–309.

Kovacs, C.S., and Kronenberg, H.M. (1997). Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. Endocr. Rev. *18*, 832–872.

Kurihara, N., Reddy, S.V., Menaa, C., Anderson, D., and Roodman, G.D. (2000). Osteoclasts expressing the measles virus nucliocapsid gene display a pagetic phenotype. J. Clin. Invest. *105*, 607–614.

Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell *93*, 165–176.

Lagasse, E., and Weissman, I.L. (1997). Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. Cell *89*, 1021–1031.

Li, J., Sarosi, I., Yan, X.Q., Morony, S., Capparelli, C., Tan, H.L., McCabe, S., Elliott, R., Scully, S., Van, G., et al. (2000). RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc. Natl. Acad. Sci. USA *97*, 1566–1571.

Liu, X., Robinson, G.W., Wagner, K.U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. *11*, 179–186.

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C.A., Jr., Shyamala, G., Conneely, O.M., O'Malley, B.W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. *9*, 2266–2278.

Neville, M.C. (1999). Physiology of lactation. Clin. Perinatol. *26*, 251–279.

Ormandy, C.J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., and Kelly, P.A. (1997). Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. *11*, 167–178.

Pollard, J.W., and Hennighausen, L. (1994). Colony stimulating factor 1 is required for mammary gland development during pregnancy. Proc. Natl. Acad. Sci. USA *91*, 9312–9316.

Robinson, G.W., Karpf, A.B., and Kratochwil, K. (1999). Regulation of mammary gland development by tissue interaction. J. Mamm. Gland Biol. Neoplasia *4*, 9–19.

Robinson, G.W., Hennighausen, L., and Johnson, P.F. (2000). Side-branching in the mammary gland: the progesterone-Wnt connection. Genes Dev. *14*, 889–894.

Roodman, G.D. (1999). Cell biology of the osteoclast. Exp. Hematol. *27*, 1229–1241.

Ross, F.P. (2000). RANKing the importance of measles virus in Paget's disease. J. Clin. Invest. *105*, 555–558.

Simonet, W.S., Lacey, D.L., Dunstan, C.R., Kelley, M., Chang, M.S., Luthy, R., Nguyen, H.Q., Wooden, S., Bennett, L., Boone, T., et al. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell *89*, 309–319.

Thomas, R.J., Guise, T.A., Yin, J.J., Elliott, J., Horwood, N.J., Martin, T.J., and Gillespie, M.T. (1999). Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology *140*, 4451–4458.

Wong, B.R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F.S., 3rd, Frankel, W.N., Lee, S.Y., and Choi, Y. (1997). TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J. Biol. Chem. *272*, 25190–25194.

Wong, B.R., Besser, D., Kim, N., Arron, J.R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999). TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol. Cell *4*, 1041–1049.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., et al. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc. Natl. Acad. Sci. USA *95*, 3597–3602.