Null Mutation of Peroxisome Proliferator-activated Receptor-interacting Protein in Mammary Glands Causes Defective Mammopoiesis*

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To investigate the role of nuclear receptor coactivator peroxisome proliferator-activated receptor-interacting protein (PRIP) in mammary gland development, we generated a conditional null mutation of PRIP in mammary glands. In PRIP-deficient mammary glands, the elongation of ducts during puberty was not affected, but the numbers of ductal branches were decreased, a condition that persisted long after puberty, indicating that the potential of ductal branching was impaired. During pregnancy, PRIP-deficient mammary glands exhibited decreased alveolar density. The lactating PRIP-deficient glands contained scant lobuloalveoli with many adipocytes, whereas the wild type glands were composed of virtually no adipocytes but mostly lobuloalveoli. As a result, PRIP mammy-deficient glands could not produce enough milk to nurse all the pups during lactation. The ductal branching of mammary glands in response to estrogen treatment was attenuated in PRIP mutant glands. Whereas the proliferation index was similar between wild type and PRIP-deficient glands, increased apoptosis was observed in PRIP-deficient glands. PRIP-deficient glands expressed increased amphiregulin, transforming growth factor-α, and betacellulin mRNA as compared with wild type glands. The differentiated function of PRIP-deficient mammary epithelial cells was largely intact, as evidenced by the expression of abundant β-casein, whey acidic protein (WAP), and WDNM1 mRNA. We conclude that PRIP is important for normal mammary gland development.

The multiple steps in mammary gland development are controlled by a combination of peptide and steroid hormones (1, 2). During puberty, accelerated ductal growth that finally fills the entire fat pad is stimulated by estrogen. Whereas the exact downstream signals responsible for estrogen-stimulated ductal growth remain elusive, analysis of gene knockout mice suggests that estrogen receptors in both stromal and epithelial cells are required for optimal ductal growth under physiological conditions (3, 4). During pregnancy, mammary epithelial cells undergo an extensive alveolar proliferation. The early lobuloalveolar proliferation requires both estrogen and progesterone, whereas prolactin is responsible for mammary gland development during late pregnancy and lactation (5, 6). Genetic ablation of estrogen and reciprocal transplantation experiments suggest that the presence of progesterone receptors in mammary epithelial cells but not in stromal cells is essential for alveolar branching and that progesterone receptors act in a paracrine fashion (7–9). Besides the essential role in normal mammary gland development, steroid hormones also play an important role in breast cancer development (10). Overexposure to estrogen is associated with increased risk of breast cancer (11). Pregnancy provides certain protection against breast cancers probably through the formation of secretory alveoli (12).

Estrogen and progesterone receptors are members of the nuclear receptor superfamily. Recent studies (13, 14) established that nuclear receptors require a combination of nuclear receptor coactivators for transcriptional functions. Coactivators, such as SRC-1 and CBP, harboring histone acetyltransferase activities, act by modifying the chromatin structure (15, 16), whereas the coactivator PBP serves as the anchor for a large multisubunit protein complex, which facilitates transcriptional initiation (17, 18). Peroxisome proliferator-activated receptor-interacting protein is one of the nuclear receptor coactivators (PRIP1/ASC-2/RAP250/TRBP/NRC) isolated by our laboratory and others (19–23). PRIP interacts with PIMT (PRIP-interacting protein with RNA methyltransferase activity), and its function is stimulated by PIMT (24, 25). PRIP is a component of a large protein complex including the trithorax group proteins, ALR-1, ALR-2, HALR, and ASH-2, and the retinoblastoma-binding protein RBQ-3 (26). In addition, PRIP is amplified and overexpressed in some breast cancers and could play a role in tumorigenesis (19). It would be important to elucidate the physiological function of PRIP in mammary gland development and to establish the contribution of PRIP to estrogen and progesterone signaling pathways during mammary gland development. However, the PRIP null mutation is lethal in the embryonic stage (27–29), which prevents the analysis of the role of PRIP in mammary gland development.

Here we report the generation of a PRIP null mutation in mammary glands. PRIP-deficient mammary glands exhibit decreased numbers of ductal branches, impaired lobuloalveolar development, severely deficient lactation, and attenuated ductal branching of mammary glands in response to estrogen treatment with relatively intact expression of milk gene proteins. These results suggest that PRIP is an important coactivator for normal mammary gland development.

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1 The abbreviations used are: PRIP, peroxisome proliferator-activated receptor-interacting protein; WAP, whey acidic protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; BrdUrd, bromodeoxyuridine; MMTV, murine mammary tumor virus; RT, reverse transcriptase; EGF, epidermal growth factor; TGF, transforming growth factor.
BrdUrd/g of body weight) 2 h before sacrifice. Mammary glands were stained overnight in hematoxylin. Samples were then cleared in xylene and mounted. For routine histological assessment, formalin-fixed mammary glands were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical analysis was performed on paraffin-embedded tissues. Sections were deparaffinized and rehydrated. After the inactivation of endogenous peroxidase activity and antigen retrieval, sections were blocked with 10% normal bovine serum in phosphate-buffered saline, followed by sequential incubation at room temperature with anti-PRIP antibodies for 3 h, biotinylated goat anti-rabbit IgG for 30 min, and finally streptavidin-linked horseradish peroxidase for 30 min, and finally counterstained with hematoxylin.

**MATERIALS AND METHODS**

**Generation of PRIP Conditional Null Mutation in Mammary Glands**—MMTV-Cre transgenic mice (30) were obtained from the Mouse Models of Human Cancers Consortium Repository at the NCI, Frederick, MD. The heterozygous Loxp-PRIP mice (27) were bred with MMTV-Cre mice to delete the DNA fragment between LoxP1 and LoxP3. The heterozygous mice with the expected deletion were interbred to generate homozygous mutants.

Whole-mount Histology and Immunostaining—Whole-mount examination of the inguinal mammary glands was performed as reported previously (31). Briefly, mammary glands were fixed in Carnoy’s fixative and stained overnight with hematoxylin. Samples were then cleared in xylene and mounted. For routine histological assessment, formalin-fixed mammary glands were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical analysis was performed on paraffin-embedded tissues. Sections were deparaffinized and rehydrated. After the inactivation of endogenous peroxidase activity and antigen retrieval, sections were blocked with 10% normal bovine serum in phosphate-buffered saline, followed by sequential incubation at room temperature with anti-PRIP antibodies for 3 h, biotinylated goat anti-rabbit IgG for 1 h, streptavidin-linked horseradish peroxidase for 30 min, and finally 3,3′-diaminobenzidine tetrahydrochloride solution for 4 min. Sections were counterstained with hematoxylin.

**Bromodeoxyuridine (BrdUrd) Staining and Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay**—Mice were given an intraperitoneal injection of BrdUrd (30 μg of BrdUrd/g of body weight) 2 h before sacrifice. Mammary glands were isolated, fixed, embedded, and sectioned. BrdUrd immunostaining was performed using an in situ cell death detection kit from Roche Applied Science as instructed. A total of 6000 cells were counted from six different sections of a sample, and final counts were expressed as the percentage of epithelial cells positive for BrdUrd or TUNEL. Only intensely stained nuclei were scored as positive.

**RNA Isolation and Semiquantitative RT-PCR**—Inguinal glands were collected from wild type and PRIP mutant mice. Mammary epithelial cells were separated from stromal cells by collagenase digestion and Percoll gradient centrifugation (32) and used for isolation of total RNA by the TRIzol (Invitrogen) method. RT-PCR was performed with the SuperScript one-step RT-PCR kit from Invitrogen as instructed. 1 μg of total RNA was reverse-transcribed with SuperScript II reverse transcriptase followed by PCR amplification consisting of 35 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min. For each 100 μl-reaction, 10 μl of the PCR product was taken out at cycles 25 and 30. 5 μl of the PCR product was separated on PAGE, which was then dried and exposed to x-ray film. The primers used are as follows: EGF, 5′-ACCAAGCAATTGTTGGTGGGAT-3′ and 5′-ATGTAAGCCTTG-GCTCCTCT-3′; amphiregulin, 5′-GTAAGCAGATGGAACACACCGGAGGAC-3′ and 5′-GTTGCCACCAGCTTGAGA-3′; amphiregulin, 5′-GTTGCCACCAGCTTGAGA-3′; betacellulin, 5′-GTAACCCGGGCATGCGTGTCTT-3′ and 5′-CTTGTGATGTTGTTCTCCTC-3′; TGF-α, 5′-CTTGTGATGTTGTTCTCCTC-3′ and 5′-CTTGTGATGTTGTTCTCCTC-3′; amphiregulin, 5′-GTTGCCACCAGCTTGAGA-3′; TGF-α, 5′-CTTGTGATGTTGTTCTCCTC-3′ and 5′-CTTGTGATGTTGTTCTCCTC-3′; amphiregulin, 5′-GTTGCCACCAGCTTGAGA-3′; TGF-α, 5′-CTTGTGATGTTGTTCTCCTC-3′ and 5′-CTTGTGATGTTGTTCTCCTC-3′; amphiregulin, 5′-GTTGCCACCAGCTTGAGA-3′.
CCA-3; β-actin, 5'-CCATCTACGGGGCTATGCT-3' and 5'-GCAAGTTAGGTTTTGTCAAAGA-3'.

Northern Blot Analysis—10 μg of total RNA was separated through the formaldehyde denaturing agarose gel and was transferred onto a nylon membrane (Bio-Rad). Filters were hybridized with [32P]dCTP-labeled cDNA probes including casein, WDNM1, and WAP.

RESULTS

Conditional Null Mutation of PRIP in Mammary Glands—As PRIP null mutation results in an embryonic lethality that precludes studying the role of PRIP in mammary gland development, a conditional null mutation in mammary glands was generated by crossing mice with the LoxP integrated recombinant PRIP gene and MMTV-Cre transgenic mice. MMTV-Cre specifically expressed Cre recombinase in mammary epithelial cells, which deleted exon 7 of the PRIP gene flanked by two LoxP sites (Fig. 1A). The deletion of the PRIP exon 7 led to a reading frameshift and generated a stop codon right after the fusion between exon 6 and exon 8. The successful deletion of the PRIP gene in mammary epithelial cells was revealed by PCR using primers specifically for the deleted and undeleted gene (Fig. 1B). RT-PCR showed that PRIP mRNA from mammary epithelial cells with the deleted PRIP gene was absent (Fig. 1C). Immunostaining with anti-PRIP confirmed that mammary epithelial cells with the deleted PRIP gene did not express the PRIP protein (Fig. 1D).

Defective Mammpoiesis during Puberty, Pregnancy, and Lactation—Mammary glands at different developmental stages were examined in age- and weight-matched wild type (A and B), 10-week-old (C–F), 15-week-old (G and H) wild type or PRIP mutant mice. Note that the PRIP deficiency (B, D, F, H) caused defective branching from early puberty (B) to the end of puberty (D, F) and persisted after puberty (H). E and F represent higher magnification of the gland structures in panels C and D, respectively.

FIG. 3. Morphology of PRIP-deficient glands on the 15th day of pregnancy. The fourth inguinal mammary glands from wild type (A and C) and PRIP mutant mice (B and D) were isolated and whole-mounted. Note that PRIP-deficient glands had underdeveloped lobuloalveolar structures with smaller and fewer alveoli. C and D represent higher magnification of the glands in panels A and B, respectively.

elongation. However, the number of ductal branches and the density of ducts were greatly reduced in PRIP-deficient mammary glands (Fig. 2). Histological analysis revealed no appreciable difference between wild type and PRIP-deficient glands except for the fact that PRIP-deficient glands contained fewer ducts (data not shown). By 10 weeks, ductal growth almost reached the edge of the fat pad in both wild type and PRIP mutant mice with greatly decreased branching observed in
PRIP mutant mammary glands (Fig. 2). The mammary duct completely fills the mammary fat pad in mature virgin mice and ceases active growth afterwards. An examination of mammary glands by whole-mount revealed that the extent of ductal branching was still substantially reduced in 15-week-old PRIP mutant females (Fig. 2), indicating that the potential of branching was compromised in PRIP-deficient mammary glands. During pregnancy, mammary glands undergo further ductal branching, lobuloalveolar proliferation and differentiation. Whole-mount preparation of glands from mice on the 15th day of pregnancy revealed that PRIP deficiency caused impairment of lobuloalveolar development (Fig. 3). The density of alveoli in PRIP-null glands was markedly reduced as compared with that in wild type glands (Fig. 3). However, the morphology of the alveolus, as revealed by hematoxylin and eosin section, was indistinguishable between wild type and PRIP-null glands (data not shown). Mammary glands complete lobuloalveolar development and functional differentiation during lactation. The lobuloalveoli from PRIP-deficient glands were still much less abundant and smaller than those from wild type glands as revealed by whole-mount (Fig. 4). PRIP-deficient glands on the 8th day of lactation revealed numerous adipocytes; in contrast, virtually no adipocytes were present in wild type glands (Fig. 4). PRIP mutant mice delivered normal sized litters but only two or three pups could survive. Most pups died within 48 h after birth with no milk detected in their stomachs. However, pups could be fostered by wild type females, indicating that PRIP-deficient glands are unable to produce sufficient quantities of milk to nurse pups because of defective mammary gland development.

**Attenuation of Ductal Branching of Mammary Glands in Response to Estrogen Treatment**—To examine mammary gland development in response to estrogen treatment, mice were ovariectomized, and estrogen pellets were implanted. Three weeks later, uterine weight was increased 11-fold in both wild type and mutant mice, confirming the successful implantation of the estrogen pellets. The whole-mount of the fourth pair of mammary glands from four wild type and four PRIP mutant mice consistently demonstrated that PRIP-deficient glands exhibited much less extensive ductal branching as compared with wild type glands (Fig. 5 as the representative). Therefore, PRIP is required for efficient ductal branching of mammary glands in response to estrogen.

**Proliferation and Apoptosis of PRIP-deficient Mammary Epithelial Cells**—To determine whether the retarded mammapoiesis was caused by altered proliferation and survival of mammary epithelial cells, DNA synthesis and apoptosis were examined in the terminal end buds of inguinal mammary glands from both wild type and PRIP mutant mice. Six-week-old wild type and PRIP-deficient mice were injected with bromodeoxyuridine to label the proliferating cells in the mammary glands. Immunostaining with anti-BrdUrd revealed no quantitative differences in the mitotic index between wild type and PRIP-deficient mammary glands (Fig. 6). The apoptotic index was determined by TUNEL assay. A slight increase in apoptosis was found in the terminal end buds from PRIP-deficient glands as compared with wild type mammary glands (Fig. 6), raising the possibility that abnormal apoptosis contributes to the impaired ductal branching.

**Altered Expression of Growth Factors in PRIP-deficient Glands**—ERBB signaling pathways are essential for mammary gland development (33, 34). EGF-like growth factors including amphiregulin, EGF, TGF-α, and betacellulin are the ligands for ERBB receptors. To understand the underlying mechanism for defective mammapoiesis, the levels of amphiregulin, EGF, TGF-α, and betacellulin mRNA from PRIP-deficient glands were examined by semiquantitative PCR. The level of EGF mRNA from wild type glands was slightly higher than that from PRIP-deficient glands (Fig. 7). However, PRIP-deficient glands expressed markedly increased amphiregulin (Areg), TGF-α, and betacellulin (Btc) mRNA (Fig. 7), indicating that...
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PRIP-deficient mammary epithelial cells may have impaired response to growth factors.

Expression of Milk Protein Genes in PRIP-deficient Glands—Whereas lobuloalveolar development was retarded, the morphology of individual alveolus from PRIP-deficient lactating glands was indistinguishable from that of wild type alveolus. This prompted us to investigate the differentiated functions of mammary epithelial cells. We examined the mRNA expression of the milk protein genes β-casein, WAP, and WDNM1 from mammary glands on the 8th day of lactation. Northern blot showed that PRIP-deficient glands still expressed high levels of β-casein, WAP, and WDNM1 (Fig. 8). Whereas the expression level of WDNM1 from PRIP-deficient glands was slightly higher than that from wild type glands, the expression of β-casein and WAP was reduced in PRIP-deficient glands.

DISCUSSION

To define the role of PRIP in mammary gland development, we created a conditional PRIP null mutation in mammary glands. PRIP-deficient glands showed a normal elongation of ducts but a decreased number of ductal branches during puberty, and this condition remained long after puberty, indicating that the potential of ductal branching was impaired. PRIP-deficient glands exhibited decreased density of alveoli during pregnancy. The lactating PRIP-deficient glands contained decreased numbers of lobuloalveoli with many adipocytes and failed to nurture all pups. This study demonstrated that PRIP is pivotal for normal mammary gland development.

PRIP-deficient mammary epithelial cells expressed abundant mRNAs of milk protein genes, but these mice appeared unable to produce enough milk to nurse all pups. Therefore, the major defect leading to reduced milk production is most probably the impaired side branching, which generated too few and smaller alveoli to produce sufficient quantities of milk rather than the mildly affected differentiated function of mammary epithelial cells.

ERBB signaling pathways activated by EGF-like factors are essential for mammary gland development. We found that expression of several of the EGF-like factors was increased in PRIP-deficient glands. The defect in PRIP-deficient glands should not be attributed to the lack of these EGF-like factors. Other growth factors may be the targets for PRIP protein and remain to be identified. Another possibility is that the PRIP-deficient mammary epithelial cells have an intrinsic defect, which causes an inefficient response to growth factors for ductal branching and alveologenesis. Consistent with this hypothesis, microarray studies found that STAT5a and HIF-1 mRNAs were significantly decreased in PRIP-deficient glands.3 A gene knock-out experiment revealed that STAT5 is essential for lobuloalveolar development (35). Loss of HIF-1 was demonstrated to impair mammary epithelial differentiation and lipid secretion, resulting in failure of lactation (36). Therefore these two genes are good candidates whose decreased expressions are responsible for the mammary gland defect associated with PRIP deficiency.

Ductal growth during puberty and the side branching and alveologenesis during pregnancy are stimulated by estrogen.
Semiquantitative RT-PCR was performed with the addition of 35S-band. RNA loading was demonstrated by the intensities of the 28S mRNA dATP. For each PCR reaction, 5 μl of the PCR products at 25, 30, and 35 cycles were resolved on PAGE, which was then exposed to x-ray film. β-Actin served as a loading control of an equal amount of RNA for PCR.

and progesterone, respectively. In PRIP mutant mice, ductal branching and alveolar development were still present but severely impaired. Moreover, the ductal branching stimulated by direct estrogen treatment was attenuated in mutant mice. These phenotypes of PRIP-deficient glands were consistent with those from mammary glands with compromised function of estrogen and progesterone receptors, although it is possible with those from mammary glands with compromised function severely impaired. Moreover, the ductal branching stimulated and alveolar development were still present but

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