Impaired differentiation and lactational failure of *Erbb4*-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5

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Summary

The ERBB family of type 1 receptor tyrosine kinases and their ligands have crucial functions during mammopoiesis, but the signaling networks that ultimately regulate ERBB activity in the breast have remained elusive. Here, we show that mice with Cre-lox mediated deletions of both *Erbb4* alleles within the developing mammary gland (*Erbb4*$^{Flox/Flox}$*Wap-Cre*) fail to accumulate lobuloalveoli or successfully engage lactation at parturition owing, in part, to impaired epithelial proliferation. Analysis of the mammary differentiation factor STAT5 by immunohistochemistry and western blot revealed a complete ablation of STAT5 activation in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary epithelium at parturition. Consistent with disrupted STAT5 function, *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands at parturition failed to express the mammary epithelial differentiation marker NPT2B. Defects in epithelial functional differentiation at parturition were accompanied by a profound reduction in expression of the STAT5-regulated milk genes *casein beta* and *whey acidic protein*. We propose that ERBB4 functions as an essential mediator of STAT5 signaling, and that loss of STAT5 activity contributes to the impaired functional differentiation of mammary glands observed in mice containing conditional *Erbb4* deletions.

Key words: Differentiation, ERBB, Lactation, Mammary gland development, STAT5, Tissue specific gene deletion, Mouse

Introduction

The majority of mammary gland development occurs postnatally, and is regulated during adulthood through the complex and coordinated activities of systemic hormones and locally synthesized growth factors. Although, the crucial contribution of the steroid hormones estrogen and progesterone, and the pituitary hormone prolactin, to mammopoiesis has been extensively documented (Hennighausen and Robinson, 1998), the exact influence of growth factors and their receptors on mammary gland development remains to be elucidated. However, accumulating evidence suggests that local expression and activity of the ERBB family of receptor tyrosine kinases and their ligands directly influence multiple stages of mammopoiesis (reviewed by Stern, 2003; Troyer and Lee, 2001).

The ERBB family consists of four receptors, EGFR, ERBB2/HER2/NEU (referred to here as ERBB2), ERBB3 and ERBB4, which are activated through binding of EGF-like ligands and subsequent receptor dimerization (Yarden and Sliwkowski, 2001). Although expression of ERBB receptors and EGF-like ligands can be detected at multiple stages of mammary gland development (Schroeder and Lee, 1998), recent biochemical experiments and genetic models suggest that one or more of the heregulin (HRG) receptors, which include ERBB2, ERBB3 and ERBB4, regulate mammopoiesis during crucial stages of pregnancy and lactation. For example, enhanced receptor activation profiles within mammary tissue during pregnancy and lactation indicate active ERBB receptor signal transduction during these developmental stages (Schroeder and Lee, 1998). Furthermore, we addressed the roles of ERBB2 and ERBB4 during mammary development through the independent overexpression of dominant-negative mutant forms of these receptors within the mammary glands of transgenic mice (Jones and Stern, 1999; Jones et al., 1999). These experiments suggested that ERBB2 and ERBB4 contribute to the function of milk-producing alveolar structures during pregnancy and lactation, respectively. Interestingly, mice with mutations affecting expression or processing of the ERBB4 ligands, heregulin α (HRGα; NRG1 – Mouse Genome Informatics) (Li et al., 2002) or heparin-binding epidermal growth factor (Hb-EGF) (Yu et al., 2002) also exhibit defects in alveolar functional development. These genetic results,

coupled with the fact that ERBB2 is an orphan receptor and as such must be activated through heterodimer formation with a ligand-bound ERBB family member, raise the possibility that ligand-activated ERBB4 plays an important regulatory role in the mammary gland.

STAT5 belongs to the family of signal transducers and activators of transcription (STAT), and is an ERBB-activated signaling protein required for mammary gland development. In the mammary gland, STAT5 is believed to be activated following a cascade of events involving binding of the lactogenic hormone prolactin (PRL) to the prolactin receptor (PRLR) (reviewed by Hennighausen et al., 1997). Patterns of STAT5 expression and activation are tightly coupled to epithelial proliferation and differentiation during pregnancy (Liu et al., 1996). Indeed, the essential role of STAT5 activity in mammary development and lactogenesis was confirmed in mice containing genetic disruptions of the STAT5 isoforms, STAT5A or STAT5B or both (Liu et al., 1997; Miyoshi et al., 2001; Teglund et al., 1998). Recently, members of the ERBB family have also been shown to activate STAT5 (Jones et al., 1999; Kloth et al., 2002; Olayioye et al., 2001). Similar to the PRLR, ERBB4 phosphorylates STAT5A at the regulatory amino acid Y694 in a STAT5A SRC-homology 2 (SH2) domain-dependent manner (Jones et al., 1999). Furthermore, ERBB4 phosphorylates STAT5A at a tyrosine(s) in addition to at Y694 (Jones et al., 1999), raising the intriguing possibility that ERBB4 regulates novel STAT5 activities through multiple phosphorylation events.

To date, experiments designed to examine the function of ERBB receptors during mammary gland development have generated compelling functional data (reviewed by Stern, 2003; Troyer and Lee, 2001); however, mechanistic information is required to firmly establish the contribution of ERBB signaling to mammopoiesis. Although we have previously reported a mammary gland phenotype in mice expressing a dominant-negative ERBB4 protein (Jones et al., 1999), pervasive ERBB-heterodimer formation means that a dominant-negative mutant receptor could, theoretically, inhibit signaling by all co-expressed ERBB family members. Analysis of ERBB4 function in the developing breast is further hampered because genetic deletion of *Erbb4* alleles results in an embryonic lethal phenotype (Gassmann et al., 1995). To overcome the limitations of dominant-negative mutant receptors and the early lethality of *Erbb4*-null embryos, we defined the function of ERBB4 in the mammary gland by deleting both epithelial *Erbb4* alleles using a CRE-LOX recombination strategy (Gu et al., 1994). Our results indicate that ERBB4 functions in the pregnant mammary gland by regulating STAT5 induced epithelial differentiation, and we demonstrate that ERBB4 is essential for the successful engagement of lactation. We propose a new mechanism for STAT5 regulation in the developing breast and provide crucial evidence implicating the ERBB family as essential local mediators of mammary gland development.

Materials and methods

Cloning of replacement construct into ERBB4-targeting vector

A λ-phage clone containing the coding sequence of the second exon of the *Erbb4* gene was obtained from Martin Gassmann, University of Basel (Gassmann et al., 1995). Plasmids containing unique restriction digest fragments of this phage were used for designing a targeting construct and for cloning approximately 15 kb of genomic DNA, including the entire coding sequence of the second exon and flanking intronic sequence, into the targeting vector pFLRT. In the pFLRT-ERBB4 targeting construct, 2 loxP sites flank exon 2, whereas downstream is the positive neomycin (neo) selection marker flanked by 2 frt. The targeting vector pFLRT was tested to ensure generation of the appropriate recombination-induced products after exposure to CRE recombinase.

Embryonic stem cells and the generation of homologous recombinant clones

Following an established protocol (Nagy et al., 1993), R1 embryonic stem (ES) cells were transfected with the pFLRT-ERBB4-targeting construct linearized at a unique restriction site in the vector sequence. Cells were plated on dishes containing mitomycin C-treated embryonic fibroblasts (EMFI) and grown in LIF-supplemented ES media. After positive/negative selection of FLRT-ERBB4 with G418 and FIAU, clones that had successfully undergone homologous recombination with the ERBB4-targeting construct (floxed) were identified by PCR, and confirmed by Southern digests of genomic DNA hybridized with a ^{32}P-labeled neo probe and an external probe (A probe) generated by PCR of genomic sequence contained upstream of and outside the targeting construct. ES cell clones that were confirmed to have undergone homologous recombination were frozen in liquid nitrogen and used for blastocyst injection.

Germline transmission of the floxed ERBB4 allele

Following an established protocol (Klein et al., 1993), floxed ES cell clones were resuspended in phosphate buffered saline at 4°C in preparation for injection into blastocysts harvested from C57Bl/6 females. Under microscopic control, ~20-30 R1 cells were microinjected into each of 12-16 blastocysts. After injection, ~6-10 blastocysts were transferred surgically into the uterus of timed pseudopregnant CD1 females. Ten to 14 days after birth (approximately 30 days after injection), chimeras were detected by their dominant agouti coat color. At 8 weeks of age, chimeric males were bred with C57Bl/6 females to establish germline transmission of the targeted allele. Tail genomic DNA was extracted and tested by PCR for presence of the homologous recombinant floxed allele. The male agouti-colored offspring that were heterozygous for the floxed allele (*Erbb4*$^{Flox/+}$) were saved for breeding to female mice that were heterozygous for the floxed allele (*Erbb4*$^{Flox/+}$). Twenty-five percent of offspring from this mating have two floxed alleles (*Erbb4*$^{Flox/Flox}$) and were used in later matings to generate the tissue-specific deletion of the *Erbb4* gene. Functional transcription of the floxed *Erbb4* allele was tested by RT-PCR of mRNA extracted from tissues obtained from *Erbb4*$^{Flox/Flox}$ mice. One *Erbb4*$^{Flox/Flox}$ line was used for the analysis described in this report.

Crosses and genotype analysis

All mouse strains were crossed and maintained in a FVB background. Genomic DNA was isolated from mouse tail biopsies and genotyped by PCR, exactly as described elsewhere (Jones and Stern, 1999). A 210 bp fragment in the *Wap-Cre* allele was amplified using the forward primer (5′-TAGAGCTGTGCCAGCCTCTTC-3′) and the reverse primer (5′-CATCACTCGTTGCATCGACC-3′). Control (*Erbb4*$^{+/+}$*Wap-Cre*) and experimental (*Erbb4*$^{Flox/Flox}$*Wap-Cre*) mice were generated by crossing mice with the identical genotype *Erbb4*$^{+/Flox}$*Wap-Cre*/+. The extent of CRE-mediated excision of *Erbb4* exon 2 was determined by Southern blot analysis of 6 μg of mammary gland DNA, digested with *Bam*HI and probed with a ^{32}P-labeled *Erbb4* gene fragment harboring exon 2. The *Erbb4* exon 10 ^{32}P-labeled probe 5′-GCCTCTGAAGGAAATCAGTGCGGG-3′ representing nucleotides (nt) 87377-87400 from the mouse *Erbb4* gene was used as an internal control.

Whole-mount staining of mouse mammary glands

The entire number four inguinal mammary gland was excised. The tissue was spread onto a glass microscope slide, fixed in acidic ethanol and stained in carmine solution exactly as described previously (Jones et al., 1996).

Prolactin injections

PRL (National Hormone and Pituitary Program, Torrance, CA), at a concentration of 5 μg/g body weight, was injected intraperitnoeally (ip) into biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18. After 15 minutes the mice were sacrificed and mammary tissue was processed for immunohistochemistry.

Progesterone implants

Progesterone with biodegradable carrier (Innovative Research of America) was administered to pregnant *Prlr*$^{-/-}$ and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice as described previously (Binart et al., 2000).

Tissue preparation for histological analysis

For Hematoxylin and Eosin staining and immunohistochemistry, a portion of the number four inguinal mammary gland was spread onto a glass microscope slide and fixed with freshly prepared 4% paraformaldehyde in PBS at 4°C overnight. Fixed tissue was embedded in paraffin wax and 6 μm sections were dried onto Snowcoat X-tra slides (Surgipath) using standard procedures.

Immunohistochemistry

Immunohistochemical detection of ERBB4, STAT5 and STAT5 phosphorylated at Y694 (phospho-STAT5) was performed as described elsewhere (Jones et al., 1999) with the following modifications. To detect phospho-STAT5, the primary antibody, goat anti-P-STAT5 (Santa-Cruz Biotechnology), was diluted between 1-2 μg/ml and the biotinylated rabbit anti-goat (Vector Labs) secondary antibody was diluted to 15 μg/ml. Detection of expression of NKCC1 and NPT2B by immunofluorescence has been described elsewhere (Miyoshi et al., 2001). Injection of mice with Bromodeoxyuridine (BrdU) Cell Labeling Reagent (Amersham), BrdU immunohistochemistry, and statistical analyses were performed exactly as described elsewhere (Li et al., 2002). Significant differences between data sets was determined by calculating the means and standard deviations of at least 250 epithelial nuclei from at least four independent animals at each time point. The Student's *t*-test was performed at each developmental time point. In all experiments, the DAB substrate was prepared fresh before use by adding hydrogen peroxide to 0.03% in 50 mM Tris (pH 7.6) containing 1.7 mM 3′-diaminobenzidine tetrahydrochloride (Sigma).

Sections were lightly counterstained in Hematoxylin (Polysciences) according to the manufacturer's instructions, then dehydrated in ethanol, cleared in xylene and coverslipped with Permount (Fisher).

RNA isolation and northern blot analysis

Total mammary gland RNA was isolated by TRIzol (Invitrogen) extraction, according to the manufacturer's instructions, using 200 mg of tissue from the number four inguinal mammary gland that was previously snap-frozen in liquid nitrogen and stored at –80°C. Expression of β-casein, WAP and α-lactalbumin was detected in 10 μg of total mammary gland RNA by northern blot exactly, as described previously (Li et al., 2002).

Immunoprecipitation and western blot analysis

Tissue protein lysates were prepared from mammary glands and immunoprecipitated proteins were analyzed by western blot essentially as described elsewhere (Schroeder and Lee, 1998), with the following modifications. The Triton X-100 lysis buffer contained 1 mM phenylmethylsulfonyl fluoride and Complete (Roche Diagnostics) as protease inhibitors, and the phosphatase inhibitors 10 mM NaF, 1 mM sodium orthovanadate and Phosphatase Inhibitor Cocktail II (Sigma). Immunoprecipitation and western blot analysis was performed as described elsewhere (Jones et al., 1999), using the same primary antibodies described for immunohistochemistry. Proteins containing phosphotyrosine residues were detected using the primary antibody p-Tyr (Santa-Cruz Biotechnology).

Results

Mammary gland deletion of ERBB4

To identify the function of ERBB4 in the developing mouse mammary gland, we employed a CRE-LOX recombination strategy to delete both *Erbb4* alleles in the mammary epithelium. A floxed *Erbb4* mouse was generated by inserting two *loxP* sites in introns 1 and 2 of the *Erbb4* locus (Fig. 1A-C). To generate tissue-specific deletion of *Erbb4* in mouse mammary epithelium, one floxed *Erbb4* mouse line was crossed with *Wap-Cre* transgenic mice (Wagner et al., 1997). Within the mammary gland, the *Wap-Cre* transgene is initially expressed during pregnancy and is active only in the epithelium (Wagner et al., 1997; Xu et al., 1999). Southern blot analysis of mammary gland DNA from a nulliparous and two lactating *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice revealed a significant level of *Erbb4* exon 2 excision at day 12 of lactation (L12; Fig. 1D, top panel). As predicted *Erbb4* exon 10 was not affected by CRE activity (Fig. 1D, bottom panel). Although epithelial WAP-CRE expression is limited during the first pregnancy, we expect uniform WAP-CRE mediated recombination in mammary epithelium of lactating uniparous and pregnant biparous mice (Wagner et al., 2002). As a consequence of epithelial-restricted CRE expression, mesenchymal *Erbb4* will not be altered and, therefore, Southern blot analysis will reveal a small degree of intact *Erbb4* exon 2. We therefore determined the extent of ablated ERBB4 expression in parous animals by immunohistochemistry using an antibody directed against the C terminus of human ERBB4. In control *Erbb4*$^{+/+}$*Wap-Cre* mice, ERBB4 protein was localized within nuclei of mammary epithelial cells during lactation (L, days 1 and 14; Fig. 2A,C) and the second pregnancy (P, day 18; Fig. 2E). Membrane staining of ERBB4 was consistently observed in control mammary tissue at mid to late lactation (Fig. 2C, arrowhead). By contrast, the number of epithelial nuclei staining positive for ERBB4 protein in mammary tissue from *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice was reduced to 40-60% of controls at L1 (Fig. 2B) and no staining of epithelial nuclei was observed by L14 (Fig. 2D) and P18 of the second pregnancy (Fig. 2F). CRE-mediated recombination at mammary epithelial *Erbb4* loci following the first pregnancy resulted in ablated ERBB4 expression during the subsequent pregnancy. Stromal elements continue to express ERBB4 at L14 (Fig. 2D, arrow), illustrating the epithelial specificity of *Wap-Cre* expression. Ablation of ERBB4 expression in mammary tissue from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice was confirmed by western blot analysis of tissue lysates at L1 (Fig. 7I).

Biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice fail to lactate

Eighty-three percent (15/18) of litters born to biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mothers died within two days of birth. Although three litters survived, the average weight of 14-day-old pups nursed by *Erbb4*$^{Flox/Flox}$*Wap-Cre* mothers was 23% less than that of pups from similar sized litters nursed by biparous *Erbb4*$^{+/+}$*Wap-Cre* mothers (6.03 g versus 7.85 g,

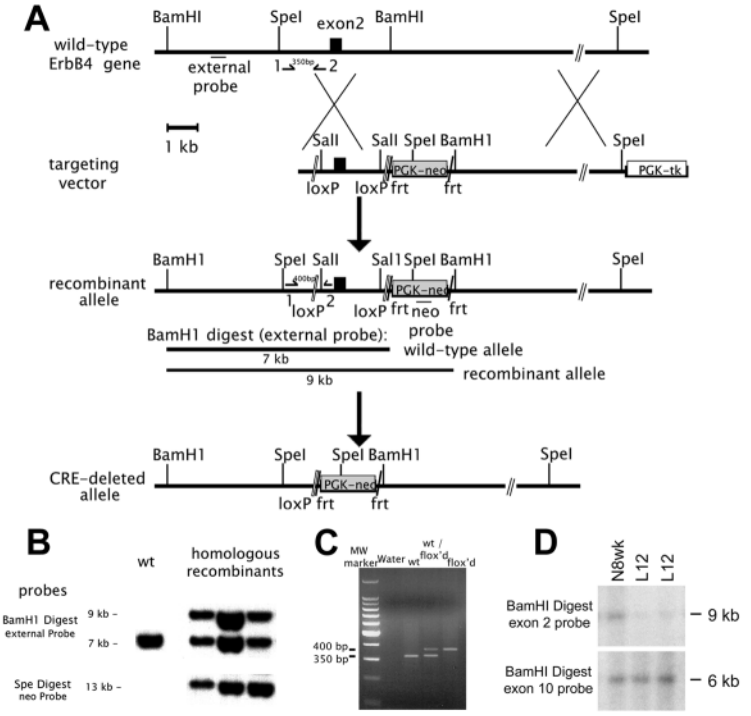

Fig. 1. Conditional deletion of *Erbb4* in the mammary gland. (A) Schematic representation of the wild-type ERBB4 locus, targeting vector, recombinant allele and CRE-deleted allele. The targeting vector replaces the coding region of exon 2 (black box) with a *loxP*-flanked exon 2. A *frt*-flanked PGK neomycin-resistance (neo) cassette (gray box) was included for positive selection, and a PGK thymidine kinase (tk) cassette (white box) was included for negative selection. The PGK-neo cassette was not removed in the CRE-deleted allele. An external genomic probe and an internal *neo* probe were used for screening embryonic stem cells. The position of PCR primers (1 and 2) are indicated by arrowheads. (B) Southern-based analysis of genomic DNA from embryonic stem cells. The 9 kb and 7 kb *Bam*H1-digested fragments correspond to the targeted and wild-type allele, respectively. (C) PCR analysis of DNA extracted from tails of mice derived from matings between mice heterozygous for the wild-type (wt) allele and the recombinant (floxed) allele, showing the 350 bp wild-type band and the 400 bp floxed band. (D) Southern blot analysis showing CRE-mediated excision of *Erbb4* exon 2 in mammary glands from 8-week-old nulliparous (N8wk) mice and two L12 *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice. The 9 kb and 6 kb *Bam*H1-digested fragments were detected using an exon 2 probe and the internal control exon 10 probe, respectively.

P<0.002). When nursed by foster *Erbb4*$^{+/+}$*Wap-Cre* mothers, pups born to biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* dams developed normally. These observations suggest that biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice have a defect in mammary gland development and/or function resulting in inadequate milk production.

ERBB4 contributes to lobuloalveolar development

To determine the impact of ERBB4 ablation on mammary gland development, we examined the number four inguinal mammary glands from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* and *Erbb4*$^{+/+}$*Wap-Cre* mice at multiple stages of pregnancy and lactation. Development of the normal breast during pregnancy and through parturition is characterized by a dramatic increase in epithelial proliferation, which results in the accumulation of lobuloalveolar structures. Fully developed lobuloalveoli with differentiated epithelium become the milk-producing structures during lactation. Mammary tissue from *Erbb4*$^{+/+}$*Wap-Cre* mice accumulated lobuloalveoli at P13.5 of the second pregnancy (Fig. 3A, arrow), culminating in extensive lateral and terminal lobuloalveolar outgrowths by P18.5 (Fig. 3C, arrow). At parturition, engorged lobuloalveoli (Fig. 2E, arrow) masked the underlying ductal system (Fig. 2E, arrowhead). By contrast, a reduction in lobuloalveolar outgrowth was observed in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P13.5 (Fig. 3B, arrow), the earliest pregnancy time-

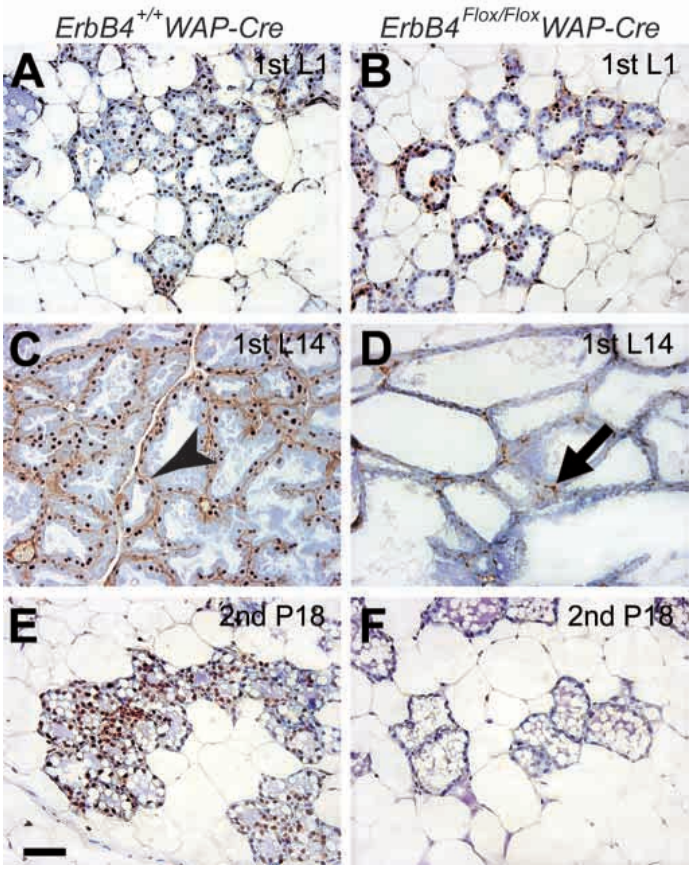

Fig. 2. Immunohistochemical localization of ERBB4 expression. Paraffin wax-embedded number 4 inguinal mammary glands from *Erbb4*$^{+/+}$*Wap-Cre* (A,C,E) and *Erbb4*$^{Flox/Flox}$*Wap-Cre* (B,D,F) mice, at L1 (A,B), L14 (C,D) and P18 of the second pregnancy (E,F), were stained for ERBB4 expression by IHC. Nuclear ERBB4 protein can be detected in *Erbb4*$^{+/+}$*Wap-Cre* mammary glands and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands at L1. Membrane staining of ERBB4 at L14 is indicated by the arrowhead in C and stromal expression is indicated by the arrow in D. Scale bar: 50 μm.

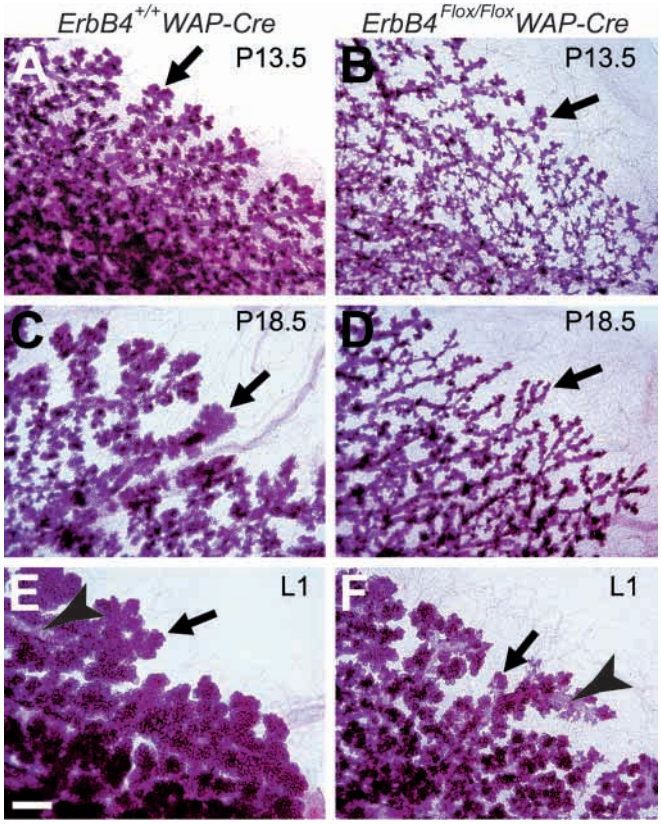

Fig. 3. Impaired mammary gland development in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice. Wholemounts of carmine stained mammary glands from biparous *Erbb4*$^{+/+}$*Wap-Cre* (A,C,E) and *Erbb4*$^{Flox/Flox}$*Wap-Cre* (B,D,F) mice at P13.5 (A,B), P18.5 (C,D) and L1 (E,F). Alveolar clusters are indicated by arrows and normal distention of mammary ducts is indicated by arrowheads. Scale bar: 500 μm.

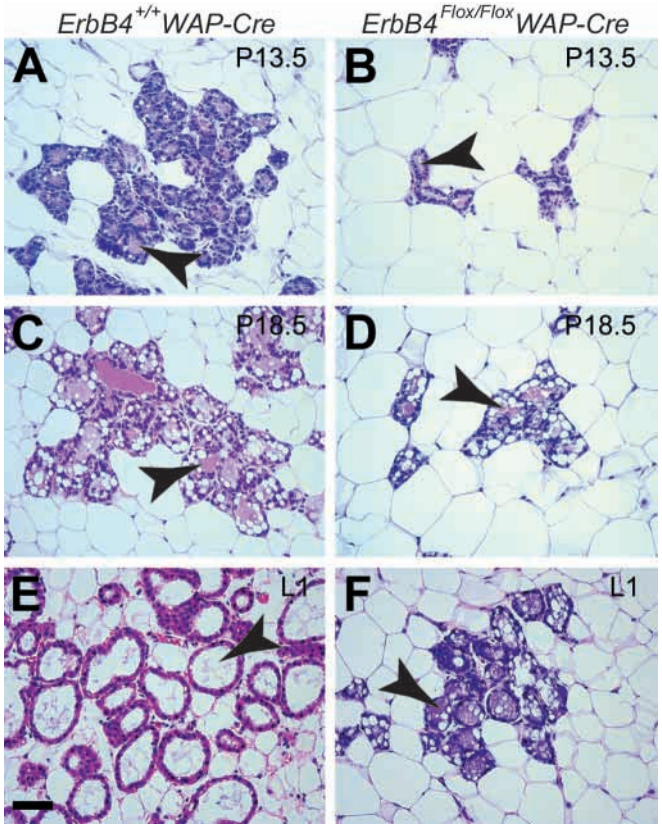

Fig. 4. Condensed alveolar structures in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice. Histological analysis of Hematoxylin and Eosin stained paraffin wax-embedded biparous *Erbb4*$^{+/+}$*Wap-Cre* (A,C,E) and *Erbb4*$^{Flox/Flox}$*Wap-Cre* (B,D,F) mammary glands at P13.5 (A,B), P18.5 (C,D) and L1 (E,F). Abnormal secretory activity is indicated by the accumulation of lumenal lipids in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands at parturition. Arrowheads indicate alveolar lumens. Scale bar: 50 μm.

point examined. By P18.5 large regions of mammary tissue exhibited sparse lobuloalveolar expansion with ducts bearing few lateral and terminal alveoli (Fig. 3D, arrow). At parturition, fully distended ducts were observed (Fig. 3F, arrowhead); however, they bore few engorged lobuloalveoli (Fig. 3F, arrow). Mammary glands from *Erbb4*$^{Flox/Flox}$*Wap-Cre* dams that did not support litters underwent extensive involution by L3 and were completely regressed by L10 (data not shown).

Histology of mammary glands from *Erbb4*$^{+/+}$*Wap-Cre* mice during pregnancy revealed large clusters of alveoli, which showed accumulation of lumenal secretory products at P13.5 (Fig. 4A, arrowhead) and synthesis of secretory lipids at P18.5 (Fig. 4C, arrowhead). At parturition, the mammary gland from biparous *Erbb4*$^{+/+}$*Wap-Cre* mice was filled with distended lobuloalveoli, indicating that lactation was successfully engaged (Fig. 4E, lumen indicated by arrowhead). Histological analysis of mammary tissue from *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice revealed a dramatic reduction in the number of alveoli accumulating during pregnancy (Fig. 4B,D). Although the alveoli in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue were condensed they appeared to accumulate normal lumenal secretory products at P13.5 (Fig. 4B, arrowhead) and secretory lipids at P18.5 (Fig. 4D, arrowhead). However, at L1, *Erbb4*$^{Flox/Flox}$*Wap-Cre* alveoli remained condensed and continued to accumulate lumenal secretory lipids (Fig. 4F, arrowhead). Accumulation of secretory lipids is usually observed during pregnancy within alveoli harboring undifferentiated epithelium (Fig. 4C). These results suggest that secretory epithelium in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue fails to undergo terminal differentiation at parturition.

ERBB4 mediates proliferation of alveolar epithelium

In the normal breast, proliferation of alveolar epithelium occurs throughout pregnancy and the early post-partum period. We used in situ bromodeoxyuridine (BrdU) incorporation to determine whether defective alveolar development in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice was a result of impaired epithelial proliferation. BrdU incorporation was determined by immunohistochemistry of paraffin wax-embedded mammary glands. When compared with *Erbb4*$^{+/+}$*Wap-Cre* mammary glands at the same developmental time points, a 20% reduction in BrdU-positive epithelial cells was observed in mammary tissue from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at both P13 and P18 (Fig. 5C). In concordance with lactational failure at parturition, a dramatic 65% reduction in BrdU-labeled cells

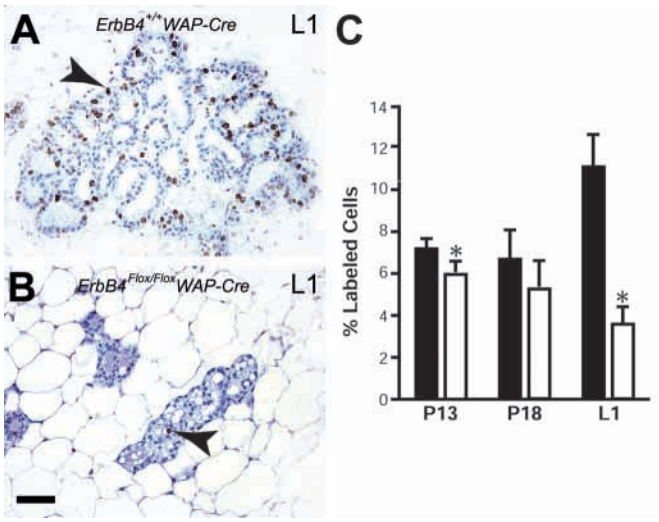

Fig. 5. *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice exhibit defects in mammary epithelial proliferation. Immunohistochemical detection of in situ BrdU incorporation in biparous *Erbb4*$^{+/+}$*Wap-Cre* control (A) and *Erbb4*$^{Flox/Flox}$*Wap-Cre* (B) mice at L1. Arrowheads indicate BrdU-labeled nuclei. (C) A significant reduction in the percentage of BrdU-labeled cells was observed in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands at P13 ($t[8]=4.41$, $P<0.01$) and L1 ($t[8]=9.11$, $P<0.001$), but not at P18 ($t[13]=1.71$, $P<0.20$). Significant differences within each data set are represented by asterisks. Scale bar: 50 μm.

was observed in mammary glands from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at L1 (Fig. 5C; also compare Fig. 5A and B), which suggests that reduced epithelial proliferation contributes to the defects in lobuloalveolar development observed in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice.

Functional differentiation of mammary epithelium requires ERBB4

Cellular specification and functional differentiation of mammary epithelium in *Erbb4*$^{+/+}$*Wap-Cre* and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice was evaluated by immunohistochemical detection of the mammary ductal cell marker sodium/potassium/chloride co-transporter (NKCC1; SLC12A2 – Mouse Genome Informatics), and the mammary differentiation marker sodium phosphate co-transporter type IIb (NPT2B; SIC34A2 – Mouse Genome Informatics), respectively.

We have previously observed NKCC1 expression at high levels on the basolateral membrane of mammary ductal epithelium from nulliparous mice. The specification of ductal epithelium to a secretory phenotype is accompanied by a diminution of NKCC1 levels during pregnancy and at parturition (Miyoshi et al., 2001). Likewise, relatively high levels of NKCC1 expression were observed in biparous *Erbb4*$^{+/+}$*Wap-Cre* and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary epithelium at P13 (Fig. 6A,B; arrowheads), and lower levels were observed at P18 (Fig. 6C,D). NKCC1 expression was dramatically reduced at L1 (Fig. 6E,F). These results indicate that ductal epithelium from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice successfully undergoes cellular specification, forming alveolar secretory epithelium. Smooth muscle actin (SMA) was expressed within myoepithelial cells of all mammary glands examined (Fig. 6A-F)

We have previously demonstrated that NPT2B is preferentially expressed on the apical membrane of differentiated secretory epithelium in the lactating mammary gland (Miyoshi et al., 2001). As expected, expression of NPT2B was not detected at P13 (Fig. 6G,H), but was observed at the apical surface of some lumenal epithelium of mammary glands from biparous *Erbb4*$^{+/+}$*Wap-Cre* mice at P18 (Fig. 6I, arrowhead). A low level of NPT2B expression was also detected in mammary glands from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18 (Fig. 6J, arrowhead). At L1, high levels of NPT2B expression were detected on the apical surface of secretory epithelium in biparous *Erbb4*$^{+/+}$*Wap-Cre* mammary glands (Fig. 6K, arrowhead). In striking contrast, alveolar epithelium of biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue at L1 failed to express NPT2B (Fig. 6L), which indicates a critical defect in terminal differentiation. β-catenin was expressed on epithelial membranes of all mammary glands examined (Fig. 6G-L).

ERBB4 regulates STAT5 activation

The defects in lobuloalveolar accumulation, absence of epithelial NPT2B expression and lactational failure observed in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice are similar to the mammary gland phenotypes described for *Stat5a*-null mice (Liu et al., 1997; Miyoshi et al., 2001; Teglund et al., 1998). To determine whether ERBB4 is required for the functional activation of STAT5 in mammary epithelium, we examined STAT5A phosphorylation at the regulatory amino acid Y694 (phospho-STAT5) by immunohistochemistry and western blot.

Prominent nuclear staining of phospho-STAT5 was observed within alveolar epithelium of biparous *Erbb4*$^{+/+}$*Wap-Cre* mammary glands at P13.5 (Fig. 7A, arrowhead), P17.5 (Fig. 7C) and L1 (Fig. 7E). Immunohistochemistry using an antibody that detects both phosphorylated and unphosphorylated STAT5 populations (STAT5) indicated that, as expected, the majority of STAT5 protein was localized within epithelial nuclei of *Erbb4*$^{+/+}$*Wap-Cre* mammary glands at L1 (Fig. 7G, arrowhead). Although phospho-STAT5 was detected within alveolar epithelium of biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue at P13.5, the majority of the activated STAT5 protein remained cytoplasmic (Fig. 7B, inset arrow), with less than 50% of nuclei showing phospho-STAT5 staining (Fig. 7B, inset arrowhead). Strikingly, phospho-STAT5 immunohistochemistry failed to reveal activated STAT5 within ERBB4-deficient mammary epithelium at either P17.5 (Fig. 7D) or L1 (Fig. 7F). Furthermore, STAT5 protein was expressed but was excluded from nuclei of *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary epithelium at L1 (Fig. 7H, arrow).

Inactivation of STAT5 in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue was confirmed by the analysis of ERBB4 and STAT5 proteins immunoprecipitated from biparous *Erbb4*$^{+/+}$*Wap-Cre* and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue at L1. Anti-ERBB4 immune complexes, analyzed by western blot with an ERBB4 antibody, confirmed the absence of ERBB4 in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue (Fig. 7I, ERBB4/ERBB4). Western blot analysis of anti-STAT5 immune complexes, probed with a STAT5 antibody, revealed equivalent amounts of STAT5 protein in control and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands (Fig. 7I, STAT5/STAT5). In addition, phosphorylation of STAT5

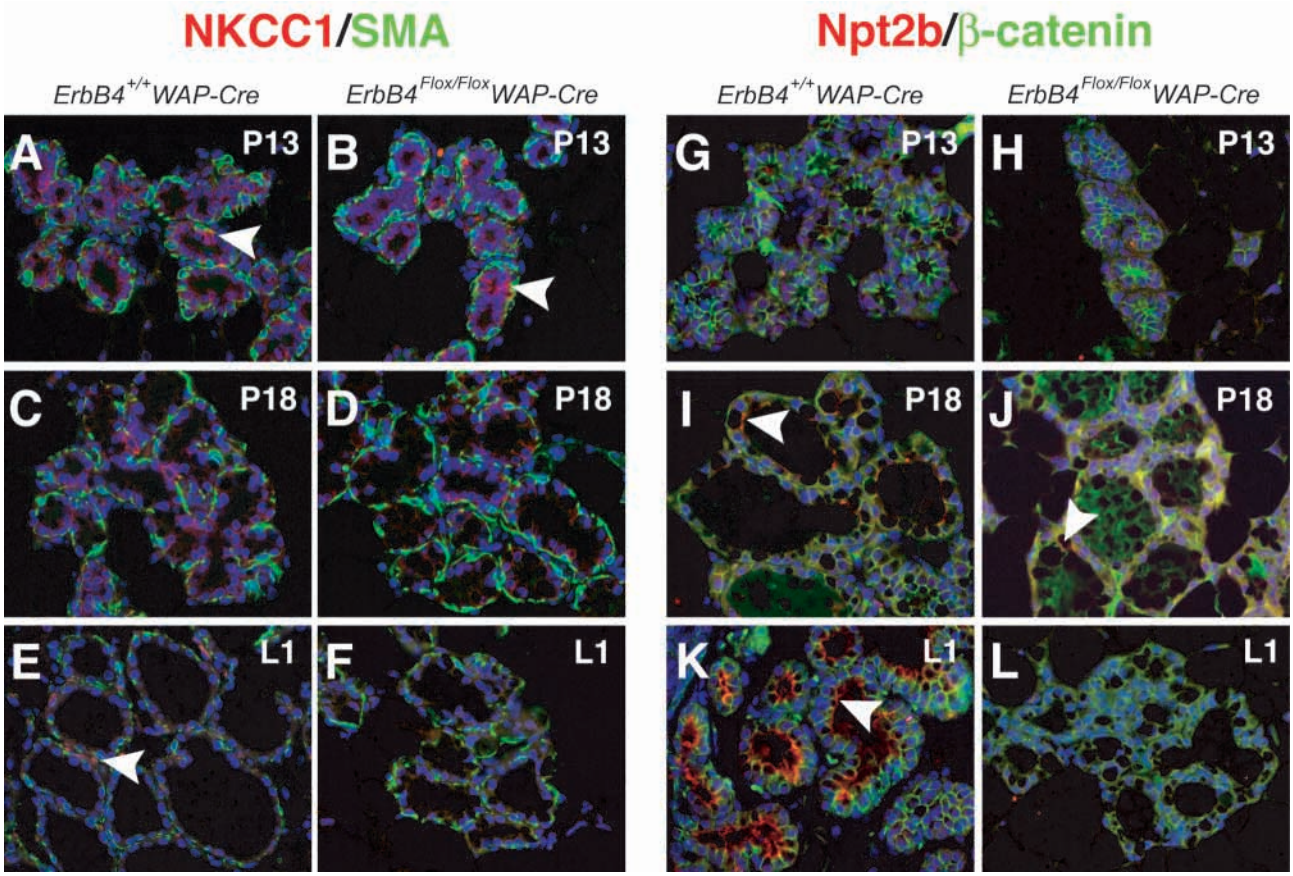

Fig. 6. *Erbb4*$^{Flox/Flox}$*Wap-Cre* secretory epithelium fails to express the differentiation marker NPT2B. Immunohistochemical staining of NKCC1 (red) and SMA (green) in mammary glands of biparous *Erbb4*$^{+/+}$*Wap-Cre* and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice, at P13 (A,B), P18 (C,D) and L1 (E,F). Immunohistochemical staining of NPT2B (red) and β-catenin (green) in mammary glands of biparous *Erbb4*$^{+/+}$*Wap-Cre* and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice, at P13 (G,H), P18 (I,J) and L1 (K,L). Lack of NPT2B staining in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands at L1 indicates a defect in epithelial differentiation. Arrowheads (A,B,E) indicate NKCC1 staining and arrowheads (I,K) indicate NPT2B staining of the apical surface of secretory epithelium.

immunoprecipitated from control lysates was demonstrated by western blot analysis using both a phosphotyrosine antibody (Fig. 7I, STAT5/P-tyr) and a specific antibody that recognizes STAT5 phosphorylated at Y694 (Fig. 7I, STAT5/P-STAT5). Consistent with immunohistochemical results, STAT5 protein present in STAT5 immune complexes prepared from *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary tissue lacked detectable tyrosine phosphorylation when probed with the phosphotyrosine antibody (Fig. 7I, STAT5/P-tyr) or the antibody specific for STAT5 phosphorylated at Y694 (Fig. 7I, STAT5/P-STAT5). The relative epithelial cell populations in *Erbb4*$^{+/+}$*Wap-Cre* control and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary gland lysates was compared by probing 50 μg of total lysate with an antibody specific for keratin 18 (Fig. 7I, NA/K18). Taken together, the immunohistochemical and western blot data implicates ERBB4 as a crucial mediator of STAT5 activation during late pregnancy and at parturition.

ERBB4-null mammary glands fail to express STAT5 regulated milk genes

STAT5 transactivates the expression of several milk genes, including *casein beta* (*csnb*) and *Wap*, which harbor canonical STAT5 binding γ-interferon activation sites (GAS) within their promoters (Rosen et al., 1999). STAT5 function in mammary glands from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice was assessed by northern blot analysis of milk-gene expression. As predicted high levels of β-casein and WAP expression, with lower levels of α-lactalbumin expression, were detected in mammary glands from biparous *Erbb4*$^{+/+}$*Wap-Cre* mice at L1 (Fig. 8; lanes 1,2). By contrast, expression of β-casein and WAP was drastically reduced in mammary tissue from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at L1, whereas expression of α-lactalbumin appeared unaffected by the absence of ERBB4 (Fig. 8; lanes 3,4). Detection of GAPDH (GAPD – Mouse Genome Informatics) expression confirmed equal RNA loading in each lane (Fig. 8; lanes 1-4). Impaired expression of *csnb* and *Wap*, two genes directly regulated by STAT5, demonstrates that STAT5 function is impaired in biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at L1. Although the α-lactalbumin gene also harbors a GAS element (Rosen et al., 1999), consistent with our observations, direct regulation of α-lactalbumin by STAT5 lacks experimental confirmation. Taken together, these results indicate that the inability of *Erbb4*$^{Flox/Flox}$*Wap-Cre* dams to support their young is, in part,

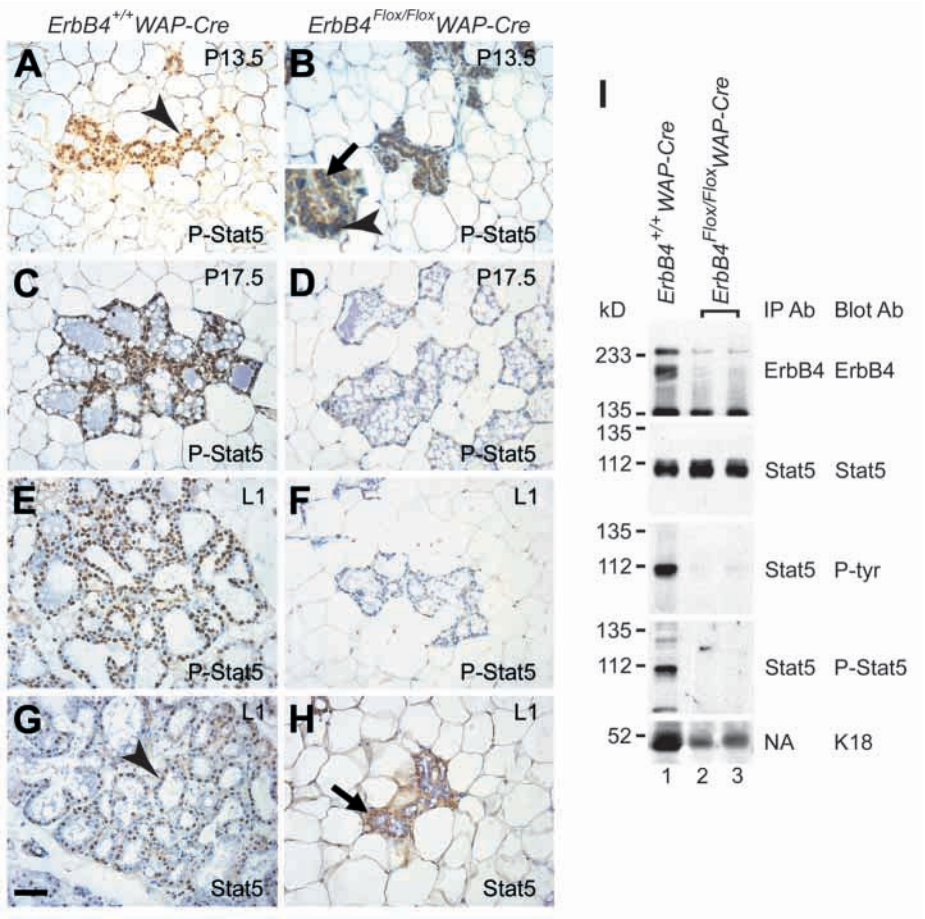

Fig. 7. STAT5 activation is abolished in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands. Immunohistochemical analysis of biparous control *Erbb4*$^{+/+}$*Wap-Cre* (A,C,E,G) and *Erbb4*$^{Flox/Flox}$*Wap-Cre* (B,D,F,H) paraffin wax-embedded mammary glands, at P13.5 (A,B), P17.5 (C,D) and L1 (E,F), for STAT5 activation using an affinity-purified antibody directed against STAT5 phosphorylated at the regulatory amino acid Y694 (A-F; P-STAT5). A STAT5 antibody was used in immunohistochemistry of paraffin wax-embedded mammary glands to identify both phosphorylated and inactive STAT5 populations (G,H; STAT5). The inset in B is a higher magnification view of positive P-STAT staining. Arrowheads and arrows indicate positive nuclear and cytoplasmic staining, respectively. Scale bar: 50 μm. (I) Expression of inactive STAT5 in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary glands was confirmed by western blot analysis of mammary gland protein lysates. STAT5 and ERBB4 were immunoprecipitated from *Erbb4*$^{+/+}$*Wap-Cre* control and *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary gland protein lysates prepared from biparous mice at L1. ERBB4 immune complexes were probed with an ERBB4 antibody (I; ERBB4/ERBB4). Phosphorylation of immunoprecipitated STAT5 protein was determined by western blot analysis using a phosphotyrosine antibody (I; STAT5/P-tyr) and an antibody specific for STAT5 phosphorylated at Y694 (I; STAT5/P-STAT5). The relative epithelial cell population was determined by probing 50 μg of total mammary gland lysate with an antibody specific for keratin 18 (I; NA/K18).

caused by the impaired expression of STAT5-regulated genes that encode essential milk proteins.

PRLR is dispensable for STAT5 activation at late pregnancy

Signaling through the prolactin receptor (PRLR) also contributes to STAT5 activation and lobuloalveolar development (Gallego et al., 2001; Miyoshi et al., 2001). Disengaged PRLR signaling as a result of ablated ERBB4 expression would therefore provide a plausible explanation for the lack of STAT5 function in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mammary epithelium. Physiological levels of serum PRL and expression of PRLR in mammary glands of pregnant *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice suggested that the crucial components of PRLR signaling were not affected by the deletion of *Erbb4* (data not shown). To confirm intact PRLR signaling in the absence of ERBB4, we performed an acute activation of STAT5 by injecting PRL into *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18. Mock injection of *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18 failed to activate mammary STAT5 (Fig. 9A,B). By contrast, mammary gland lysates contained high levels of activated STAT5 (Fig. 9A), and prominent immunohistochemical staining of nuclear phospho-STAT5 was observed within mammary epithelium from PRL-injected *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice (Fig. 9C), which indicates that PRLR signaling was intact in the absence of ERBB4 but that it remained inactive.

Intact PRLR signaling in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice suggests that essential ERBB4 and PRLR signaling pathways are non-overlapping and raises the possibility that PRLR is dispensable for STAT5 activation at late pregnancy. However, defective embryo implantation in females containing a null

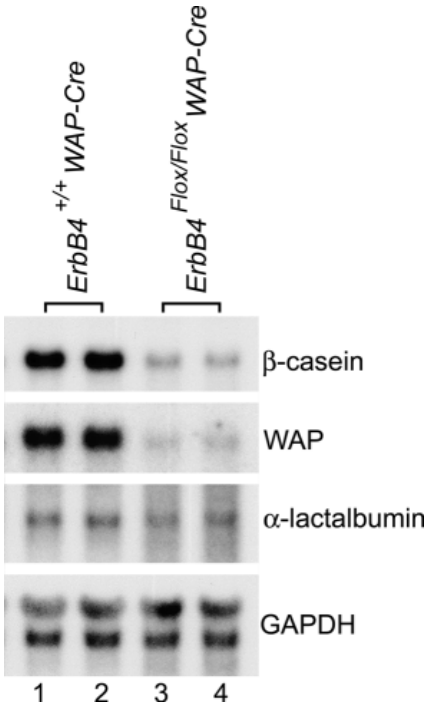

Fig. 8. Reduced milk-gene expression in *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice. Northern blot analysis of milk gene expression in biparous control *Erbb4*$^{+/+}$*Wap-Cre* (lanes 1,2) and *Erbb4*$^{Flox/Flox}$*Wap-Cre* (lanes 3,4) mice at L1. Total mammary gland RNA was isolated at L1 and subjected to northern blot analysis using probes specific for the milk genes β-*casein*, *Wap* and α-*lactalbumin*. A GAPDH probe served as a control for RNA loading.

mutation of the *Prlr* gene has hampered in vivo analysis of PRLR-mediated STAT5 activation in parous mammary tissue (Ormandy et al., 1997). Recently, we have demonstrated that progesterone administration to *Prlr*$^{-/-}$ mice rescues embryo implantation and maintains pregnancy to P19.5 (Binart et al., 2000). Using this model system we performed phospho-STAT5 immunohistochemistry to determine the level of STAT5 activation in *Prlr*-null mammary tissue. Progesterone treatment of pregnant biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice failed to activate significant levels of STAT5 at P18 (Fig. 9D), which demonstrates that progesterone treatment alone does not directly result in STAT5 activation. Despite severe defects in alveolar development, immunohistochemical staining of mammary tissue from progesterone rescued *Prlr*-deficient mice at P18 revealed pronounced nuclear staining of activated STAT5 (Fig. 9E). Taken together, these results suggest that STAT5 activation can bypass PRLR signaling, and suggest a novel pathway with ERBB4 as the obligate mediator of STAT5 activation at late pregnancy and parturition.

Discussion

ERBB4 is essential for normal breast function

Several lines of experimental evidence indicate that expression of the ERBB family of receptors and ligands contributes to normal breast function (Stern, 2003; Troyer and Lee, 2001). However, the exact contribution of individual ERBB receptors to breast development, and the downstream mechanisms that regulate ERBB activity in breast epithelium, have not been characterized. We have identified an essential function for the ERBB family member ERBB4 during pregnancy-induced mammary differentiation and lactation. We propose that

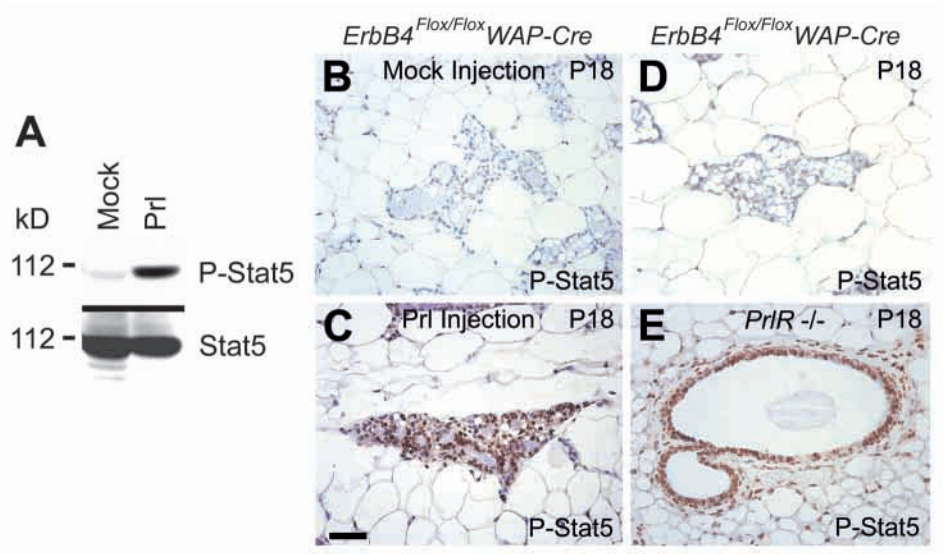

Fig. 9. PRLR signaling in mammary glands at late pregnancy. *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18 were either mock injected or injected with PRL at 5 μg/g body weight for 15 minutes. Mammary gland lysates were analyzed by western blot for phospho-STAT5 (A, top panel) and total STAT5 protein (A, bottom panel). In addition, paraffin wax-embedded mammary glands from mock- (B) or PRL (C)-injected *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18 were stained for phospho-STAT5 by immunohistochemistry. Detection of PRL-stimulated STAT5 activation in mammary glands from biparous *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at P18 (A,C) indicates intact PRLR signaling. Pregnancy was rescued in *Prlr*$^{-/-}$ mice by the administration of progesterone. Mammary glands from progesterone-treated *Erbb4*$^{Flox/Flox}$*Wap-Cre* control mice (D) and progesterone-rescued *Prlr*-deficient mice (E) at P18 were embedded in paraffin wax and stained for phospho-STAT5 by immunohistochemistry. Phospho-STAT5 immunohistochemical staining of progesterone-rescued *Prlr*-null mammary glands indicates PRLR-independent STAT5 activation at late pregnancy. Scale bar: 50 μm.

ERBB4 is an obligate mediator of STAT5 function during late pregnancy and that the loss of STAT5-induced milk-gene expression directly contributes to the lactational failure observed in mice with conditional *Erbb4* deletions.

Our results demonstrating the essential contribution of ERBB4 signaling to breast development are corroborated by recent experiments from Martin Gassmann and colleagues (Tidcombe et al., 2003). Heart defects associated with embryonic lethality of mice with deletions of both *Erbb4* alleles were rescued by expressing ERBB4 under the control of a cardiac-specific promoter ($ERBB4^{-/-}HER4^{heart}$). The $ERBB4^{-/-}HER4^{heart}$ mice survived to adulthood; however, they failed to lactate at parturition (Tidcombe et al., 2003). Severe lactational defects in uniparous $ERBB4^{-/-}HER4^{heart}$ mice underscores the essential contribution of ERBB4 signaling to pregnancy-induced mammary development and supports the conclusion that the absence of a lactation phenotype in uniparous $Erbb4^{Flox/Flox}$*Wap-Cre* mice results from incomplete WAP-CRE mediated deletion of *Erbb4* during the first pregnancy.

There is limited phenotypic overlap between the biparous $Erbb4^{Flox/Flox}$*Wap-Cre* mice described herein and our previous description of mammary gland defects in mice overexpressing a dominant-negative ERBB4 transgene (ERBB4ΔIC). Indeed, significant differences in the severity and temporal presentation of lobuloalveolar defects were observed. Most noteworthy is that condensed lobuloalveoli at mid-lactation was a distinct feature of ERBB4ΔIC-expressing mammary glands (Jones et al., 1999). However, this phenotype was present in less than 5% of the mammary gland and ERBB4ΔIC failed to impact mammary gland function. Interpretation of results from ERBB4ΔIC mice is complicated because dominant-negative ERBB proteins suffer from non-specific pan-dominant effects, potentially attenuating signaling through all four ERBB receptors. Indeed, despite complete ablation of epithelial ERBB4 expression during the first lactation (see Fig. 2D), a mid-lactation phenotype was not observed in uniparous $Erbb4^{Flox/Flox}$*Wap-Cre* mice. The lack of phenotypic overlap between ERBB4ΔIC and uniparous $Erbb4^{Flox/Flox}$*Wap-Cre* mice at mid-lactation underscores the fact that ERBB4ΔIC harbors non-specific activity and directly impacts mammary developmental pathways in addition to ERBB4. Our current genetic experiments clearly indicate that the essential contribution of ERBB4 signaling to breast development and lactation occurs during pregnancy and at parturition.

Multiple ERBB4 activities in the mammary gland

Our current results indicate that ERBB4 has essential functions during pregnancy-induced epithelial proliferation and during the differentiation of secretory epithelium at parturition. This functional dichotomy suggests that activated ERBB4 couples to divergent signaling pathways during breast development. Ligand-induced ERBB heterodimerization represents an important mechanism driving ERBB signal diversification (reviewed by Alroy and Yarden, 1997). However, it remains unclear whether ERBB4 regulates development as a signaling homodimer or through heterodimerization with other ERBB family members. Indeed, all four ERBB receptors are highly phosphorylated within the mammary gland at parturition (Schroeder and Lee, 1998), and each may therefore contribute to ERBB4 signaling at this developmental stage. For example, *waved 2* mice, harboring a mutant EGFR with reduced tyrosine kinase activity (Luetteke et al., 1994), exhibit impaired alveolar development and lactational defects (Fowler et al., 1995) similar to those seen in ERBB4-deficient mammary glands.

Experimental evidence implicates ERBB2 as the central mediator of ligand-induced signaling through the ERBB family (Graus-Porta et al., 1997) and, as such, it may contribute to ERBB4 function in the developing mammary gland. In support of this, we have previously reported alveolar developmental defects at parturition in transgenic mice expressing a dominant-negative ERBB2 receptor (ERBB2ΔIC) (Jones and Stern, 1999). Analysis of milk-gene expression by in situ hybridization revealed reduced levels of β-casein and WAP transcripts in alveolar epithelium expressing ERBB2ΔIC (F.E.J. and D. Stern, unpublished). In addition, mice lacking the ERBB4 ligand HRGα exhibit impaired epithelial proliferation during pregnancy and reduced β-casein expression at parturition (Li et al., 2002). Phenotypic overlap between *Hrgα*-null, ERBB2ΔIC-expressing and ERBB4-deficient mammary glands at parturition underscores a possible role for HRGα-driven ERBB2/ERBB4 signaling during pregnancy-induced epithelial proliferation and functional differentiation.

ERBB4 regulates STAT5 function

The mammary gland phenotype observed in $Erbb4^{Flox/Flox}$*Wap-Cre* mice was reminiscent of observations reported for *Stat5a*-null mice (Liu et al., 1997; Miyoshi et al., 2001; Teglund et al., 1998). Loss of ERBB4 or STAT5A expression results in the accumulation of histologically identical lobuloalveolar defects during pregnancy, and a failure to lactate at parturition. The extent of phenotypic overlap observed in $Erbb4^{Flox/Flox}$*Wap-Cre* and *Stat5a*-null mice suggests that the ERBB4 and STAT5A signaling pathways are directly coupled during functional differentiation of breast epithelium. In support of this, we demonstrate by both immunohistochemistry and western blot analysis complete ablation of STAT5 activation in mammary epithelium from $Erbb4^{Flox/Flox}$*Wap-Cre* mice at late pregnancy and parturition. Similar to STAT5A-null mice, ERBB4-deficient mammary epithelium fails to express the differentiation marker NPT2B and exhibits a dramatic reduction in the expression of the STAT5-regulated milk genes *csnb* and *Wap*. Our suggestion that ERBB4 directly activates STAT5 in the pregnant mammary gland is further supported by our previous results demonstrating a physical interaction between ERBB4 and STAT5, which resulted in phosphorylation of the STAT5 protein at the regulatory amino acid Y694 and at additional novel tyrosine residue(s) (Jones et al., 1999).

Pregnancy-induced functional differentiation of mammary epithelium requires both ERBB4 and PRLR (Ormandy et al., 1997). Interestingly, loss of either ERBB4 or PRLR leads to ablation of STAT5 activation (Gallego et al., 2001; Miyoshi et al., 2001), which suggests that these two pathways cooperate to activate STAT5. Our previous results identified an early role for PRLR signaling in cell fate determination during the pregnancy-induced transition from ductal to secretory alveolar epithelia. PRLR- and STAT5-null epithelium retained expression of the ductal epithelial marker NKCC1 (Miyoshi et al., 2001; Shillingford et al., 2002). By contrast, dramatically reduced expression of NKCC1 indicates that

ERBB4-deficient mammary epithelium successfully undergoes pregnancy-induced cell specification (see Fig. 6). However, despite evidence of intact PRLR signaling (see Fig. 9), ERBB4-null epithelium lacks functional STAT5 and fails to express the epithelial differentiation marker NPT2B. Based upon our current understanding, we propose a novel mechanism for STAT5 regulation that first requires PRLR signaling at early pregnancy during STAT5-regulated cellular specification. Then at late pregnancy, ERBB4 supplants PRLR and functions as the obligate mediator of STAT5-induced epithelial differentiation and lactation. This model is supported by our results demonstrating STAT5 activity in mammary glands of *Erbb4*$^{Flox/Flox}$*Wap-Cre* mice at early pregnancy, and by evidence that PRLR is dispensable for STAT5 activation at late pregnancy. The molecular switch between PRLR and ERBB4 as the obligate STAT5-regulating receptor may be driven by enhanced ERBB4-ligand expression at late pregnancy, and/or by altered STAT5 function mediated by novel ERBB4-induced STAT5 phosphorylation events (Jones et al., 1999).

Conclusions

In summary, we propose that ERBB4 functions as an essential mediator of STAT5 activation during mammopoiesis and lactogenesis, thereby ensuring the milk production crucial for survival of the offspring. Our results, describing the function of ERBB4 signaling during normal breast development, have important implications for breast cancer. Despite the essential contribution of ERBB4 signaling to normal breast function, the majority of breast tumors selectively extinguish ERBB4 expression (Kew et al., 2000; Srinivasan et al., 2000; Srinivasan et al., 1998). When expressed, ERBB4 is associated with favorable clinicopathological factors and a differentiating tumor phenotype (Kew et al., 2000; Knowlden et al., 1998; Srinivasan et al., 2000). These clinical observations support our model of ERBB4 as a crucial mediator of epithelial differentiation. However, we also demonstrate that ERBB4 signaling contributes to the proliferation of breast epithelium. The exact mechanisms that differentially regulate ERBB4 mitogenic and differentiation responses in breast epithelium will be an area of intense investigation. As we and other laboratories attempt to marshal ERBB4 differentiation signals as a therapeutic approach to inhibit breast cancer progression, ultimate success will depend on our ability to disengage ERBB4-induced proliferation.

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