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Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development

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SUMMARY

Targeted mice lacking functional EGF or amphiregulin (AR) were derived and bred to the TGFα-knockout to generate mice lacking various combinations of the three ligands. In contrast to EGF receptor (EGFR) knockout mice, triple null mice lacking half of the EGFR ligand family were healthy and fertile, indicative of overlapping or compensatory functions among EGF family members. Nevertheless, pups born to triple null dams frequently died or were runted, suggesting a mammary gland defect. Comparison of individual and combinatorial knockouts established that specific loss of AR severely stunted ductal outgrowth during puberty, consistent with dramatic expression of AR transcripts in normal developing ducts. Surprisingly, loss of all three ligands did not significantly affect cellular proliferation, apoptosis, or ERK activation within terminal end buds. Following pregnancy, most AR single null females, but few triple null females could nurse their young, revealing collaborative roles for EGF and TGFα in mammopoiesis and lactogenesis. In triple null glands, alveoli were poorly organized and differentiated, and milk protein gene expression was decreased. Additionally, Stat5a activation was frequently reduced in AR single and combinatorial nulls in association with impaired lactation. Collectively, our results provide genetic confirmation of a requirement for EGFR signaling throughout the development of the mouse mammary gland, and reveal stage-dependent activities for different EGFR ligands. Finally, the additional loss of growth factors from pups nursed by triple null dams further worsened their survival and growth, establishing functions for both maternal- and neonatal-derived growth factors.

Key words: Gene targeting, EGF, Amphiregulin, EGF receptor, Mammary gland, Terminal end bud, Ductal morphogenesis

INTRODUCTION

The epidermal growth factor receptor (EGFR) is an integral membrane tyrosine kinase that is activated upon binding of a family of polypeptides that includes epidermal growth factor (EGF), transforming growth factor-alpha (TGFα), amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC), and epiregulin (EPR) (reviewed by Lee et al., 1995). These ligands share a conserved, three disulfide-loop structure (the EGF-like motif) that is released by proteolytic cleavage of transmembrane precursor proteins. EGFR (erbB1) and its ligands are components of an extended signaling superfamily that contains three related receptors (erbB2, erbB3, erbB4) (reviewed by Riese and Stern, 1998), and multiple additional ligands harboring an EGF-like sequence (the neuregulins) (Wen et al., 1994; Carraway III et al., 1997). There is growing evidence, principally from cell culture, of interactions among superfamily members through both receptor heterodimerization (reviewed by Heldin, 1995) and the binding of individual ligands to multiple erbB proteins (Beerli and Hynes, 1996; Pinkas-Kramarski et al., 1996; Riese et al., 1996).

In vitro responses to mammalian EGFR ligands include altered cell proliferation, differentiation, survival and motility. However, physiological roles for mammalian EGFR ligands have not been well defined. As a step toward elucidating these, we and others have used gene targeting to produce mutant mice lacking either the EGFR ligand, TGFα, or EGFR itself. Loss of TGFα produces wavy hair, accompanied by aberrant hair shaft morphology and deranged follicle patterning (Luetteke et al., 1993; Mann et al., 1993). It also delays eyelid migration and fusion in late gestation, predisposing to open eyes at birth and a spectrum of subsequent eye anomalies (Luetteke et al., 1993; Mann et al., 1993; Berkowitz et al., 1996). The characterization of these knockout mice led to the discovery that the similar phenotypes of spontaneous mouse mutants, *waved-1* (*wa-1*) and *waved-2* (*wa-2*), are due to reduced expression of a normal TGFα transcript and a point mutation in the EGFR tyrosine kinase, respectively (Luetteke et al., 1994; Fowler et al., 1995; Berkowitz et al., 1996). In addition to the aforementioned hair and eye abnormalities, ablation of the mouse EGFR induces peri-implantation through perinatal death depending on the genetic background of the host mice (Sibilia and Wagner, 1995; Threadgill et al., 1995; Miettinen

et al., 1995). This variable lethality is associated with either placental defects or impaired epithelial maturation in several tissues of neonates, including skin, lung and gastrointestinal tract, indicating that EGFR and its ligands have broad roles in development.

The rodent mammary gland is one of several epithelial tissues in which the EGFR system is believed to be crucial. Experimental evidence includes findings that when EGFR ligands are exogenously administered by pellet implantation or ectopically expressed from transgenes, they are potent growth, differentiation and survival factors for mammary gland epithelium (reviewed by Schroeder and Lee, 1997). Additionally, the six EGFR ligands, together with the four erbB receptors, are all naturally expressed in the mammary gland in overlapping patterns that nevertheless show some stage- and cell-type specific distinctions (Schroeder and Lee, 1998). Moreover, female *wa-2* mice harboring the aforementioned EGFR kinase mutation exhibit deficient lactation as a consequence of sparse lobuloalveolar development (Fowler et al., 1995), and ductal outgrowth is retarded in transgenic mice expressing a truncated, dominant negative form of EGFR (Xie et al., 1997). Unfortunately, confirmation of these various results in the EGFR knockout mice has been complicated by their early lethality.

As a further step toward understanding requirements for the various EGFR ligands, we have now derived mice that lack EGF or AR. Additionally, by breeding to our TGFα-null line, we have also generated the various double and triple null mice lacking up to half of the EGFR ligand family. Analysis of these combinatorial mutants confirms the importance of the EGFR system in both the developing and differentiating mammary gland. Specifically, the present study reveals a distinct and essential role for AR in mammary ductal morphogenesis, and supporting roles for EGF and TGFα in lactogenesis.

MATERIALS AND METHODS

Gene targeting

Genomic clones of the EGF and AR genes were isolated from a 129Sv mouse λ library (Stratagene, La Jolla, CA) by screening with a 1.9 kb mouse EGF cDNA (a gift from Graeme Bell, Univ. Chicago, Chicago, IL) or a 0.5 kb *Pst*I-*Bam*HI genomic AR fragment (probe in Fig. 1A) derived from pMoAR (a gift from Greg Plowman, Sugen, Redwood City, CA). For the EGF targeting vector, 0.8 kb *Hin*cII-*Eco*RI and 4.7 kb *Hin*dIII-*Sal*I fragments were inserted, respectively, into the unique *Bam*HI and *Hin*dIII sites of pNTK (a gift from Richard Mortensen, Harvard Medical School, Boston, MA). This targeting plasmid contains the neomycin resistance and Herpes Simplex virus thymidine kinase genes driven by the phosphoglycerate kinase promoter (Mortensen et al., 1992). For the AR targeting vector, 2.5 kb *Bam*HI-*Eco*RI and 5.5 kb *Hin*dIII fragments were inserted respectively into the *Bam*HI and *Hin*dIII sites of pNTK. Either E14 or R1 embryonic stem cells were electroporated with 20 μg of *Xho*I-linearized EGF or AR constructs and subjected to positive-negative selection with G418 and gangcyclovir as previously described (Luetteke et al., 1993). Drug-resistant ES clones were screened for homologous recombination by Southern blot analysis of genomic DNAs digested with *Msc*I (EGF) or *Xba*I (AR) using 5′ probes from the respective genes (Fig. 1A). After verification of correct targeting with additional Southern analyses, ES clones were karyotyped, microinjected into 3.5-day-old C57BL/6J blastocysts, and implanted into the uterine horns of pseudopregnant CD-1 foster mothers. Chimeras were crossed to C57BL/6J partners; thus, subsequent generations of the mice were on a mixed background of 129 and C57BL/6J strains.

Tissue harvest and analysis

Mice were killed by CO_2 asphyxiation. Six- to twelve-week-old virgin females were injected with 10 μl/g body weight BrdU (Amersham, Arlington Heights, IL) 2 hours prior to being killed. The right 3rd thoracic and 4th inguinal mammary glands were fixed in 10% formalin for 48-72 hours and processed for paraffin embedding. Sections (5 μm) prepared by the UNC-CH Histopathology Core Lab were stained with hematoxylin and eosin or subjected to immunohistochemistry using alkaline phosphatase-conjugated BrdU antibody (Cell Proliferation Kit, Boehringer Mannheim, Indianapolis, IN) or TUNEL assay (Apotag Peroxidase Kit, Oncor, Gaithersburg, MD). The left 3rd and 4th glands were whole mounted, fixed overnight in Carnoy's, and defatted through 3 changes of acetone. Specimens were rehydrated and stained overnight in 0.2% carmine, 0.5% aluminum potassium sulfate. Slides were dehydrated to absolute ethanol, cleared in xylene, permanently mounted, and imaged.

For analyses of pregnant or lactating mammary glands, 5- to 6-month-old dams were killed, and the extent of epithelial penetration noted. Third glands were harvested for whole mount and histological examination as described above. One 4th gland was removed and snap frozen in liquid nitrogen for total RNA, while the other was immediately homogenized in lysis buffer (Schroeder and Lee, 1998), aliquoted, and stored at –80°C. Total protein was assayed using Coomassie dye (Bio-Rad Laboratories, Hercules, CA). Samples (1 mg) were immunoprecipitated with 1 μg/ml Stat3 or Stat5a antibody (Santa Cruz, Santa Cruz, CA) and 25 μl protein G agarose (Life Technologies, Gaithersburg, MD). Immune complexes were washed three times, denatured and resolved by 10% SDS-PAGE. Gels were immunoblotted (Schroeder and Lee, 1998) with anti-phosphotyrosine RC20 (Transduction Laboratories, Lexington, KY). Stripped blots were reprobed with 0.1 μg/ml anti-Stat3 or Stat5a.

RNA analyses

For northern blots, tissues were homogenized in 4 M guanidinium isothiocyanate, and total RNA isolated by centrifugation through CsCl. Samples (10 μg) were electrophoresed through 1.0% agarose gels containing formaldehyde, and transferred to ZetaProbe (Bio-Rad Laboratories, Hercules, CA). Immobilized RNA was hybridized with milk protein cDNAs (kindly provided by Jeffrey Rosen, Baylor College of Medicine, Houston, TX). RNA loading and integrity were assessed by comparing ribosomal RNA staining. RT-PCR of AR was performed (Luetteke et al., 1993) using the following primers: P1: 5′-ATGAGAACTCCGCTGCTACCGCTG-3′; P4: 5′-ATAACGATGCC-GATGCCAATAGCT-3′; P5: 5′-ACCCTGCATTGTCCTCAGCTA-3′.

For in situ analyses, 4% paraformaldehyde-fixed, paraffin sections (10 μm) were deparaffinized, treated for 30 minutes with 10 μg/ml proteinase K in 0.05 M Tris (pH 8.0), 0.05 M EDTA, acetylated for 10 minutes in 0.1 M triethanolamine (TEA)/0.27% (v/v) acetic anhydride, and dehydrated through ethanol. Sections were hybridized with 10^7 cts/minute of ^{35}S-labeled antisense or sense probe for 18 hours at 54°C in 50% formamide, 2.5× Denhardt's, 0.6 M NaCl, 10 mM Tris, 1 mM EDTA, 0.1% SDS, 10 mM DTT, 0.5 mg/ml *E. coli* tRNA, and 10% dextran sulfate. Following the hybridization, slides were washed five times for 15 minutes each in 4× SSC at 25°C, treated with 20 mg/ml RNase A for 30 minutes at 37°C, then washed four times in 2× SSC/1 mM DTT at 25°C and three times in 0.5× SSC/1 mM DTT at 54°C. Slides were dehydrated in alcohol and exposed to β-max film (Amersham Life Sciences, Arlington Heights, IL) for 2 days to gauge exposure times. They were then dipped twice in Kodak NTB2 photo emulsion and stored for 3 days to 4 weeks at 4°C. Developed slides were stained with hematoxylin and examined by bright- and dark-field microscopy. The AR probe was a *Dra*II/*Eco*RV fragment of the mouse AR cDNA (bases 573-904). The TGFα probe was described previously (Berkowitz et al., 1996), while the EGF

probe was an *Xba*I/*Pst*I fragment (bases 3489-3850) from EGF cDNA. The EGFR probe was derived from cDNA sequences encoding the extracellular domain (bases 1552-1857; Luetteke et al., 1994), while the HB-EGF probe was a 0.4 kb *Bam*HI fragment from exon 2. Microscopic image analysis was performed with the aid of the UNC-CH Department of Pathology's Microscopy Services Laboratory.

RESULTS

Derivation of EGF and AR null mice

To inactivate the mouse EGF and AR genes, targeting vectors (Fig. 1A) were designed to replace most or all of the EGF-like motif with the neomycin resistance cassette. Deletion of EGF exon 20 eliminates the first two disulfide loops, and any transcriptional readthrough or splicing to exon 21 leads to a frameshift. Deletion of AR exons 3 and 4 eliminates all three disulfide loops along with the aminoterminal heparin-binding region and the transmembrane domain of the precursor, and splicing of exon 2 to exon 5 similarly results in a frameshift. Targeting constructs were electroporated into E14 (EGF) or R1 (AR) embryonic stem (ES) cells, and G418 and gancyclovir-resistant clones screened by Southern blot. Two of three EGF targeted ES clones and one of two AR targeted ES clones microinjected into blastocysts developed into strong chimeras capable of germline transmission of the mutant alleles. Intercrosses of F_1 heterozygotes yielded offspring with a normal Mendelian distribution of genotypes for EGF (20% EE, 55% Ee, 25% ee) and AR (27% AA, 50% Aa, 23% aa); the upper and lower case of the first letter of the growth factor name denote wild-type and mutant alleles, respectively. Southern blots (not shown) of mouse tail DNAs verified the expected shifts of diagnostic *Msc*I (EGF) and *Xba*I (AR) restriction fragments located within the affected regions of the genes (Fig. 1A, arrows).

Northern blots revealed negligible expression of EGF transcripts in salivary gland and kidney of homozygous mutants (ee), and western blots failed to detect both the mature 6 kDa species and higher molecular mass forms in salivary glands of EGF null mice (Fig. 1B). In the case of AR, northern

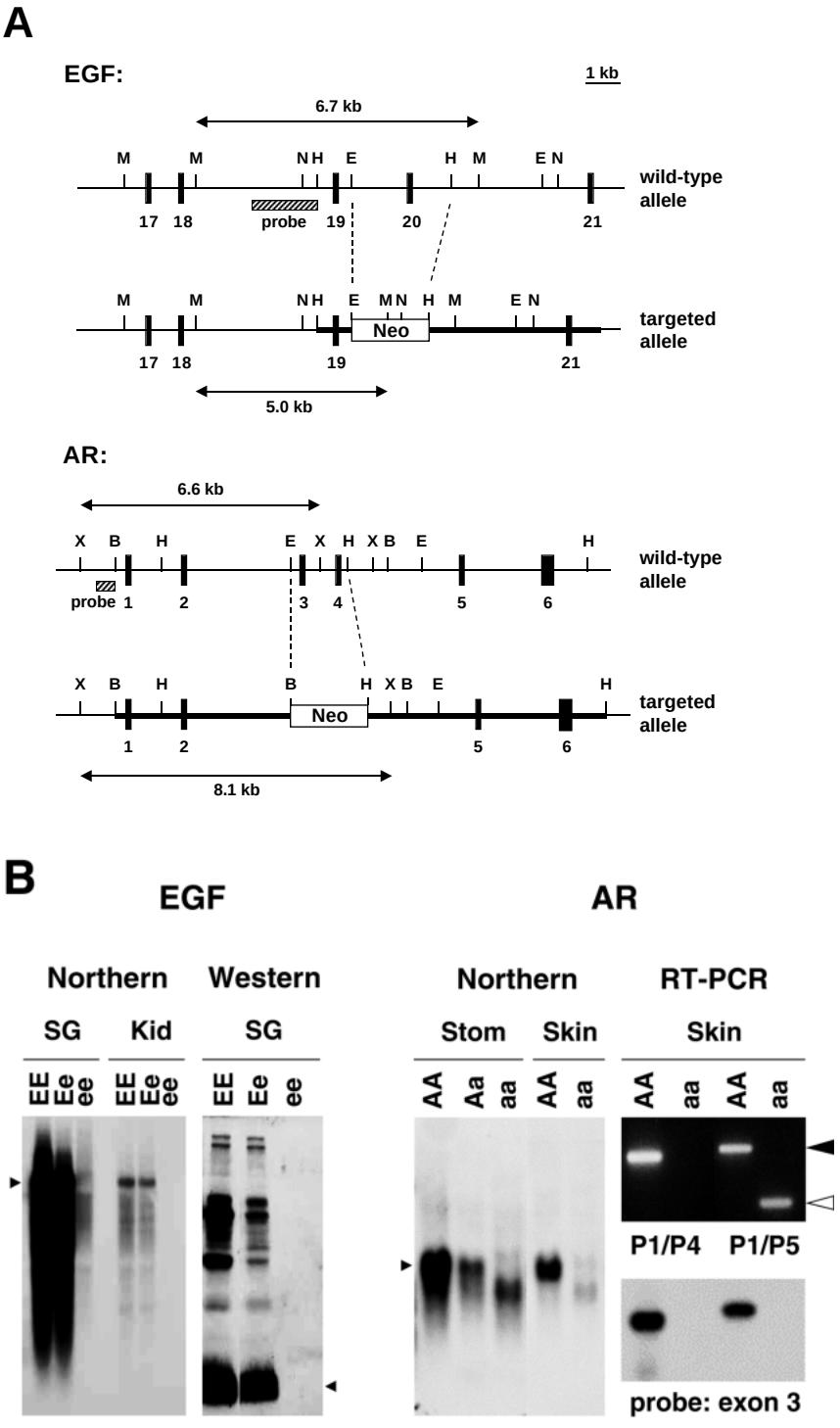


Fig. 1. Targeted inactivation of EGF and AR. (A) Schematics show wild-type and targeted mouse EGF and AR alleles with exons numbered (vertical bars). The 5′ and 3′ regions of homology in the targeting vectors are indicated by heavy lines, and dashed lines indicate regions replaced by Neo. Useful restriction sites are shown, and hatched boxes depict probes used to confirm homologous recombination. Arrows indicate diagnostic restriction fragments. Restriction sites: M, *Msc*I; H, *Hin*dIII; E, *Eco*RI; N, *Nco*I; X, *Xba*I; B, *Bam*HI. (B) Expression analyses. For EGF, total RNA (20 μg) and protein (200 μg) from adult salivary gland (SG) and/or kidney (Kid) were subjected to northern and western blot, respectively, using cloned cDNA fragments or rabbit anti-mouse EGF (Upstate Biotechnology Inc., Lake Placid, NY) as probes. Arrowheads indicate normal transcript (4.8 kb) and mature EGF protein (6 kDa). For AR, the northern of total RNA from adult stomach and neonatal skin was hybridized with AR cDNA. Arrowhead indicates the normal transcript of 1.4 kb. For RT-PCR, total neonatal skin RNAs were amplified using primers derived from preserved (P1, P5) or deleted (P4) AR exons. Products from indicated primer pairs were Southern blotted with an exon 3 oligo. Arrowheads denote the wild-type 764 bp (solid) and targeted 400 bp (open) products, respectively.

Table 1. Birth and growth rates of EGF and AR single null offspring

Genotype	Litters	Pup survival	Pup body weight (g)		
			1 week old	2 weeks old	3 weeks old
AAEETT	5	31/32 (97%)	4.2±0.14	7.2±0.16	9.1±0.16
AAeeTT	9	62/64 (97%)	3.7±0.08	6.8±0.10	8.0±0.11
aaEETT	8	64/64 (100%)	3.9±0.13	7.0±0.19	9.0±0.24

Wild-type or targeted partners were mated at 2-3 months of age, and their offspring counted and weighed weekly until weaning. Genotype refers to both parents and pups. Data shown is mean body weight and standard error of the mean.

blots of stomach and neonatal skin revealed decreased but significant levels of a smaller transcript in aa mice. The residual AR transcript was investigated by RT-PCR using primers from preserved (P1, P5) or targeted (P4) exons. With P1/P4 primers, an amplified product of the expected size was obtained from wild-type but not from aa tissues. In contrast, primer pair P1/P5, which flanks the deleted exons, yielded products from both genotypes. However, the product generated from aa skin (open arrowhead) was smaller and did not hybridize to an exon 3 probe, consistent with the loss of coding sequences. Sequence analysis of cloned RT-PCR products verified that the aa transcript reflected precise splicing of exon 2 to exon 5, predicting a frameshift and premature termination (data not shown).

EGF and AR null mice displayed no overt phenotype, and did not exhibit the hair or eye defects observed in TGFα deficient mice. Moreover, screening of lung, kidney and gastrointestinal tract did not disclose gross or histological abnormalities. EGF and AR homozygous mutants were also fertile, and survival and growth rate of single null pups born to the respective single null parents were not significantly different from wild-type values (Table 1). This contrasts with mild (10%) and severe (50-70%) growth retardation reported, respectively, in TGFα null (Luetteke et al., 1993) and EGFR null neonates (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995).

Triple null mice lacking TGFα, EGF and AR are viable

The viability and fecundity of the individual knockout lines allowed us to generate combinatorial knockout mice and thereby explore possible functional redundancy or compensation among these EGFR ligands. EGF and TGFα null mice were mated to obtain double heterozygotes (EeTt), which were intercrossed to obtain the double homozygotes (eett). These mice were mated to AR single null mice to derive triple heterozygotes (AaEeTt). From 68 litters born to AaEeTt parents, we obtained 5 triple null (aaeett) and 6 wild-type (AAEETT) pups out of a total of 424 offspring, close to the fraction (1/64) predicted by Mendelian segregation. Surprisingly, triple null mice lacking half of the EGFR ligand family survived to maturity. Consistent with the loss of TGFα, they exhibited wavy fur and whiskers. In comparison to TGFα single null mice, they showed an increased penetrance of eye defects (80-90% versus 40-50%), as well as accelerated hair and weight loss, dermatitis, and skin ulceration with aging. Otherwise, aaeett mice displayed no overt phenotype and have survived for over one year.

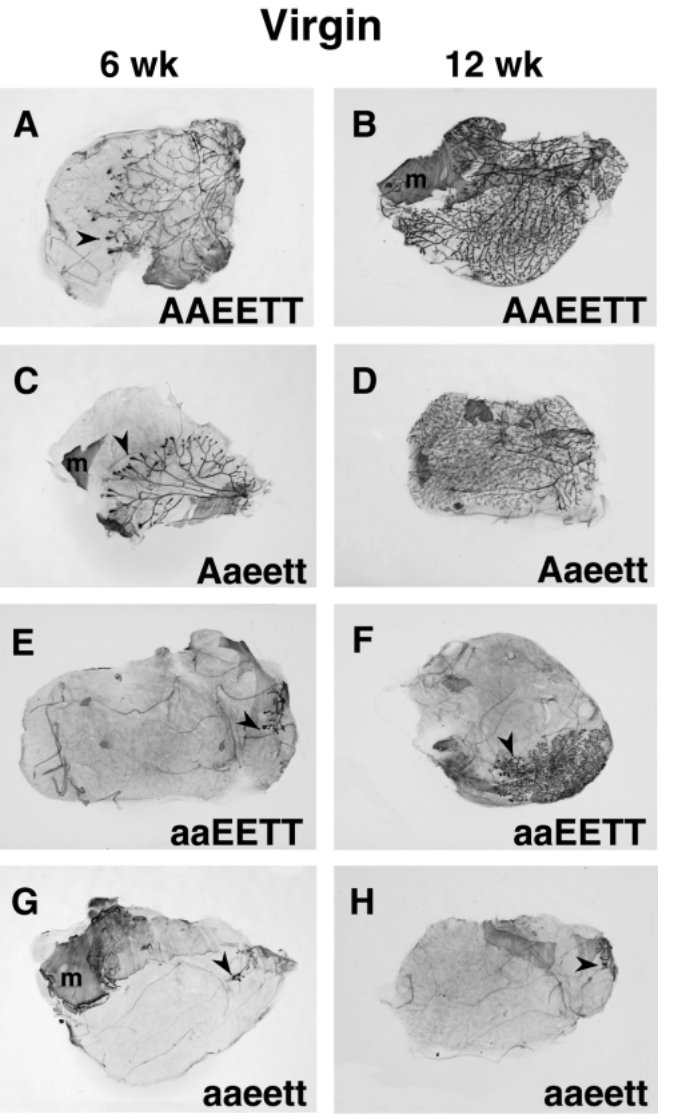


Fig. 2. Defect in ductal outgrowth in glands from virgin AR null female mice. Whole mounts of thoracic glands were prepared from pubescent (A,C,E,G) and mature (B,D,F,H) virgin females of the indicated genotypes. Note the lack of ductal outgrowth in 6-week-old (6 wk) AR single and triple null glands. In 12-week-old females, ductal arborization is complete in wild-type and EGF/TGFα double null glands, but minimal in AR null glands. Arrowheads indicate TEBs. m, muscle. Magnification: 10×.

AR null virgin females display a mammary gland phenotype

Matings between double and triple null partners demonstrated that both sexes of all combinatorial genotypes were fertile. However, pups born to most double or triple null dams lacking AR (aaee, aatt, aaeett) often died within 2 days postpartum. Despite constant nurturing and suckling, these pups had little or no milk in their stomachs. Perinatal morbidity was most prevalent among the first litters of mothers between 2-3 months of age. Nursing competence generally improved with age and parity, but surviving offspring were usually growth retarded. These observations suggest that AR deficiency is associated with impaired mammary gland development and/or function,

and potentially aggravated by the additional loss of EGF, TGFα, or both.

Mammary gland development during puberty is characterized by progressive outgrowth and branching of a ductal system throughout the subcutaneous fat pad. This process is driven by dynamic epithelial structures, denoted terminal end buds (TEBs), that disappear once the fat pad is fully arborized. To monitor this stage, we compared whole mounts of virgin glands of various genotypes. Glands from pubescent (6 week) AAEETT and Aaeett females displayed active ductal elongation, led by numerous bulbous TEBs (Fig. 2A,C; arrowheads). In striking contrast, ducts in pubescent aaEETT and aaeett glands barely extended beyond the rudimentary anlage, though a few TEBs were visible (Fig. 2E,G; arrowheads). In mature (12 week) virgins, AAEETT and Aaeett ductal trees completely permeated the fat pads and lacked TEBs (Fig. 2B,D), but those in aaEETT and aaeett glands were still underdeveloped and contained persistent TEBs (Fig. 2F,H; arrowheads). Although their penetration of the adipose stroma was impeded, ducts in the aaEETT gland had undergone substantial lateral branching as evidenced by increased epithelial density (Fig. 2F). These results demonstrate a specific and unique requirement for AR during ductal morphogenesis in the mouse mammary gland. EGF and TGFα are clearly dispensable for this process, though inspection of numerous specimens suggested that additional loss of these ligands may further exacerbate the delay in ductal outgrowth in the absence of AR (compare Fig. 2F and H).

Since TEBs formed in AR-deficient mammary glands, but failed to advance at a normal rate, we investigated their cellular architecture and activity. AR and other EGFR ligands promote epithelial cell proliferation and survival in the mouse mammary gland (Coleman et al., 1988; Matsui et al, 1990; Sandgren et al, 1995), and hence we hypothesized that the balance between DNA synthesis and apoptosis might be altered in the TEBs of knockout mice. Accordingly, sections of wild-type and triple null virgin glands were assayed for BrdU incorporation and terminal deoxytidyl transferase dUTP nick end labeling (TUNEL) by immunohistochemistry. Because TEBs were rarely seen in 6-week-old triple null glands, we compared wild-type specimens at 6 weeks of age with the persistent TEBs present in triple null virgins at 6, 8, 10 and 12 weeks of age. TEBs from both genotypes exhibited the classic shape and structure, including a leading cap cell layer (cc), and a dense neck region containing multiple layers of body cells (bc) trailed by the nascent duct. In glands of both genotypes, DNA synthesis (BrdU incorporation; Fig. 3A,B) was prominent in the cap cell layer and outer body cells, whereas apoptosis (dUTP incorporation; Fig. 3C,D) was more prevalent in body cells proximal to the lumen of TEBs (Fig. 3A-D). BrdU labeling indices in the two genotypes were not significantly different (20.9±2.3% for AAEETT [13 TEBs, 6-8 weeks]; 21.4±2.4% for aaeett [17 TEBs, 6-12 weeks]), and were similar to previously reported values (Humphreys et al., 1996). Apoptosis levels, which ranged from 3 to 15 percent (average = 7.9±2.7% for AAEETT TEBs; 7.4±2.8% for aaeett TEBs), were also comparable between the two genotypes but slightly lower than the 11% value reported for 5-week-old Balb/c mice (Humphreys et al., 1996). In accord with normal levels of cell proliferation and elimination, the mean sizes of TEBs were nearly identical: 233±27 cells and 234±19 cells for AAEETT and aaeett glands, respectively. Collectively, these data unexpectedly indicate that the ductal defect in triple null females is not due to deregulation of cell growth or death.

We also examined MAP kinase activation in situ. In TEBs from both genotypes, dpERK immunostaining was concentrated in body cells immediately behind the cap cell layer, and was detected in both cytoplasm and nucleus consistent with activation-coupled translocation (Fig. 3E,F). Staining was eliminated when antibody was preincubated with the dual-phosphorylated peptide antigen, but not unphosphorylated peptide, confirming specificity (data not shown). Interestingly, despite frequently scoring positive for BrdU, cap cells were rarely positive for dpERK, while similar

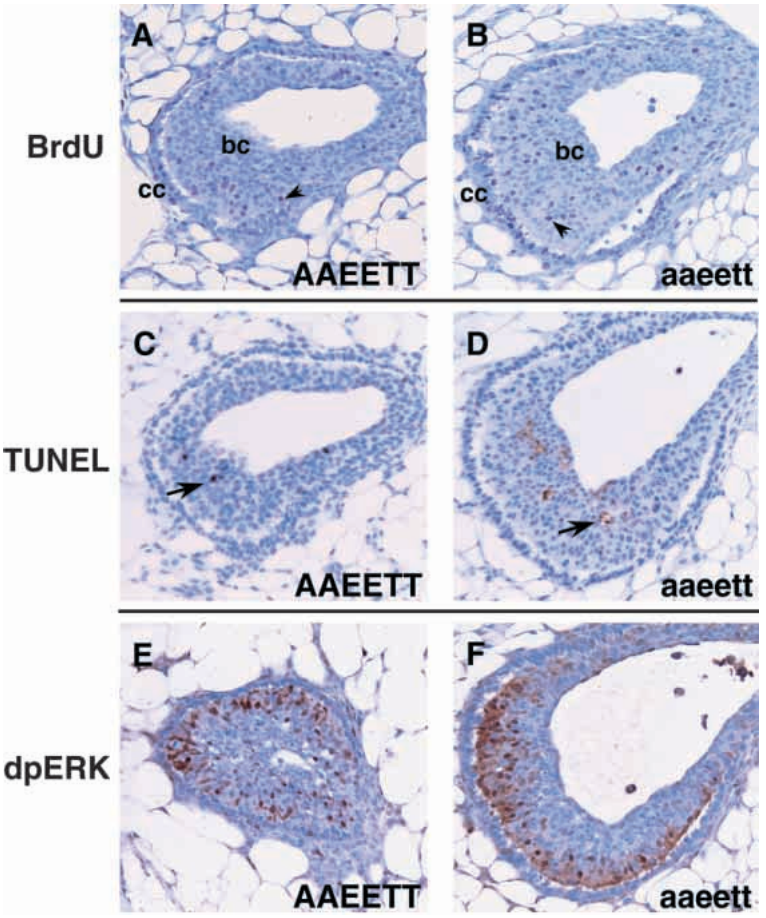
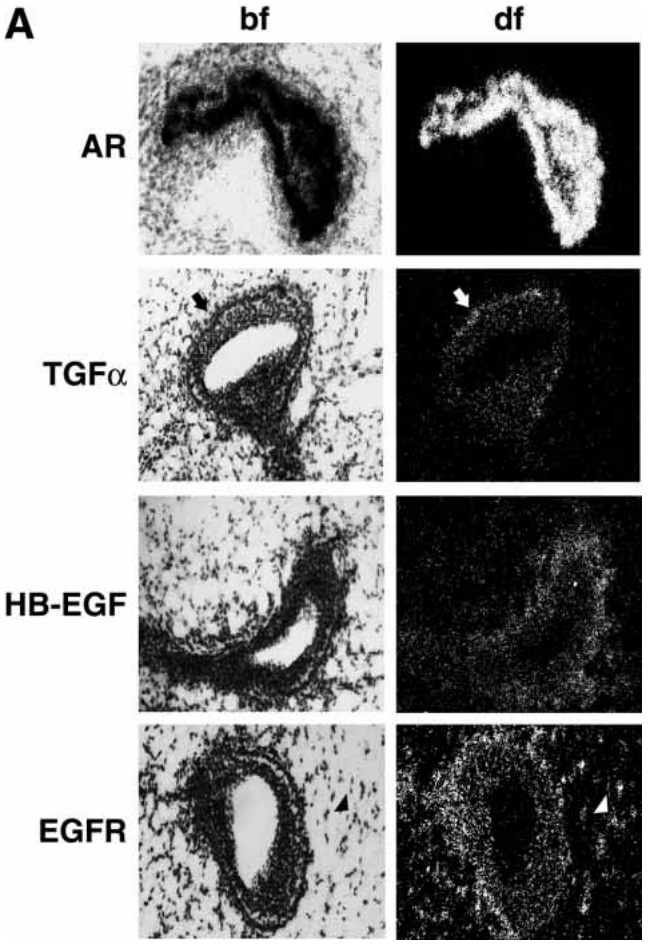


Fig. 3. DNA synthesis, apoptosis, and ERK activation in TEBs in wild-type and triple null virgin mammary glands. Immunohistochemistry was performed on serial sections from 6-week-old wild-type (A,C,E) or 6- to 12-week-old triple null virgins (8-week old shown; B,D,F) to detect BrdU incorporation (A,B), dUTP incorporation (TUNEL; C,D), and dual-phosphorylated ERKs (E,F). Arrowheads and arrows indicate labeled cells undergoing DNA synthesis and apoptosis, respectively. Mean BrdU labeling indices (± s.e.m.) were AAEETT, 20.9 ±2.3%; aaeett, 21.4±2.3%. Mean dUTP labeling indices (± s.e.m.) were AAEETT, 7.9±2.7; aaeett, 7.4±2.8%. cc, cap cells; bc, body cells. Magnification: 400×.



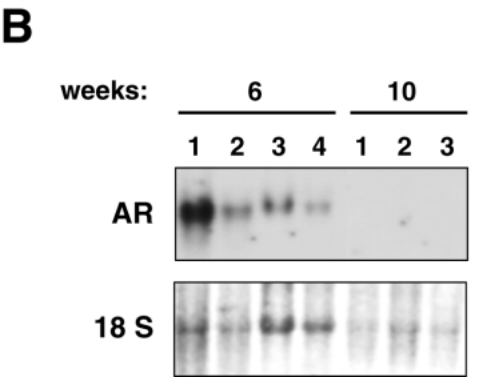


Fig. 4. Expression of EGFR and ligands in wild-type mammary glands. (A) In situ hybridization. Sections from 6-week-old virgin glands were hybridized to the indicated probes and visualized by either bright-field (bf) or dark-field (df) microscopy. Results with antisense and sense probes were compared to confirm specificity of hybridization, but only images from antisense probes are shown. Exposure times were 10 days for AR or 3 weeks for the others. Note that AR expression was especially strong, but TGFα, HB-EGF and EGFR transcripts were also prominent in TEBs with expression concentrated in the outer cell layer (arrow). Additionally, HB-EGF and EGFR transcripts were detected in fat cells (arrowhead). Magnification, 200×. (B) Northern blot analysis of AR expression in pubescent versus mature glands. The two fourth mammary glands of 6- (*n*=4) and 10- (*n*=3) week-old wild-type virgin mice were dissected and pooled, and total RNA prepared. Samples (10 μg) were electrophoresed, transferred to membrane, and probed with AR cDNA. The 18S rRNA was visualized by methylene blue staining to monitor loading and integrity of RNA. The 1.4 kb AR mRNA is indicated. Numbers above lanes refer to individual mice.

regions of body cells stained with both antibodies (compare Fig. 3A,B to E,F). Anti-dpERK also stained ductal epithelia in developing glands and periductal stroma in both pubescent and mature virgin glands (Schroeder and Lee, 1998; data not shown). Importantly, while there was variation in intensity in both genotypes, dpERK reactivity of these various cellular compartments was not appreciably different between wild-type and triple-null glands.

AR transcripts are highly expressed during ductal morphogenesis

Our previous RT-PCR analyses indicated that several EGFR ligands, including TGFα, BTC, HB-EGF, and AR, are all expressed in the developing mammary gland, albeit in different temporal patterns (Schroeder and Lee, 1998). To understand the unique requirement for AR, we compared expression of AR and related ligands by in situ hybridization (Fig. 4A). These analyses revealed dramatic hybridization of AR antisense, but not sense, probe to sections of 6-week-old wild-type virgin mammary gland (Fig. 4A; data not shown). Hybridization was localized in punctate patterns to epithelial cells of ducts and TEBs. Northern blots revealed temporal regulation of AR expression with transcripts detected in pubescent but not mature virgin glands (Fig. 4B). These results are generally consistent with the previously reported detection of AR protein, though we did not detect AR transcripts in stroma (Kenney et al., 1995).

Transcripts for other EGFR ligands were also detected by in situ hybridization, albeit with much longer exposures (3 weeks versus 3 days for AR). This suggests that the unique requirement for AR in ductal morphogenesis may be due in part to its relatively high level of expression. TGFα and HB-EGF transcripts were specifically detected in the epithelium of ducts and TEBs and concentrated near the leading cap cell layer, though HB-EGF transcripts were also found in stromal fat (Fig. 4A). In contrast, EGF transcripts were not detected in either pubescent or mature virgin glands, but were readily localized to alveolar epithelium in the day 2 lactating gland (not shown). This matches our previous RT-PCR finding that EGF expression was uniquely induced during lactogenesis while expression of the other ligands declined (Schroeder and Lee, 1998). Finally, receptor transcripts were predominantly localized to ducts and TEBs of juvenile glands, particularly in the outermost cell layer of TEBs (Fig. 4A). In agreement with our previous immunostaining results (Schroeder and Lee, 1998), EGFR expression was also readily observed in adipocytes (arrowhead) and periductal stroma. Receptor expression was similarly localized in 10-week-old wild-type and triple null glands. In the lactating gland, EGFR transcripts were prevalent in alveolar epithelial cells, but were only weakly detected in fat (data not shown).

Lobuloalveolar development is abnormal in female mice lacking AR, EGF, and TGFα

Pregnancy-induced mammopoiesis and lactogenesis are

characterized by further ductal branching, lobuloalveolar proliferation and differentiation, and milk production (reviewed by Hennighausen and Robinson, 1998). To assess alterations at this stage, whole mounts were prepared from thoracic glands of 5- to 6-month-old uniparous females at one day postpartum. Wild-type and EGF/TGFα double null glands completed lobuloalveolar development, filling the entire fat pad (Fig. 5A,B). In contrast, single, double and triple null glands lacking AR displayed only partial penetration of the fat pad (Fig. 5C-E), consistent with the ductal outgrowth defect in young virgins. These restricted regions exhibited increased epithelial density, indicating lobuloalveolar growth had occurred within the limits of the stunted ductal trees. However, TEBs, atypical of this stage, often persisted at the periphery of these areas (Fig. 5C-E; arrowheads). By comparison, lactating glands from age-matched *wa-2* females (Fig. 5F) were fully traversed by ducts, but had sparser epithelium as previously reported (Fowler et al., 1995).

Although AR-deficient glands appeared grossly capable of lobuloalveolar development, conspicuous abnormalities were evident upon histological evaluation. At day 18 of pregnancy, control glands contained secretory alveoli engorged with fat droplets (Fig. 6A). In contrast, alveoli in AR-deficient glands appeared small, dense, and immature (Fig. 6B-D). Interestingly, there was a progressive decrease in the proportion of lipid-laden cells in single (B), double (C), and triple (D) null specimens, corroborating the notion that additional loss of the other ligands aggravates the defect in lobuloalveolar development. On day 2 of lactation, alveoli in AR-null glands were overcrowded and compressed (Fig. 6E vs 6F,G), consistent with the increased epithelial density observed in the whole mounts (Fig. 5A vs 5C,D). Compared to the distended alveoli of normal lactating glands (Fig. 6E), double (aaee) and triple null specimens (Fig. 6G,H) exhibited irregular, compact alveolar morphology with clusters of disorganized or undifferentiated epithelial cells (arrowhead) interspersed among areas of more synthetic alveoli resembling those of late pregnancy (Fig. 6G,H vs 6A). Finally, while these alterations were more frequent and remarkable in AR-deficient glands with minimal ductal penetration, they were also observed in double and triple null glands containing substantial (> 50%) ductal outgrowth (Fig. 6B,C,F-H). This suggests that the delay or defect in alveolar differentiation was not solely attributable to inferior ductal morphogenesis.

Because of the immature appearance of postpartum knockout glands, we compared expression of milk protein genes by northern blot. Transcripts for whey acidic protein (WAP) and β-casein were present at moderate to high levels in day 2 lactating glands from most genotypes (Fig. 7). However, WAP and to a lesser extent β-casein transcripts were variably decreased in AR-deficient glands, especially those of triple null dams. Not surprisingly, aaeett glands with minimal ductal outgrowth contained negligible levels of milk protein mRNA and could not sustain lactation (Fig. 7, asterisks). Importantly, inguinal glands from other triple null mothers whose pups survived (until day 2) and displayed 50-80% epithelial penetration, nevertheless had depressed milk protein gene expression (particularly WAP). Collectively, these data demonstrate that mammary glands lacking AR are competent in lobuloalveolar proliferation and differentiation, but that additional loss of EGF and/or TGFα compromises lactogenesis.

The morphology of alveoli and their decreased expression of WAP are reminiscent of the phenotype of Stat5a knockout mice (Liu et al., 1997). Normally, activation of Stat5a increases during lobuloalveolar differentiation, whereas activation of Stat3 increases during lobuloalveolar involution (Philp et al., 1996, Liu et al., 1996; Li et al., 1997). Moreover, EGF and AR stimulate phosphorylation of Stats by the EGFR tyrosine kinase (David et al., 1996). We therefore assayed activation of Stats 3 and 5a in day-18-pregnant and day-2-lactating mammary glands by phosphotyrosine blotting of the immunoprecipitated proteins. Although levels of Stat5a protein and activation varied among individual samples, Stat5a phosphorylation was conspicuously decreased in AR-deficient glands relative to controls (Fig. 8). Severe reductions were most prevalent in double and triple null females and correlated with aberrant morphology and impaired lactation. Stat3 phosphorylation was not observed in the same control or knockout glands, though the protein was detected (data not shown). The decrease in Stat5a activation in pregnant AR null glands, coupled with the uniform absence of Stat3 activation

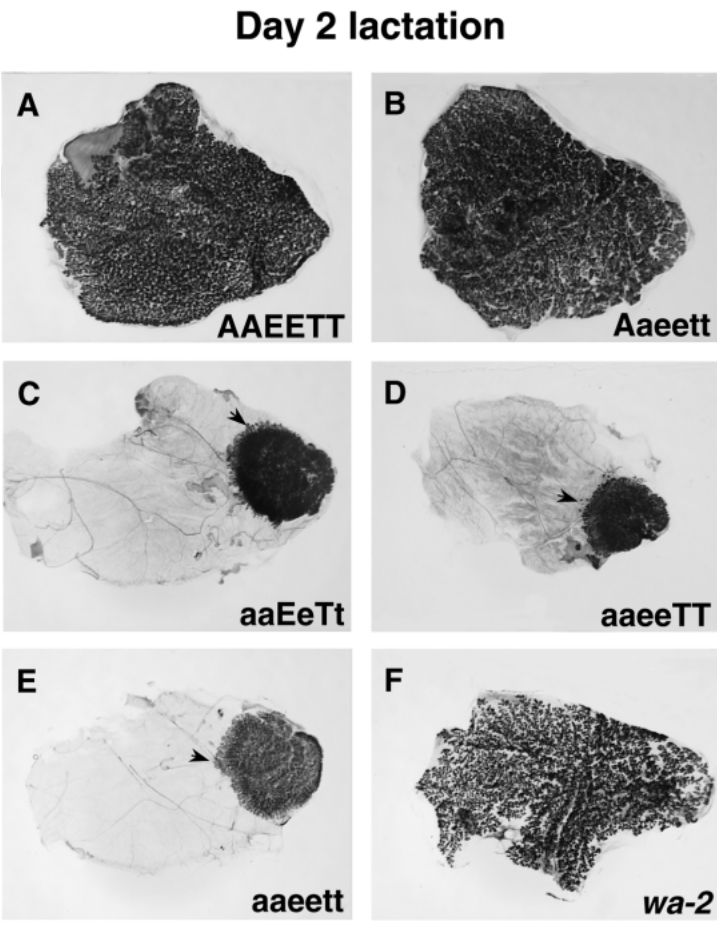


Fig. 5. Abnormal lobuloalveolar development in lactating glands from AR null mice. Whole mounts of thoracic glands were prepared from 5- to 6-month-old uniparous females of the indicated genotypes the day after parturition. Arrowheads mark boundaries of limited epithelial development with persistent TEBs. Note the increased epithelial density in C and D, and decreased epithelial density in F. Magnification: 10×.

in postpartum glands, indicates immature alveolar differentiation rather than premature involution. Finally, these data provide evidence of a physiological relevance for activation of Stats in response to EGF or AR.

Maternal and neonatal growth factors both influence pup survival and growth

Surviving pups born to double or triple null parents were usually growth retarded. This could result from malnutrition due to the mothers' impaired mammary gland function and/or depletion of milk-borne ligands, as well as elimination of growth factors from the pups themselves. To evaluate the contribution of neonatal ligands, we compared the survival and growth of triple hemizygous versus triple null litters nursed by triple null mothers. To generate the requisite litters, we bred 5- to 6-month-old triple null females, since lactation improved with aged mothers. Fig. 9 shows that triple hemizygous pups (AaEeTt) born to triple null mothers displayed reduced survival (86% vs. 95%) and body weight (70% at 1 week; compare 1st and 3rd bars) when compared with wild-type litters born to wild-type mothers. However, pup survival and growth rate declined drastically when the pups also lacked the growth factors. Thus, the survival of triple null pups reared by triple null mothers was 25%, and the average weight of survivors was only 42% of the wild-type value (compare 1st and 5th bars). For all groups, similar weight trends were observed at 2 and 3 weeks of age. We performed a comparable analysis with aaee double null mice (2nd and 4th hatched bars of each weekly set). Again, survival and growth rate of double hemizygous pups (AaEeTT) born to aaee double null mothers were moderately reduced in comparison to wild-type controls, but were remarkably worse in aaee double null pups. Further corroboration of the importance of neonatal growth factors was obtained from preliminary crossfostering experiments (not shown). Adopted triple null pups nursed by wild-type foster mothers were 15-20% growth-retarded relative to their wild-type littermates. Collectively, these results suggest that EGFR ligands produced both maternally and neonatally collaborate to ensure the health and growth of offspring. Removal of both sources of growth factors, or growth factor responsiveness (i.e., the EGFR knockout) severely compromises perinatal development.

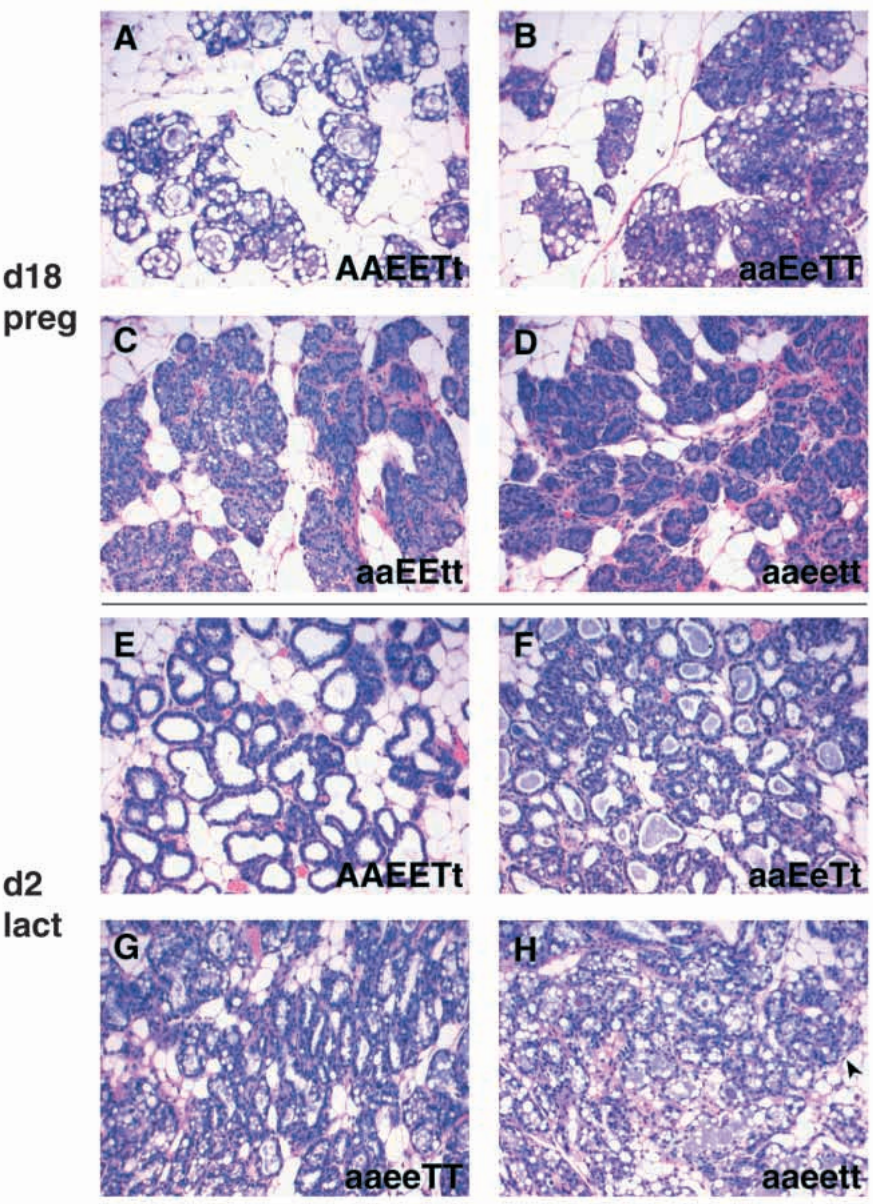


Fig. 6. Morphology of pregnant and lactating mammary glands. Thoracic glands from 5- to 6-month-old uniparous females of the indicated genotypes were removed the day before or after parturition, fixed in formalin, sectioned, and stained with hematoxylin and eosin. Gross estimations of epithelial penetration of the fat pads were (A) 100%, (B) 66%, (C) 50%, (D) 25%, (E) 100%, (F) 50%, (G) 75%, (H) 66%. For lactation specimens, pup survival in the respective litters at the time of sacrifice was (E) 5/5, (F) 4/4, (G) 3/7, (H) 5/5. Note the decreasing accumulation of lipid in alveoli from A to D and the compact, immature alveolar morphology in F-H. Magnification: 100×.

DISCUSSION

EGF family growth factors are hypothesized to participate in multiple developmental, physiological and pathological processes, based on endogenous expression and exogenous effects in culture systems or animal models. Consistent with a broad range of ligand activities, ablation of the EGF receptor gene in mice causes embryonic to perinatal lethality from pleiotropic abnormalities (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Surviving neonates suffer from hair and eye defects, respiratory distress, growth retardation, and progressive wasting due to aberrant proliferation and/or differentiation in epithelia, particularly the skin, lung and gastrointestinal tract. In light of this severe phenotype, the viability, fertility, and longevity of our triple null mice (aaeett),

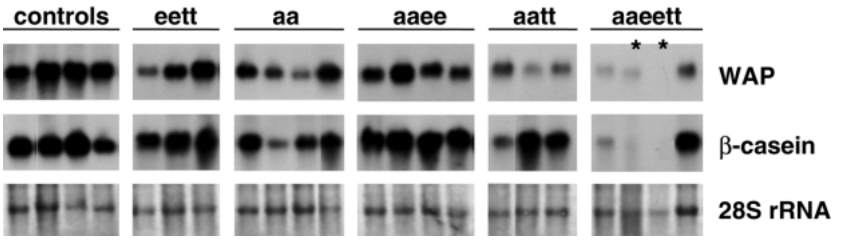


Fig. 7. Expression of milk protein genes in control and targeted mice. At day 2 of lactation, inguinal mammary glands were harvested from uniparous females of the indicated genotypes, and total RNAs prepared as described in Materials and Methods. Samples (10 μg) were analyzed by northern blot for WAP and β-casein expression using cloned cDNA probes. Staining of 28S rRNA was used to verify equivalent loading and integrity of RNA. TGFα, EGF or AR genotypes not listed are either wild type or heterozygous for the targeted allele.

lacking half of the known ligand family, is surprising. It also contrasts with the drastic consequences of mutations in individual ligands for the *Drosophila* EGFR (Schweitzer and Shilo, 1997), suggesting that the mammalian EGF family has evolved a high degree of functional redundancy or compensation. In some contexts, this premise is supported by overlapping spatial and temporal patterns of expression. Additionally, the natural actions of these growth factors may be cooperative or cumulative, as evidenced by the aggravation of ligand-specific problems in the eyes, skin and mammary glands of our combinatorial mutants. Furthermore, the potential for EGF family members to activate other erbB receptors may be relevant to the comparison of phenotypes in receptor versus ligand knockout mice. The targeted agonists (AR, EGF, TGFα) only bind EGFR, but induce its heterodimerization with erbB2 and erbB3 in vitro (Beerli and Hynes, 1996). The remaining EGFR ligands, betacellulin, HB-EGF, and epiregulin, also bind erbB4 (Riese et al., 1996; Elenius, et al., 1997; Riese and Stern, 1998). These intricate receptor interactions could modify, amplify, or diversify signaling pathways in vivo, but their physiological significance has yet to be established.

Our results provide important genetic proof of a need for EGFR signaling in two stages of mammary gland development. Furthermore, comparison of our knockouts clearly establishes that AR is the critical ligand in this tissue. The unique requirement for AR in ductal morphogenesis could be due to its intense expression in ducts and TEBs. Additionally, the potency of AR may differ since it has a lower affinity for the EGFR than EGF or TGFα (Shoyab et al., 1989). Furthermore, AR binds heparin, and heparan sulfate proteoglycans modulate its mitogenic action (Shuger et al., 1996). This potential regulation of AR availability or activity by extracellular matrix proteins may be relevant to epithelial-stromal interactions crucial for ductal outgrowth. Differential proteolytic processing of the AR precursor could also control its distribution or presentation to receptors. Finally, AR may evoke distinctive signaling outputs by favoring association of select erbB receptor dimers. On the other hand, implantation of pellets containing AR, EGF, or TGFα initiates ductal outgrowth in virgin glands, and TGFα appears to be the most potent agonist (Snedeker et al., 1991; Jones et al., 1996; Kenney et al., 1996). However, important distinctions in the physiological actions of EGFR ligands may be masked at pharmacological doses. Whether the function of these growth factors in the mammary gland arise from differences in intrinsic expression or bioactivity is being addressed by knock-in substitution.

It is intriguing that respective roles for TGFα and AR in hair follicle and mammary duct morphogenesis similarly involve penetration of epidermis-derived structures through underlying adipose tissue, processes dependent on epithelial-mesenchymal interactions (Hardy, 1992; Cunha and Hom, 1996). Skin graft (Hansen et al., 1997) and mammary gland transplant experiments (Sebastian et al., 1998; Wiesen et al., 1999) with EGFR null tissues showed that the receptor is required in epithelial cells for hair follicle function, but in stromal cells for ductal outgrowth. In both contexts, the ligands (TGFα and AR) are expressed in adjacent epithelial cells suggesting paracrine (or juxtacrine) receptor activation. Interestingly, ablation of neither growth factor significantly

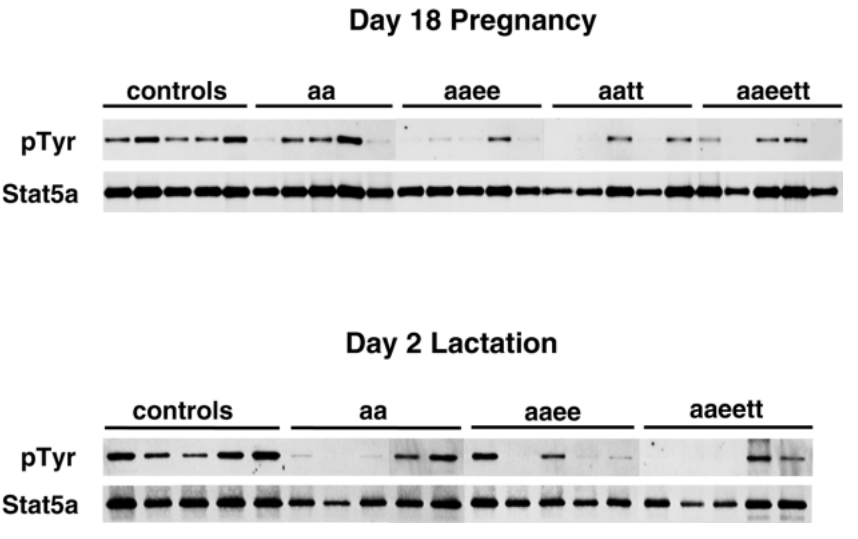


Fig. 8. Activation of Stat5a in mammary glands of control and AR null mice. At day 18 of pregnancy or day 2 of lactation, inguinal mammary glands were harvested from uniparous females of the indicated genotypes and immediately homogenized in cold lysis buffer containing protease and phosphatase inhibitors. Aliquots (1 mg) of total protein were immunoprecipitated with Stat5a antibody, resolved on 10% SDS-PAGE, and immunoblotted with phosphotyrosine antibody RC20. Blots were stripped and reprobed with the Stat5a antibody. Exposure times following chemiluminescent detection were 1 hour for p-Tyr and 20 seconds for Stat5a.

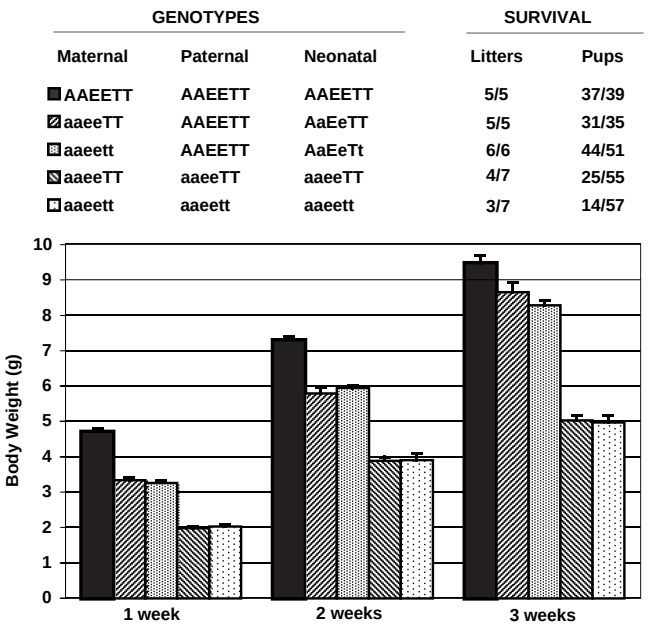


Fig. 9. Influence of maternal and neonatal growth factor deficiency on pup survival and growth. Wild-type, double-null, and triple null partners at 5 to 6 months old were mated to generate control or null neonates as indicated in the accompanying table. Offspring were weighed at weekly intervals until weaning. Bars show mean body weight of surviving pups of each neonatal genotype (from each type of cross) with standard error of the mean shown. Solid bars, pups of wild-type (AAEETT) mothers; hatched bars, pups of double-null (aaeeTT) mothers; stippled bars, pups of triple-null (aaeett) mothers. Note that the last two bars of each weekly set reveal the greatly reduced (by 50-60%) body weight of double and triple null pups born to double and triple null mothers, respectively.

affects the proliferation of the cells that produce or respond to them. Rather, the nature of the AR and TGFα null phenotypes suggests abnormal epithelial cell migration or adhesion. While the mechanism by which these ligands mediate these processes are not understood, candidate signaling pathways include integrins and their downstream targets (e.g., focal adhesion kinase and other non-receptor tyrosine kinases). Interactions between integrins and basement membrane or matrix proteins influence proliferation, adhesion, and differentiation of mammary epithelial cells (Streuli and Edwards, 1998) and α6β4 and laminin are implicated in branching morphogenesis in culture (Stahl et al., 1997). Since EGFR ligands enhance phosphorylation or expression of these and other integrin subunits and thereby stimulate motogenesis (Mainiero et al., 1996), this could be the mode of AR action. Finally, AR may also regulate the synthesis or secretion of matrix proteases necessary for ductal invasion of the stroma (Rudolph-Owen and Matrisian, 1998; Kondapaka et al., 1997).

Although the impediment in ductal morphogenesis was the most consistent and distinct mammary phenotype, lobuloalveolar development was also frequently impaired in AR-deficient glands, especially in uniparous double or triple null females. This could reflect a tissue architectural constraint resulting from inferior ductal outgrowth. However, markers of differentiation and milk production declined more when EGF and TGFα were also missing, even in glands with ample ductal penetration. Thus, further loss of these ligands compounds the lactation problem. This finding is compatible with the normal expression of EGF and TGFα and activation of EGFR during pregnancy and lactation (Schroeder and Lee, 1998). The precise nature of the EGFR-dependent signal(s) involved in mammopoiesis and lactogenesis is unknown. Mice homozygous null for the Stat5a gene, or heterozygous for a mutant prolactin receptor gene, also failed to lactate following their first pregnancy due to defective lobuloalveolar development (Liu et al., 1997; Ormandy et al., 1997). Since both EGFR ligands and prolactin induce Stat5a phosphorylation (David et al, 1996), deficiency in the latter could account for the similar mammary gland defects. Lobuloalveolar development during pregnancy was also impaired in cyclin D1 knockout mice (Fantl et al., 1995; Sicinski et al., 1995), but this phenotype appears to result from an earlier block in epithelial proliferation rather than differentiation. The mammary gland phenotype of AR null mice most closely resembles that of mice lacking the βB subunit of activins and inhibins, with respect to retarded ductal elongation, persistent TEBs, deficient alveolar differentiation, and insufficient lactation (Robinson and Hennighausen, 1997). Thus, proper development requires a balance or interplay between both positive and negative regulators produced by epithelial and stromal cells. Finally, the amelioration of mammary gland function with increasing age and parity is a phenomenon also described in the prolactin receptor heterozygotes (Ormandy et al., 1997) and Stat5a knockouts (Liu et al., 1998). These observations underscore the adaptive plasticity of the mammary gland. Perhaps the sensitivity of this dynamic tissue to multiple growth factors and hormones, many of which are locally expressed, allows the emergence of auxillary signaling pathways.

Our studies also demonstrate the importance of both maternal and neonatal sources of these growth factors for optimal pup survival and growth. The growth retardation in offspring of triple null mothers likely results, in part, from inadequate quantity or quality of milk, secondary to defective mammary gland development. However, the remarkable reduction in survival and body weight when the pups themselves were also triple null (Fig. 9), coupled with the runting and wasting of receptor knockout pups (Sibilia and Wagner, 1995; Threadgill et al., 1995; Miettinen et al., 1995), supports roles for both endogenous and milk-borne growth factors in perinatal development. The abundance of EGF in mouse milk (Beardmore and Richards, 1983; Brown et al., 1989) has fostered the hypothesis that EGF exerts cytoprotective or proliferative influences in the gastrointestinal tract of suckling young (Playford and Wright, 1996). Multiple EGFR ligands are also expressed in several gastrointestinal epithelia, albeit at different sites and levels (Barnard et al., 1995), and may contribute to gut development. Although the health and growth of EGF single null pups born to EGF single null mothers casts doubt on some conventional roles ascribed to this factor, its absence clearly aggravates the lactation problem in AR null mothers, and worsens survival and growth rate in their pups. We propose that the poorer prognosis for double (aaee) or triple null pups born to double or triple null dams reflects a synergism of disadvantages resulting from elimination of multiple ligands from multiple sources. Thus, these neonates would not only be undernourished due to a low milk supply, but also deprived of maternal and internal growth

factors that may functionally cooperate or compensate for each other. The availability and viability of the individual and combinatorial ligand knockouts will facilitate future efforts to test this notion.

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