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Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin

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Summary

Epithelial-mesenchymal crosstalk is essential for tissue morphogenesis, but incompletely understood. Postnatal mammary gland development requires epidermal growth factor receptor (EGFR) and its ligand amphiregulin (AREG), which generally must be cleaved from its transmembrane form in order to function. As the transmembrane metalloproteinase ADAM17 can process AREG in culture and *Adam17*^{-/-} mice tend to phenocopy *Egfr*^{-/-} mice, we examined the role of each of these molecules in mammary development. Tissue recombination and transplantation studies revealed that EGFR phosphorylation and ductal development occur only when ADAM17 and AREG are expressed on mammary epithelial cells, whereas EGFR is required stromally, and that local AREG administration can rescue *Adam17*^{-/-} transplants. Several EGFR agonists also stimulated *Adam17*^{-/-} mammary organoid growth in culture, but only AREG was expressed abundantly in the developing ductal system in vivo. Thus, ADAM17 plays a crucial role in mammary morphogenesis by releasing AREG from mammary epithelial cells, thereby eliciting paracrine activation of stromal EGFR and reciprocal responses that regulate mammary epithelial development.

Keywords

Mammary gland; Branching morphogenesis; Metalloproteinase; ADAMs; TNF α converting enzyme; ERBB; Stromal-epithelial interactions; Epidermal growth factor receptor; Mouse

Introduction

Branching morphogenesis is a fundamental process of organogenesis. The mammary gland, unlike other branched tissues, undergoes most of its morphogenesis during adolescent development (Wiseman and Werb, 2002). In mice, the rudimentary ductal tree that forms during late embryogenesis undergoes just enough growth to keep pace with normal body growth until puberty, at which point robust branching morphogenesis begins. Bulbous terminal end buds (TEBs) form at the tips of the ducts and penetrate the mammary fat pad, new primary ducts form by bifurcation of the TEBs, and secondary side-branches sprout laterally from the

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Supplementary material

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trailing ducts until the entire fat pad is filled by an extensive system of branched ducts by 8–10 weeks of age. The mature ductal tree, in turn, forms the foundation for future phases of mammary development and function. Thus, alveolar structures develop along the entire ductal tree during pregnancy, produce milk throughout lactation, and regress during post-weaning mammary involution, leaving the ductal tree intact and ready for further rounds of lobuloalveolar expansion and differentiation.

Epidermal growth factor receptor (EGFR/ERBB1) is a transmembrane tyrosine kinase that is activated upon binding EGF, transforming growth factor α (TGF α), amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPIR) or epigen (EPGN), each of which is expressed as a transmembrane precursor that is proteolytically shed from the cell surface (Harris et al., 2003). Once occupied, EGFR dimerizes with another EGFR monomer or one of three related receptors, ERBB2, ERBB3 or ERBB4. Notably, mammary development is impaired in waved 2 mutant mice that harbor a kinase-impaired EGFR (Fowler et al., 1995; Sebastian et al., 1998) and in transgenic mice that express a mammary-targeted, dominant-negative EGFR (Xie et al., 1997). *Egfr* mRNA and protein are abundant in mammary stroma (Luetke et al., 1999; Schroeder and Lee, 1998). Indeed, EGF induces EGFR phosphorylation in gland-free fat pads (Sebastian et al., 1998) and significantly more ¹²⁵I-EGF binds to stromal cells surrounding TEBs than to any other area of the developing gland (Coleman et al., 1988). Notably, *Egfr*^{-/-} glands show impaired ductal outgrowth when grown under the renal capsules of host mice, and when wild-type or *Egfr*^{-/-} ducts are surgically recombined with fat pads of the same or opposite genotype, the ducts grow regardless of genotype if the stroma contains EGFR, but not if it lacks EGFR. This indicates that stromal rather than epithelial EGFR is essential for ductal development (Wiesen et al., 1999). Nevertheless, *Egfr*^{-/-} transplants do undergo alveolar differentiation in response to prolactin from nearby pituitary isografts, suggesting that EGFR is essential for ductal, but not alveolar, development.

The importance of EGFR also means that one or more of its ligands must influence mammary development. At least six EGFR agonists are expressed during mammary development, but only AREG is strongly upregulated at puberty and dramatically downregulated during and after pregnancy (D’Cruz et al., 2002; Schroeder and Lee, 1998), a pattern consistent with the importance of EGFR in post-pubertal mammary development. Indeed, ductal outgrowth is severely impaired in triple-null mice lacking AREG, EGF and TGF α , which are lactation incompetent, and variably impaired in mice lacking only AREG (Luetke et al., 1999). As neither ductal outgrowth nor lactation is affected by elimination of EGF, TGF α , HB-EGF or BTC alone or in various combinations (Jackson et al., 2003; Luetke et al., 1999), AREG must be uniquely required for this process.

Like all EGFR ligands, AREG is expressed as a transmembrane precursor that is generally cleaved and released to activate its receptor. Extensive data indicate that various members of the ADAM (a disintegrin and metalloproteinase) family of cell surface enzymes, including ADAM17 (TNF α -converting enzyme or TACE), are responsible for the release of EGFR ligands, including AREG, in vitro (Hinkle et al., 2004; Sahin et al., 2004; Sunnarborg et al., 2002) and even cell contact-dependent juxtacrine activation of EGFR may require ADAM17-mediated processing of EGFR agonists (Borrell-Pages et al., 2003). However, no genetic evidence supporting a role for ADAM17 as a physiological AREG sheddase has yet been provided. By contrast, *Adam17*^{-/-} mice (Peschon et al., 1998a; Shi et al., 2003) display the altered eyelid, hair and whisker development of TGF α -deficient mice (Luetke et al., 1993; Mann et al., 1993), the aberrant heart valve development of HB-EGF-null and uncleavable HB-EGF knock-in mice (Iwamoto et al., 2003; Jackson et al., 2003; Yamazaki et al., 2003), and the broad epithelial defects and perinatal lethality of *Egfr*^{-/-} mice (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Moreover, studies using single-, triple- and

quadruple-gene knockout mice lacking ADAM9, ADAM12, ADAM15 and/or ADAM17 show that only ADAM17 is responsible for the eyelid and heart phenotypes and is thus required for efficient processing of TGF α and HB-EGF in these tissues (Sahin et al., 2004).

Although the above data suggest that ADAM17 processes EGFR ligands in certain embryonic situations, its role in mediating paracrine crosstalk between differing cell-types postnatally remains unexplored. In this study, we use tissue recombination methods to show that EGFR is indeed required in the stroma of the developing mammary gland, whereas the requirement for AREG to form a competent ductal tree rather than an inadequate bush resides in the epithelium, and that ADAM17, which can process AREG, is also required in the epithelium in a paracrine pathway that is essential for normal branching morphogenesis.

Materials and methods

Mammary transplantation

Adam17^{-/-} and *Areg*^{-/-} mice were generated on a mixed 129/C57Bl-6 genetic background and *Egfr*^{-/-} mice were generated from heterozygous breeding pairs on an out-bred 129SV/J \times Swiss Black-CD1 background. Unless otherwise indicated, timed pregnant females were sacrificed 18 days after the appearance of a vaginal plug (gestational day E18.5) and the uteri and embryos removed under sterile conditions. Genotypes were determined from tail DNA using published PCR protocols (Luetteke et al., 1999; Shi et al., 2003; Wiesen et al., 1999) and by the presence of open eyelids in *Adam17*^{-/-} and *Egfr*^{-/-} embryos. Intact female nude mice were from Charles River.

Recombined mammary transplants were prepared as previously described (Wiesen et al., 1999). The rudimentary ductal tree was microdissected from the abdominal 4 fat pad, trimmed of excess stroma and placed onto a gland-free embryonic or neonatal fat pad. The recombined epithelium and stroma were allowed to adhere to one another by overnight culture on solidified agar plates containing 0.5% Bacto agar (Difco), 10% fetal bovine serum, 100 U/ml penicillin G and 100 μ g/ml streptomycin in DME-H16 medium enriched with 6 mM L-glutamine. Recombined and non-recombined glands were placed under the renal capsules of nude mice with intact ovaries and allowed to grow for 2–6 weeks with or without subcutaneous 1.7 mg 60-day slow-release 17 β -estradiol pellets or adjacent 10 μ g 21-day AREG micropellets (Innovative Research of America). Embryonic mammary glands were also transplanted to surgically cleared host fat pads as described elsewhere (Wiesen et al., 1999). Morphometry was performed on digital images of carmine-stained mammary whole mounts using FoveaPro3. All experiments were performed in accordance with protocols approved by the UCSF and UNC Committees on Animal Research.

Mammary organoid culture

Embryonic and neonatal mammary organoids were prepared in a similar manner to that described for adult organoids (Simian et al., 2001). Rudimentary ductal trees were microdissected from the surrounding stroma, pooled and swirled at 100 rpm for 30 minutes at 37°C in DMEM/F12 medium containing 0.2% collagenase A (Sigma), 0.2% trypsin (Life Technologies), 5% fetal bovine serum, 5 μ g/ml insulin and 50 μ g/ml gentamicin. The resulting suspension was spun at 300 g for 5 minutes and the pellets gently agitated at room temperature for 2 minutes in DMEM/F12 with 40 U/ml DNase I (Sigma). Cell clusters and dissociated single cells were pelleted at 300 g, washed with DMEM/F12, re-centrifuged and gently resuspended in ice-cold growth factor-reduced Matrigel (Becton Dickinson). The suspended organoids were transferred to 48-well plates over a thin cell-free layer of Matrigel and allowed to gel at 37°C. Following gel formation, 300 μ l of basal medium (5 mg/ml insulin, 5 mg/ml transferrin, 5 μ g/ml selenium, 100 U/ml penicillin G and 100 μ g/ml streptomycin in DMEM/

F12) was added with or without growth factors, and the organoids cultured in a humidified 5% CO₂ incubator at 37°C. Growth was observed over 7 days in the presence or absence of 5 nM murine TNF α or human AREG, HB-EGF or NRG1 β 1 (R&D Systems), or 5 nM rat TGF α or human EGF, FGF1, FGF2, FGF7 or FGF10 (Sigma).

Expression profiling

TEB, duct and distal stroma regions of mammary glands 2–5 were independently microdissected from anesthetized 5-week-old β -actin-GFP reporter mice (Jackson Laboratory) using a Leica MZFLIII fluorescence microscope. RNA was extracted with Trizol Reagent (Tel-Test), reverse transcribed in the presence of amino-allyl-dUTP, coupled to CyScribe dyes (Amersham), and the unamplified Cy5-labeled TEB or duct cDNAs and Cy3-labeled stromal cDNAs were hybridized to 70-mer oligonucleotide microarrays with 19,500 features (Operon, mouse version 2.0), as described elsewhere (Barczak et al., 2003). Differential expression values in the text were obtained by converting the lowest normalized, log₂-transformed gene expression ratios $M = \log_2(\text{Cy5}/\text{Cy3})$ and average overall signal intensities $A = 0.5[\log_2(\text{Cy5}) + \log_2(\text{Cy3})]$ to linear values for each of six TEB versus distal stroma and six duct versus distal stroma arrays.

Protein analysis

Tissues were extracted on ice in four volumes (w/v) of 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100 buffer containing protease and phosphatase inhibitors (2 mM EDTA, 2 mM EGTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 50 μ M sodium molybdate, 2.5 mM sodium pyrophosphate and 1 mM β -glycerophosphate) by Polytron homogenization and centrifugation. Supernatant proteins were resolved by SDS-PAGE and transferred to PVDF membranes for western blots or immunoprecipitated with rabbit anti-mouse EGFR (Upstate, 1 μ g/125 μ g of protein) and protein A agarose in Tris-buffered saline containing 0.5% NP-40 and protease and phosphatase inhibitors. Immunoblotting was performed using mouse anti-phosphotyrosine (4G10, Upstate, 1:1000), rabbit anti-phosphoEGFR (Y1068, Cell Signaling, 1:1000), rabbit anti-mouse EGFR (Upstate, 1:1000), rabbit anti-mouse keratin 14 (Covance, 1:20,000), goat anti-actin (Santa Cruz, 1:2000), and HRP-conjugated donkey anti-rabbit, mouse and goat IgG secondary antibodies (Amersham, 1:2000) followed by enhanced chemiluminescence autoradiography.

Statistical analysis

Mean values are provided with standard deviations and were compared by unpaired, two-tailed *t*-tests. Array-based statistics were adjusted for multiple comparisons using the Benjamini-Hochberg method of controlling for the false discovery rate (Benjamini and Hochberg, 1995).

Results

Epithelial AREG is required for mammary development

Our prior data show that AREG is required for normal mammary morphogenesis (Luetteke et al., 1999). However, several EGFR ligands can rescue ductal development in ovariectomized and estrogen receptor α (ER α)-deficient mice (Coleman et al., 1988; Kenney et al., 2003; Kenney et al., 1996; Snedeker et al., 1991); so why are they unable to compensate for the absence of AREG in vivo? To address this, we harvested TEBs together with their immediate surrounding stroma and ducts with their own surrounding stroma from 5-week-old mice and compared their respective gene expression profiles with those of distal, epithelium-free stroma using high-density oligonucleotide arrays. Twelve arrays run on pooled samples from two mice each revealed strong AREG expression that was 13.0 ± 3.9 -fold higher in and around TEBs and

10.2±4.3-fold higher in and around ducts than in distal, epithelium-free stroma (Hochberg's $P < 0.0001$ and 0.005 , respectively) (Fig. 1). AREG was also the only EGFR ligand with a mean overall signal intensity from the TEB plus distal stroma (or duct plus distal stroma) compartments that was well above the threshold needed to distinguish true signal from noise, suggesting that other EGFR ligands may be absent or weakly expressed during ductal development (see Tables S1 and S2, and Fig. S1 in supplementary material). The only other ERBB ligand on the array that was highly expressed was the ERBB3/ERBB4 ligand neuregulin 4 (NRG4), which was more highly expressed in the stroma than in TEBs or ducts ($P < 0.0005$), though its own possible role in ductal development remains unclear. Because our method reveals relative expression in the epithelial plus adjacent stromal compartments versus distal, epithelium-free stroma, we cannot rule out the possibility that AREG is weakly expressed in the stroma or induced in the immediate periepithelial stroma. Nevertheless, our results are consistent with in situ hybridization data that show substantial and exclusive expression of AREG in the epithelium of developing ducts and TEBs (D'Cruz et al., 2002; Luetteke et al., 1999), further suggesting that although other EGFR ligands can support ductal growth in culture and in mice, only AREG is expressed at significant levels in epithelial ducts during their outgrowth.

To test whether AREG is required in the epithelium or stroma, we used recombined neonatal mammary transplants and found that wild-type epithelium grew regardless of the stromal genotype, whereas *Areg*^{-/-} epithelium showed little or no growth in either type of stroma (Fig. 2A, B). After three weeks, non-recombined *Areg*^{-/-} glands occupied ~11% of the area of paired wild-type glands (2.0 ± 0.8 versus 18.5 ± 1.9 mm²; $P = 0.0002$), and *Areg*^{-/-} epithelium grown in wild-type fat pads occupied areas that were ~22% of those occupied by wild-type epithelium in *Areg*^{-/-} fat pads (2.5 ± 2.4 versus 11.3 ± 5.3 mm²; $P = 0.004$). Thus, consistent with our array and in situ hybridization data, only epithelial AREG was required. Nevertheless, lobuloalveolar development in response to subcutaneous slow-release estradiol pellets was qualitatively and quantitatively normal regardless of the presence or absence of AREG in any tissue compartment (Fig. 2E).

Stromal EGFR is required for mammary development

Our data show that during mammary development the crucial EGFR ligand AREG comes from the epithelium. However, prior studies suggest that EGFR is enriched in the periepithelial mammary stroma (Coleman et al., 1988; Schroeder and Lee, 1998) and that stromal rather than epithelial EGFR is required for mammary epithelial development in vivo (Wiesen et al., 1999). Moreover, EGFR ligands are epithelial (and stromal) mitogens, yet epithelial EGFR is not needed for mammary epithelial development in vivo. Thus, we revisited the tissue recombination studies that led to this somewhat paradoxical finding, this time in the presence of estradiol pellets in order to also assess the role of EGFR in estrogen-induced alveolar differentiation, and again found that wild-type and *Egfr*^{-/-} epithelium grew in fat pads that contained EGFR, but not in *Egfr*^{-/-} fat pads (Fig. 2C, D). After 3 weeks, non-recombined *Egfr*^{-/-} glands occupied only ~4% of the area occupied by paired wild-type glands (0.5 ± 0.2 versus 11.2 ± 2.9 mm²; $P < 0.0001$), and wild-type epithelium grown in *Egfr*^{-/-} fat pads occupied areas that were ~11% of those occupied by *Egfr*^{-/-} epithelium in wild-type fat pads (1.3 ± 0.7 versus 11.6 ± 5.7 mm²; $P = 0.0002$). Thus, in contrast to AREG, stromal EGFR indeed is required for mammary epithelial development, whereas epithelial EGFR is dispensable. However, like AREG, EGFR was not required for estrogen-induced lobuloalveolar development (Fig. 2E).

Epithelial ADAM17 is required for mammary development

In order for AREG on the surface of mammary epithelial cells to activate EGFR in the stroma, it must be released by a protease, and that protease should be required in the same location as its putative substrate. However, each of the catalytically active ADAMs on our expression

array was highly expressed, and most, including ADAM17, had similar levels of expression in the epithelial and stromal compartments (Fig. 1). The only notable exception was ADAM9, which was ~50% less abundant in TEBs than distal stroma, a pattern that would not be expected to foster the release of epithelial AREG. Interestingly, tissue inhibitor of metalloproteinases 3 (TIMP3) and TIMP1 were inversely regulated in TEBs but not ducts. Specifically, the expression of TIMP1 was $709 \pm 213\%$ higher in TEBs than distal stroma ($P=0.0001$), whereas TIMP3 was $66 \pm 6\%$ lower in the TEBs than in the stroma ($P=0.0001$) (see Table S2 in the supplementary material). Moreover, the relative (epithelial versus stromal) and absolute (mean overall) fluorescence intensities for TIMP1 were both significantly greater in the TEBs and distal stroma than in the ducts and distal stroma ($P<0.005$), whereas the relative and absolute values for TIMP3 were significantly lower in the TEBs and distal stroma than in the ducts and distal stroma ($P<0.005$), further suggesting that TIMP1 is specifically upregulated in TEBs, while TIMP3 is specifically downregulated (see Fig. S1 in supplementary material). Because TIMP3 is the only known endogenous inhibitor of ADAM17 (Lee et al., 2004), this inverse regulation of TIMP1 and TIMP3 in TEBs would tend to de-constrain ADAM17 and to increase its net proteolytic activity in an area of active ductal invasion and branching, while limiting the activity of other TIMP1-inhibitable ADAMs. In light of this, and in particular the demonstrated ability of ADAM17 to process AREG in culture (Hinkle et al., 2004; Sunnarborg et al., 2002), we evaluated ADAM17 as a possible mediator of AREG shedding during mammary development.

Adam17^{-/-} pups, which exhibit perinatal lethality, had 65–69% fewer mammary branches and 64–68% shorter ductal trees than their wild-type littermates at E18.5 and birth ($P<0.0001$; Fig. 3). Likewise, *Egfr*^{-/-} neonates had 77% fewer branches ($P<0.005$) and 74% shorter ductal trees ($P<0.001$) than their own wild-type littermates, indicating that they too had impaired fetal mammary development (Fig. 3B, C). Two weeks after renal transplantation, *Adam17*^{-/-} glands lacked normal TEBs and had 90% less overall ductal length than contralateral wild-type glands when exogenous estradiol was absent ($P<0.0001$; Fig. 4A, I). *Adam17*^{-/-} transplants to cleared mammary fat pads also underwent little or no growth in the absence of exogenous estradiol, and even after 5 weeks, were still not significantly larger than the rudiments of newborn wild-type mice (Fig. 4E, I). When estradiol was added, the wild-type renal transplants often filled the fat pads by 3 weeks, whereas the *Adam17*^{-/-} epithelium occupied only 20–30% of the area of wild-type transplants at all time points up to six weeks ($P<0.0001$; Fig. 4B, C, J, K). Indeed, the slope of the regression line for growth of the *Adam17*^{-/-} glands in the presence of added estradiol was not significantly different from that of a flat line ($P=0.75$), again indicating that they were not catching up and that other ADAMs are unable to compensate for the absence of ADAM17. *Adam17*^{-/-} epithelium also consistently failed to grow in wild-type stroma in tissue recombination or cleared fat pad experiments, whereas wild-type epithelium grew readily in *Adam17*^{-/-} stroma ($P<0.002$; Fig. 4F, J) and cleared contralateral fat pads ($P=0.0002$; Fig. 4D, E, I). Thus, like AREG, ADAM17 is only required in the epithelium. Moreover, like AREG and EGFR, its absence had no apparent effect on estrogen-induced lobuloalveolar development (data not shown).

Local AREG administration rescues *Adam17*^{-/-} mammary development in vivo

If *Adam17*^{-/-} glands fail to develop due to a lack of proAREG processing, then exogenous slow-release AREG pellets should rescue their development. Indeed, AREG pellets yielded a 2.6 ± 0.4 -fold increase in the epithelial area of paired *Adam17*^{-/-} mammary glands if they were within ~0.75 mm of the epithelium ($P=0.001$), whereas pellets that were ≥ 1.4 mm from the epithelium had no effect on growth when compared with contralateral placebo control pellets (Fig. 4G, H, J). The failure of distant pellets to influence growth probably reflects capture of the AREG by intervening EGFR-expressing stromal cells or heparan sulfate proteoglycans, which would also be abundant between the pellets and epithelium (Schuger et al., 1996).

EGFR ligands induce branching in cultured *Adam17*^{-/-} mammary organoids

If EGFR regulates mammary development downstream of ADAM17 and AREG, then its other ligands should also foster the growth and branching of *Adam17*^{-/-} and *Areg*^{-/-} mammary epithelium in culture. Indeed, when embryonic and neonatal mammary organoids were grown in three-dimensional basement membrane gels, the wild-type, *Adam17*^{-/-} and *Areg*^{-/-} organoids underwent robust growth and branching in the presence of EGF (91±8% of organoids), TGFα (93±4%), HB-EGF (55±26%) and AREG (72±15%). By contrast, no growth was seen when insulin was the sole growth or survival factor provided or when *Egfr*^{-/-} organoids were cultured in the presence of EGFR agonists (Fig. 5). When heparin-acrylic beads saturated with AREG were embedded in Matrigel to mimic the AREG pellets in vivo, 67% of organoids within 400 μm of the pellets displayed definitive growth, whereas those that were more than 1 mm away did not grow (Fisher's exact test $P < 0.001$). This distinction may again reflect sequestration of AREG.

Because ADAM17 can cleave multiple substrates, it may also influence mammary development via other targets. TNFα, the substrate for which ADAM17 was originally named, can stimulate growth and branching of cultured mammary epithelial cells in an EGFR-independent, but metalloproteinase-dependent manner (Lee et al., 2000; Varela et al., 1997). In our study, however, TNFα failed to support organoid growth in six independent experiments (Fig. 5) and its mRNA was undetectable in developing glands (Fig. 1). Moreover, mice that lack TNFα or either of its receptors (which are also shed by ADAM17) (Peschon et al., 1998a) have no overt phenotype (Marino et al., 1997; Pasparakis et al., 1996; Peschon et al., 1998b) and are lactationally competent (J. Peschon, L. Old and G. Kollias, personal communications). Thus, TNFα is not required for mammary morphogenesis or function.

We also used genetically defined organoids to test for signaling and effector molecules that might act up- or downstream of ADAM17 and EGFR. Thus, factors that support the growth of *Egfr*^{-/-} organoids may act downstream or independently of EGFR, whereas those that stimulate the growth of wild-type, but not *Adam17*^{-/-} organoids may act upstream. Although *Egfr*^{-/-} organoids were refractory to EGFR agonists, 53±16% did grow in response to the ERBB3/ERBB4 ligand neuregulin-1-β1 (NRG1β1), as did wild-type, *Adam17*^{-/-} and *Areg*^{-/-} organoids. Interestingly, the NRG1β1-treated organoids formed large expanding mounds and folds rather than the florid sprouts induced by EGFR ligands (Fig. 5). Fibroblast growth factors FGF2/bFGF and FGF7/KGF also stimulated branching in 97±2% and 85±6% of all organoids, respectively, including *Egfr*^{-/-} organoids, whereas FGF1 and FGF10 yielded weak growth in fewer than 12% of organoids. Interestingly, the FGF2- and FGF7-treated organoids formed hollow branches that were considerably longer than those that formed in response to EGFR ligands and often had solid club-like ends that resembled TEBs. Thus, as FGF2 and FGF7 support the growth of *Egfr*^{-/-} organoids, they may act downstream of EGFR, independently regulate other aspects of ductal morphogenesis or exert compensatory effects when delivered pharmacologically.

Epithelial ADAM17, epithelial AREG and stromal EGFR are required for EGFR phosphorylation in vivo

If ADAM17 is responsible for the release of epithelial AREG and subsequent activation of stromal EGFR, then EGFR activation should only occur if each protein is expressed in the appropriate compartment. Indeed, EGFR phosphorylation was only detected on immunoblots when ADAM17 and AREG were present in the epithelium and EGFR was present in the stroma of recombined transplants (Fig. 6). Autocrine activation was not detected in transplants containing wild-type epithelium in an *Egfr*^{-/-} stroma; however, we cannot rule out the possibility that this reflects the limited glandular development that occurs in the absence of stromal EGFR. These results, as well as the increased presence of the myoepithelial cell marker

keratin 14 in transplants containing stromal EGFR and epithelial ADAM17 and AREG (Fig. 6), mirror our phenotypic observations and thus lend further credence to the conclusion that ADAM17-mediated release of epithelial AREG is required for the activation of stromal EGFR and ductal development.

Discussion

Branching morphogenesis is fundamental to the formation of many complex organs and requires constant two-way communication between developing epithelia and their surrounding stroma (Affolter et al., 2003). Indeed, bidirectional epithelial-mesenchymal crosstalk plays a crucial role in mammary development (Veltmaat et al., 2003; Wiseman and Werb, 2002). Our data support a model in which the EGFR axis is an essential mammary signaling system in which ADAM17 releases epithelial AREG, which then activates stromal EGFR, thus eliciting reciprocal responses that further orchestrate mammary epithelial development (Fig. 7). Prior studies show that ADAM17 can process AREG in culture and suggest that it is a key regulator of EGFR signaling, yet other ADAMs can also process EGFR ligands and genetic evidence that ADAM17 is responsible for the release of EGFR ligands in vivo has been limited to TGF α and HB-EGF (Blobel, 2005). Thus our study provides the first genetic evidence that ADAM17 is an essential physiological sheddase for AREG and is the first to reveal a role for ADAM17, the absence of which is perinatal lethal, in a postnatal developmental process. It is also the first to show that by liberating a ligand that is available only on epithelial cells so that it may interact with its receptor on stromal cells, ADAM17 plays an essential role in the epithelial-stromal crosstalk that drives mammary development. Thus, although juxtacrine activation of EGFR may occur in some situations (Borrell-Pages et al., 2003), the proteolytic release of AREG is absolutely essential in this paracrine setting. Moreover, our results raise new questions and possibilities concerning the cues that act upstream and downstream of this pathway.

How is ADAM17-AREG-EGFR signaling regulated?

Mammary morphogenesis begins in late fetal development, pauses after birth and resumes in response to ovarian hormones at the onset of puberty. Indeed, estrogens are essential and can restore ductal development in ovariectomized mice (Daniel et al., 1987). Moreover, tissue recombination studies show that both epithelial and stromal estrogen receptors are necessary for mammary development (Mueller et al., 2002). Notably, EGFR ligands can rescue ductal development in ER α -deficient mice (Kenney et al., 2003) and exogenous estradiol stimulates EGFR and ERBB2 phosphorylation in ovariectomized mice (Sebastian et al., 1998), suggesting that EGFR acts downstream of ER α and that it may influence mammary development in concert with ERBB2. Thus an initial action of estrogens on stromal cells may produce stromal signals that regulate the ADAM17-mediated release of AREG from epithelial cells, which then elicits EGFR-mediated stromal responses that further influence the developing epithelia.

Clearly, many inputs influence the expression and activity of ADAM17, AREG and EGFR during mammary development. For example, AREG is strongly induced by estrogens (Vendrell et al., 2004) and is the only EGFR ligand that is adequately expressed and enriched in developing mammary epithelium. However, several ADAMs are expressed during mammary development, at least two of which (ADAMs 15 and 17) can process AREG (Schafer et al., 2004). However, only ADAM17 appears to be required, as other ADAMs are unable to compensate for its absence and triple-null mice lacking ADAM9, ADAM12 and ADAM15 are fully able to nurse their pups (C. Blobel, personal communication). Thus, either ADAM17 is the only physiologic AREG sheddase or it must be regulated independently of the other available ADAMs, or both.

Several potential avenues are available for the differential regulation of ADAM17. ADAM17 is active at the cell surface, as the removal of its propeptide domain by furin-like proprotein convertases occurs in the trans-Golgi network (Srour et al., 2003). Notably, our results indicate that the only known natural inhibitor of ADAM17, TIMP3 (Lee et al., 2004), is specifically downregulated in and around invading TEBs. Thus, even though ADAM17 is ubiquitously expressed, local downregulation of its inhibitor would tend to increase its net activity in and around TEBs, thereby augmenting the local release of its only readily available substrate, AREG, and enhancing EGFR activation on nearby cells. However, the upregulation of TIMP1 in TEBs may offset the absence of TIMP3 as far as other metalloproteinases are concerned, while having no direct effect on ADAM17-mediated signaling. G-protein-coupled receptors can induce ADAM17-mediated release of AREG and transactivation of EGFR in culture (Gschwind et al., 2003; Lemjabbar et al., 2003); however, it remains unclear how they do so, whether they regulate ADAM17 in mammary epithelium or which receptor agonists may be physiologically relevant. Phosphorylation of the cytoplasmic domain of ADAM17 appears to regulate processing of some substrates (Diaz-Rodriguez et al., 2002; Fan et al., 2003), whereas the cytoplasmic domain is dispensable for the processing of others (Reddy et al., 2000). Integrin $\alpha_5\beta_1$ may also influence ADAM17 activity (Bax et al., 2004), and Eve-1/Sh3d19, which binds to the cytoplasmic domain of various ADAMs, appears to promote the processing of EGFR ligands, including AREG (Tanaka et al., 2004). Interestingly, our microarray data indicate that Sh3d19 expression mirrors that of ADAM17 in developing mammary gland.

Does EGFR act alone or in concert with other ERBB receptors?

It is unclear whether EGFR forms homodimers or heterodimers with other ERBB receptors during mammary development. One argument favoring the formation of homodimers is that EGFR is enriched in the mammary stroma, whereas ERBB2 is mainly expressed in the epithelium, ERBB3 is not detected until mammary glands mature, and ERBB4 is only expressed during pregnancy and lactation (Schroeder and Lee, 1998). Our data indicate that stromal EGFR regulates mammary development, yet ductal development is also impaired in transgenic mice that express dominant-negative EGFR in the epithelium alone (Xie et al., 1997). Although this could reflect downregulation of ERBB2 signaling, transgenic expression of dominant-negative ERBB2 causes alveolar defects that only become apparent at parturition (Jones and Stern, 1999). Nevertheless, *ErbB2*^{-/-} mammary glands do exhibit delayed ductal penetration and TEB defects when transplanted to cleared fat pads, but eventually catch up and undergo lactational differentiation (Jackson-Fisher et al., 2004). In this case, only epithelial ERBB2 is required, as the host fat pads contain ERBB2 (and EGFR). Indeed, selective ablation of ERBB2 in mammary epithelial cells yields a similar phenotype (Andrechek et al., 2005). Because ERBB2 has no known ligand, it requires a co-receptor; yet ERBB3 and ERBB4 are in short supply during ductal development and our data suggest that epithelial EGFR is expendable. Thus, epithelial EGFR-ERBB2 interactions, though not absolutely essential, may still influence the rate of ductal development, a parameter not specifically addressed in our study. Although our organotypic culture experiments show that the mutant epithelium is competent to grow and branch, the ability of the organoids to respond to EGFR ligands in culture may also reflect the possibility that epithelial EGFR signals contribute to normal ductal development in a non-essential way. Nevertheless, prior studies (Simian et al., 2001) and our examination of the current organoid cultures show that at least 12% of the cells in these organotypic cultures are stromal in origin and may, therefore, also contribute to organoid growth and branching.

EGFR may also interact with ERBB3 or ERBB4, although the latter only appears to affect lobuloalveolar development. Genetically rescued *ErbB4*^{-/-} mice develop alveolar defects during pregnancy and lactation, yet their ductal development often surpasses that of their wild-type siblings (Tidcombe et al., 2003). Likewise, mice that express a mammary-targeted,

dominant-negative ERBB4 or lack the ERBB4 ligand NRG1 α display impaired alveolar differentiation but normal ductal development (Jones et al., 1999a; Li et al., 2002). Although these alveolar effects could also involve EGFR, our data suggest that EGFR is not necessary for alveolar development in response to estradiol or prolactin (Wiesen et al., 1999). However, EGFR ligand-deficient dams do display more compact alveoli than wild-type mothers during true pregnancy and lactation, when other important stimuli, such as placental lactogens, also participate, although this could also reflect crowding as a result of impaired ductal outgrowth (Lueteteke et al., 1999). Thus, some EGFR ligands may affect lactational differentiation. Indeed, EGF is strongly upregulated during late pregnancy and lactation (D'Cruz et al., 2002; Schroeder and Lee, 1998), although any effects it might have could still be independent of EGFR, as ERBB4 has high affinity for EGF in the presence of ERBB2 (Jones et al., 1999b). Clearly, the unique growth pattern we observed in culture in response to NRG1 β 1 did not require EGFR, as it occurred in *Egfr*^{-/-} organoids. However, the implication that ERBB3 or ERBB4 can affect mammary cell growth independently of EGFR does not necessarily mean that EGFR signaling occurs independently of ERBB3 or ERBB4. Thus, it remains unclear whether EGFR and ERBB4 interact during pregnancy and lactation, whereas it is unlikely that they do so during ductal development, as ERBB4 is neither necessary nor expressed at that time.

How do AREG-activated stromal cells regulate mammary epithelial development?

Because mammary epithelial development requires stromal EGFR, reciprocal stromal-to-epithelial responses must also contribute. Other metalloproteinases undoubtedly affect branching downstream of ADAM17, as TIMP1 inhibits branching in culture and in vivo (Fata et al., 1999), even though it does not inhibit ADAM17. Moreover, broad-spectrum metalloproteinase inhibitors block organoid growth in response to EGF and KGF (Simian et al., 2001; Wiseman et al., 2003), yet the absence of ADAM17 alone does not. Notably, AREG administration induces expression of the matrix metalloproteinase (MMP) inducer EMMPRIN, MMP2 (gelatinase A) and MMP9 (gelatinase B) in cultured breast epithelial cells (Menashi et al., 2003). Moreover, the activator of latent MMP2, MMP14 (MT1-MMP), is strongly induced by EGFR activation in neonatal lung and cultured embryonic fibroblasts (Kheradmand et al., 2002), and is present at high levels in the stromal cells adjacent to invading mammary TEBs (Wiseman et al., 2003). Indeed, our data show that MMP2 and MMP3 (stromelysin 1) regulate mammary ductal morphogenesis in vivo (Wiseman et al., 2003) and that MMP14 promotes ductal development by activating MMP2 and degrading type I collagen (M. Egeblad, B. S. Wiseman, M. D. S. and Z. W., unpublished). Nevertheless, the collagen accumulation that characterizes *Mmp14*^{-/-} mammary glands is absent in *Adam17*^{-/-}, *Areg*^{-/-} and *Egfr*^{-/-} glands (M. D. S. and Z. W., unpublished), either because EGFR does not regulate MMP14 during mammary development or because collagen deposition and remodeling are not elicited in the absence of ductal development itself. Moreover, because MMP14 is membrane bound, it can influence epithelial behavior only indirectly, unless it is also shed.

Stromal FGF2 and FGF7/KGF may also act downstream of EGFR and may do so directly, as they support the growth and branching of *Egfr*^{-/-} mammary organoids. Indeed, their receptor, FGFR2B, is expressed on mammary epithelial cells and is required for the initial formation of embryonic mammary placodes, as is FGF10 (Veltmaat et al., 2003). Thus, a full understanding of their role in subsequent processes, such as branching, will require the analysis of conditional deletion models. However, no mammary phenotype has been described in FGF7-deficient mice, possibly owing to compensatory mechanisms. Nevertheless, stromal FGFs and their epithelial receptors have been shown to play critical roles in branching of the tracheal system in *Drosophila* and in mammalian lung, salivary gland and kidney branching, suggesting that similar signaling mechanisms may influence mammary branching as well (Affolter et al., 2003).

The pathway that we have elucidated is undoubtedly part of a larger cascade of signals that pass back and forth between neighboring cells of the developing mammary gland. In addition, similar pathways undoubtedly contribute to other biological processes and may be hijacked or corrupted during the onset and evolution of disease. Indeed, ADAM17, AREG, TGF α and EGFR are often upregulated in human breast cancer, with co-expression of the latter two indicating a worse prognosis (Desruisseau et al., 2004; Lendeckel et al., 2005; Umekita et al., 2000). Moreover, experimental data show that these molecules can actively contribute to the development and progression of cancer (Borrell-Pages et al., 2003; Brandt et al., 2000; Gschwind et al., 2003). Thus, a clearer understanding of the mechanisms that regulate ADAM17-AREG-EGFR signaling under normal circumstances will be crucial to overcoming them when they go awry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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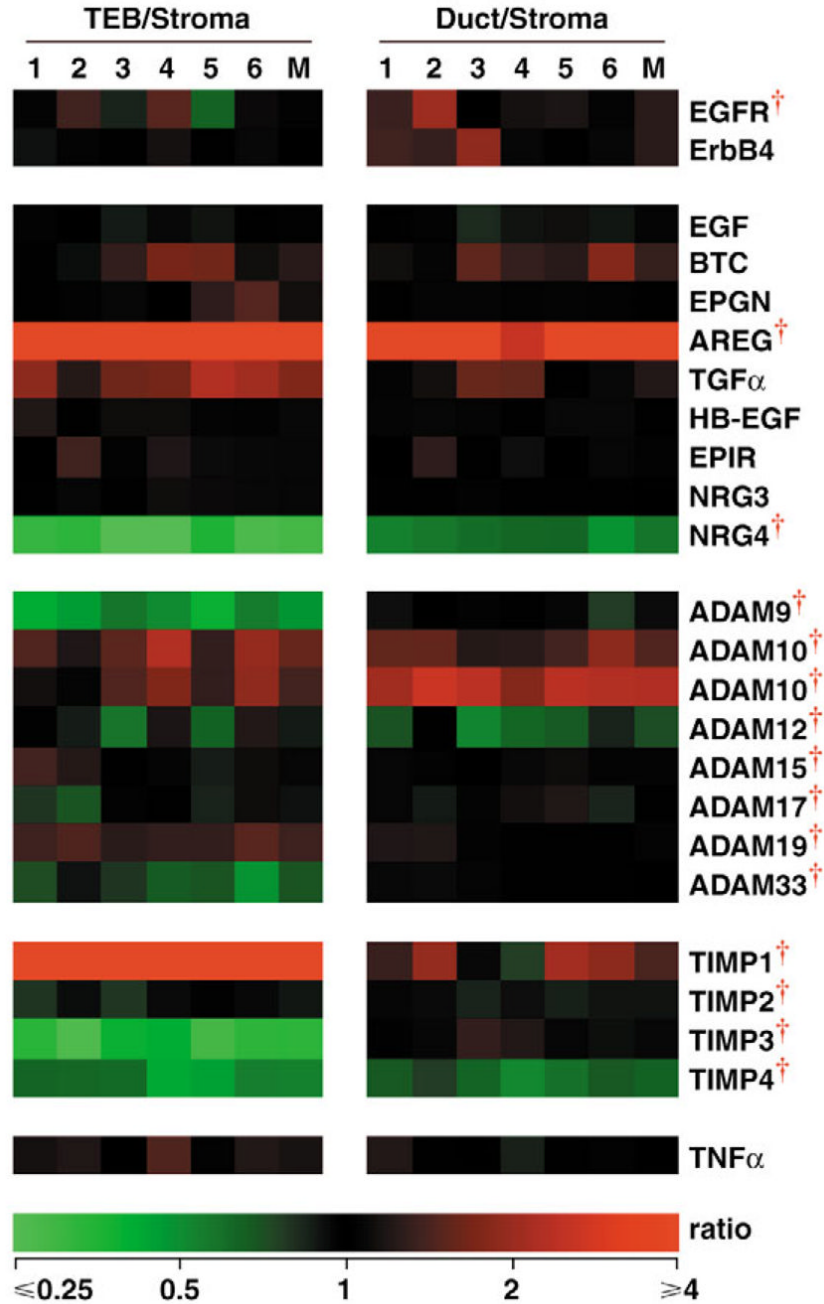


Fig. 1. Relative expression of potential interacting genes in 5-week-old mammary glands. Cy5-labeled cDNAs from microdissected TEB- or duct-containing regions and Cy3-labeled cDNAs from distal stroma were hybridized to long-oligonucleotide microarrays. TEB/stroma (Cy5/Cy3) expression ratios and duct/stroma ratios are each shown for six independent experiments (lanes 1–6) and their respective means (M) using the color scale shown, with black indicating no difference in expression, red indicating relative enrichment within the TEB or duct regions and green representing relative enrichment in the pure distal stroma. Thus, intense red indicates a greater than fourfold enrichment in and around TEBs or ducts. Relative expression levels for ADAM10 are shown for two independent oligonucleotides, whereas all other genes were

represented by a single oligonucleotide. † Genes for which the mean overall fluorescence signal $A=0.5[\log_2(\text{Cy5}) + \log_2(\text{Cy3})]$ was consistently more than eight, the level below which it was difficult distinguish true signal from noise.

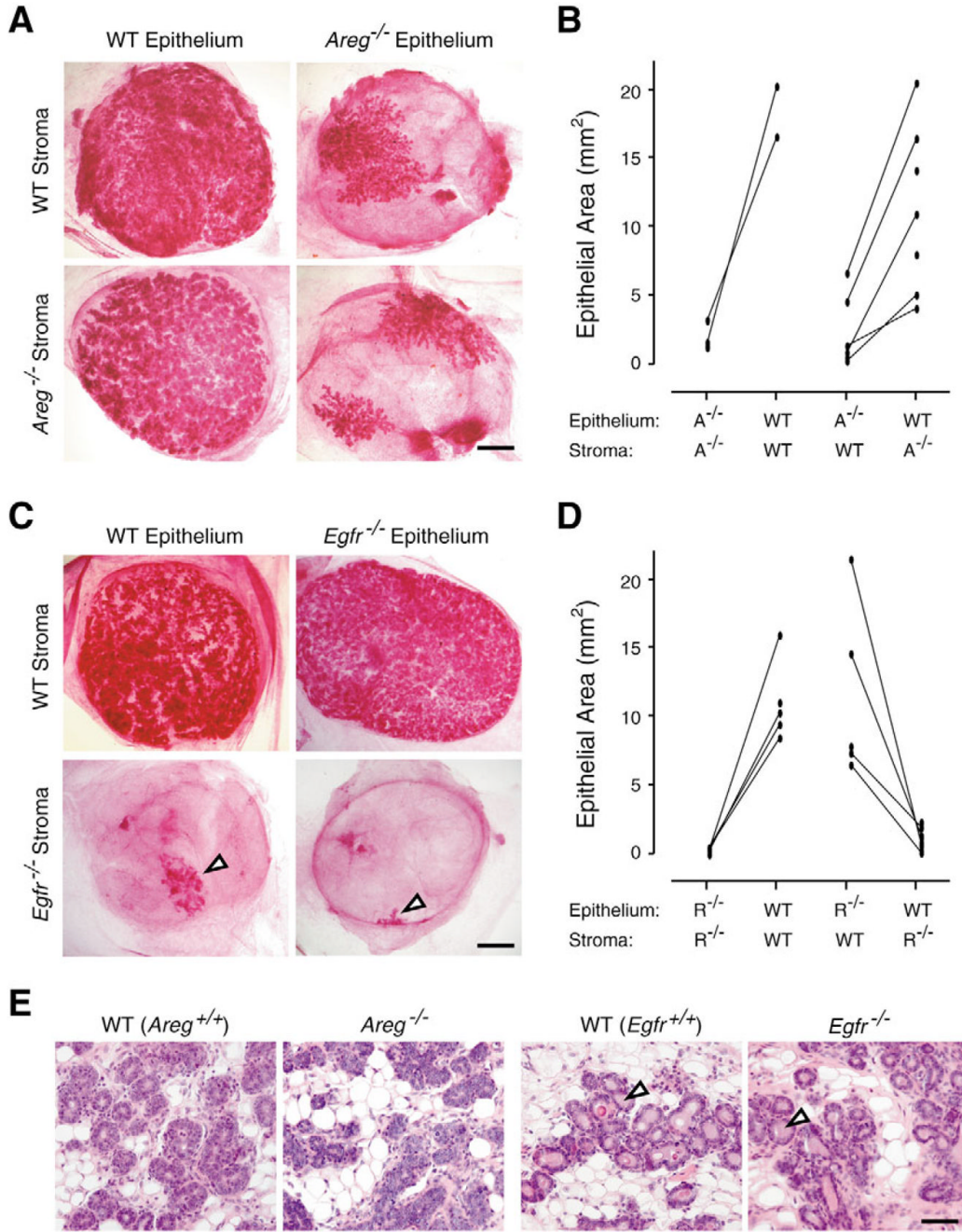


Fig. 2. Effect of AREG and EGFR deficiency on postnatal mammary development. (A) Carmine-stained whole mounts of paired wild-type, *Areg*^{-/-} (upper left and lower right panels) and recombined mammary transplants grown for 3 weeks in the presence of estradiol pellets. Scale bar: 1 mm. (B) Epithelial areas of 3-week-old, estradiol-stimulated transplants with the indicated epithelial and stromal genotypes (*A*^{-/-}, *Areg*^{-/-}; WT, wild type). Lines connect paired contralateral transplants from individual host mice and single data points represent unpaired transplants. (C) Whole mounts of wild-type, *Egfr*^{-/-} and recombined 3-week-old transplants. Scale bar: 1 mm. (D) Epithelial areas of 3-week-old transplants with the indicated epithelial and stromal genotypes (*R*^{-/-}, *Egfr*^{-/-}). (E) Histological appearance of paired transplants.

Similar alveolar morphologies and accumulation of luminal secretory products (arrowheads) were seen in paired transplants from individual hosts, regardless of the transplant genotype. Scale bar: 50 μm .

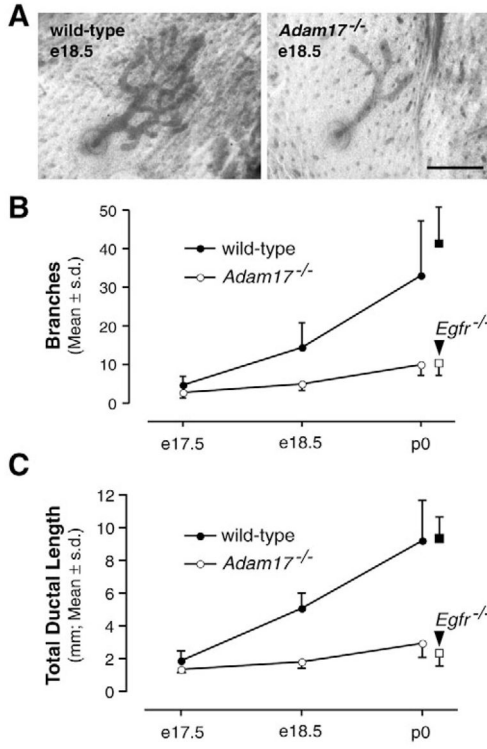


Fig. 3. Effect of ADAM17 deficiency on prenatal mammary development. (A) Carmine-stained E18.5 mammary gland/skin whole mounts. Scale bar: 500 μ m. (B) Average number of branches in the thoracic (2 and 3) mammary glands of *Adam17*^{-/-} embryos and neonates (white circles), *Egfr*^{-/-} neonates (white squares) and their respective wild-type littermates (black symbols). (C) Overall lengths of the thoracic mammary ductal trees of *Adam17*^{-/-} embryos and neonates, *Egfr*^{-/-} neonates and their wild-type littermates.

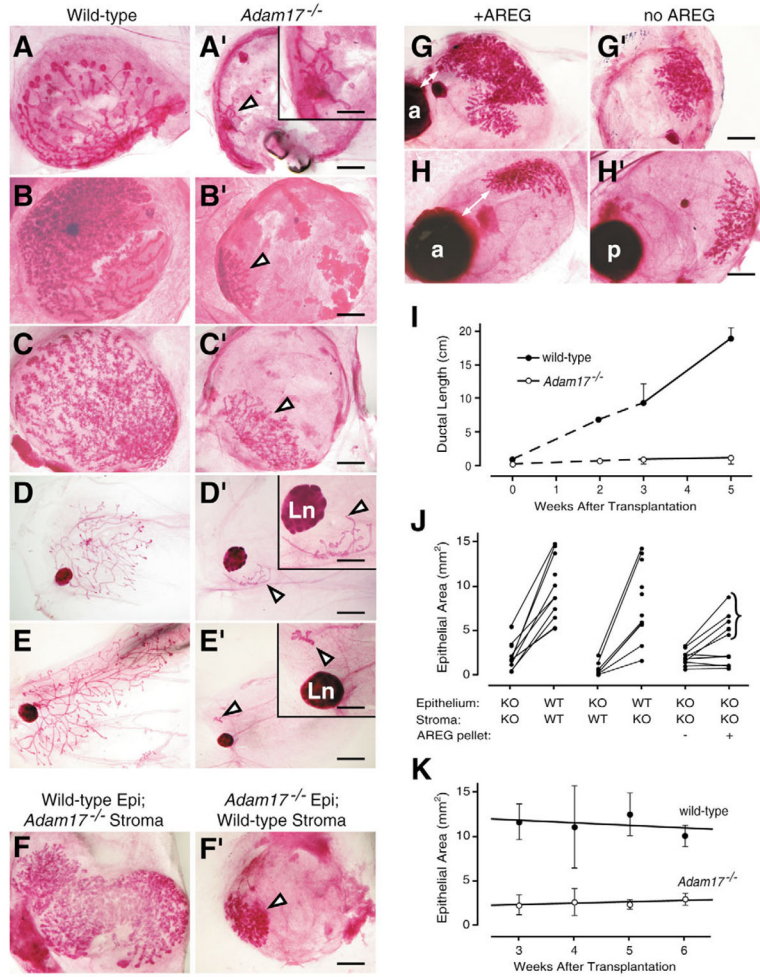


Fig. 4. Effect of ADAM17 deficiency on postnatal mammary development. (A–F) Whole mounts of paired wild-type (A–E), *Adam17*^{-/-} (A'–E') and surgically recombined (F, F') mammary glands grown under contralateral kidney capsules (A–C, F) or in surgically cleared fat pads (D, E) for 2 (A), 3 (B, D, F) or 5 (C, E) weeks with (B, C, F) or without (A, D, E) slow-release estradiol pellets. *Adam17*^{-/-} outgrowths (arrowheads) were consistently smaller than contralateral wild-type outgrowths, whereas the extent of estradiol-induced alveolar differentiation was similar in each respective host (e.g. B versus B'). (G, H) Paired *Adam17*^{-/-} transplants were also grown for 3 weeks in the presence of estradiol and adjacent AREG (a) or placebo (p) pellets. The farthest AREG pellet to yield greater-than-normal growth was ~0.75 mm from the epithelium (double-headed arrow in G), whereas the closest pellet to have no effect on growth as compared to its placebo control was ~1.4 mm from the epithelium (double-headed arrow in H). Scale bar: 500 μm in A'; 2 mm in D, D'; 2.5 mm in E, E'; 1 mm in all other panels and inserts. (I) Overall ductal lengths for newborn wild-type and *Adam17*^{-/-} mammary glands, 2-week-old renal transplants, and 3- and 5-week-old cleared fat pad grafts in the absence of added estradiol. Error bars at birth and 2 weeks are hidden by the mean data points. (J) Epithelial areas of paired (connected) and unpaired renal transplants with the indicated epithelial and stromal genotypes (KO, *Adam17*^{-/-}; WT, wild type). The non-recombined pairs on the left were grown for 3–6 weeks, whereas all other transplants were grown for 3 weeks in the presence of added estradiol. Bracketed data points are those for which AREG pellets were within ~0.75 mm of the

Adam17^{-/-} epithelium. (K) Mean epithelial areas of non-recombined mammary glands at various times after renal transplantation in the presence of E2.

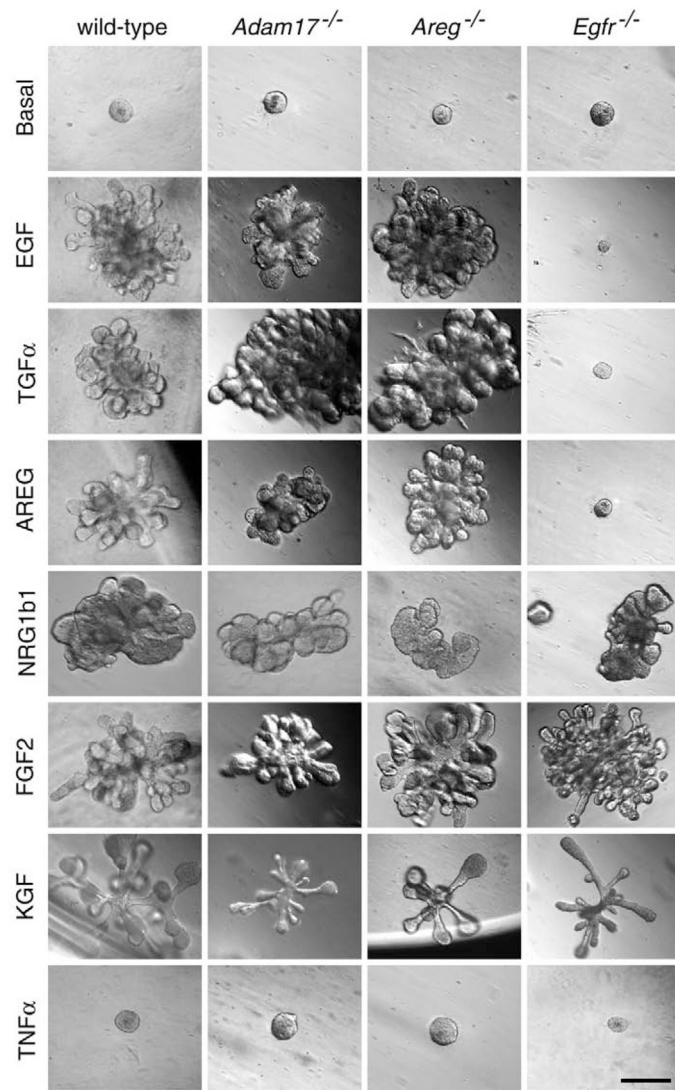


Fig. 5. Effect of growth factors on genetically defined mammary organoids in Matrigel. Hoffman modulation contrast images were taken after 7 days of growth in the presence of the indicated supplements. Scale bar: 400 μ m for KGF-treated organoids; 200 μ m for all other panels.

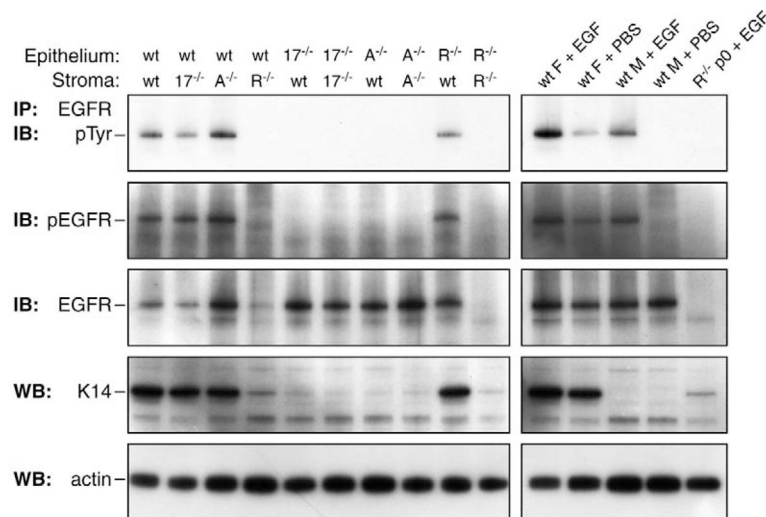


Fig. 6. EGFR phosphorylation in recombined mammary glands. Tissue extracts were immunoprecipitated (IP) with anti-EGFR antibodies, immunoblotted (IB) for phosphotyrosine (pTyr) or phosphorylated EGFR (pEGFR; Y1068), and re-probed for EGFR. Western blots (WB) for keratin 14 were run on the original extracts and re-probed for β -actin. Renal transplants with the indicated epithelial and stromal genotypes (wt, wild type; 17^{-/-}, *Adam17*^{-/-}; A^{-/-}, *Areg*^{-/-}; R^{-/-}, *Egfr*^{-/-}) were harvested 2 weeks after transplantation without exogenous estradiol. Control tissues were harvested from 6-week-old wild-type male (M) and female (F) mice, and *Egfr*^{-/-} neonates (R^{-/-} p0) 15 minutes after intraperitoneal injection of EGF (2.5 mg/kg body weight) or phosphate-buffered saline (PBS).

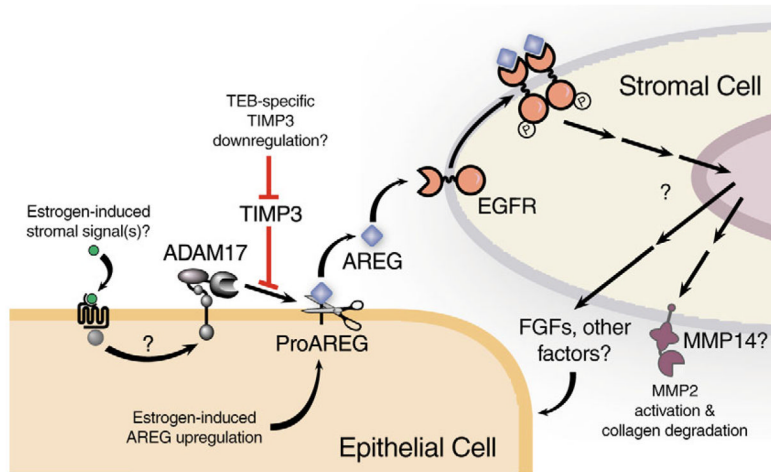


Fig. 7. Model depicting epithelial-mesenchymal crosstalk and potential modifiers of ADAM17-AREG-EGFR signaling in mammary development.