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The ADAM17–amphiregulin–EGFR Axis in Mammary Development and Cancer

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Abstract In order to fulfill its function of producing and delivering sufficient milk to newborn mammalian offspring, the mammary gland first has to form an extensive ductal network. As in all phases of mammary development, hormonal cues elicit local intra- and inter-cellular signaling cascades that regulate ductal growth and differentiation. Among other things, ductal development requires the epidermal growth factor receptor (EGFR), its ligand amphiregulin (AREG), and the transmembrane metalloproteinase ADAM17, which can cleave and release AREG from the cell surface so that it may interact with its receptor. Tissue recombination and transplantation studies demonstrate that EGFR phosphorylation and ductal development proceed only when ADAM17 and AREG are expressed on mammary epithelial cells and EGFR is present on stromal cells, and that local administration of soluble AREG can rescue the development of ADAM17-deficient transplants. Thus proper mammary morphogenesis requires the ADAM17-mediated release of AREG from ductal epithelial cells, the subsequent activation of EGFR on stromal cells, and EGFR-dependent stromal responses that in return elicit a new set of epithelial responses, all culminating in the formation of a fully functional ductal tree. This, however, raises new issues concerning what may act upstream, downstream or in parallel with the ADAM17–AREG–EGFR axis, how it may become hijacked or corrupted during the onset and evolution of cancer, and how such ill effects may be confronted.

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Abbreviations

ADAM	a disintegrin and metalloproteinase
AREG	amphiregulin
BTC	betacellulin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENMPRIN	extracellular matrix metalloproteinase inducer
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GH	growth hormone
GPCR	G-protein coupled receptor
HB-EGF	heparin-binding EGF-like growth factor
IGF	insulin-like growth factor
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
NRG	neuregulin
P13	phosphoinositide 3
PR	progesterone receptor
TACE	tumor necrosis factor alpha converting enzyme
TEB	terminal end bud
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases

Introduction

The mammary gland accomplishes its unique task of producing and delivering adequate amounts of milk from mother to newborn in part by forming an extensive tree-like network of branched ducts [1, 2]. In general, the formation

of any branched organ entails (1) the initial specification, formation and in-growth of its so-called *anlage*, (2) the initiation and outgrowth of its first rudimentary branches, (3) its spatial organization through iterative branching and remodeling events, (4) the formation of a continuous lumen, and (5) functional differentiation of its proximal and terminal elements [3]. While some of the mechanisms that underlie these processes appear to be conserved among diverse branched organs from insect trachea to the lungs and kidneys of higher organisms, many others are specific and essential to the unique form and function of each individual organ [4].

The mammary gland, unlike other branched organs, undergoes most of its branching during adolescence rather than embryonic development. Still, mammary development begins in utero with the formation of bilateral epidermal ridges, or 'milk lines', along which disk-shaped placodes form at the site of each future nipple. The placodes then invaginate to form bulb-shaped *anlagen* that penetrate their underlying mesenchyme and enter a cluster of preadipocytes that later becomes the mammary fat pad [5]. Thereafter, a small number of branches sprout from each *anlage* such that a rudimentary epithelial tree is present within the much larger fat pad at birth. This rudimentary tree grows at the same rate as other adjacent body parts until puberty, at which point robust hormone-dependent branching begins. Following puberty, bulbous terminal end buds (TEBs) form at the tips of the ducts, invade into the fat pad as the ducts elongate, and then regress once they reach the margins of the mammary fat pad [6]. New primary ducts also form by TEB bifurcation, and secondary side-branches sprout laterally from the trailing ducts until the entire fat pad of the young adult female is filled by an extensive system of branched ducts [2]. Thereafter, short tertiary branches form along the ducts in response to cyclic ovarian stimulation, resulting in a mature yet 'open' ductal tree, its open architecture leaving space for milk-producing alveoli to form during pregnancy and lactation like leaves on otherwise bare branches [7].

Each phase of mammary branching—embryonic, adolescent and adult—is uniquely regulated. Adolescent branching, for instance, requires estrogen and estrogen receptor α (ERα), adult tertiary side-branching requires progesterone and its receptor (PR), and embryonic branching is hormone-independent, since it proceeds in mice regardless of whether or not they express ERα, ERβ, PR, growth hormone (GH) receptors or prolactin receptor [8, 9]. In response to these hormonal influences, branching is coordinated by local communication between the developing epithelia and nearby stromal cells. Indeed, the importance of this local communication and, in particular, the instructive nature of the mesenchyme now seems undeniable. For instance, embryonic mammary epithelium forms salivary rather than mammary-like structures if it is recombined with salivary gland mesenchyme and then engrafted underneath the renal capsule of a host mouse, although the epithelium still retains its ability to produce milk proteins in response to prolactin [10]. Moreover, when prospective pituitary epithelium from e8.5 Rathke's pouch is transplanted together with salivary mesenchyme, it forms salivary-like structures that in this instance contain differentiated α-amylase-producing acini [11]. Likewise, epithelium from e13 mouse skin forms milk-producing mammary ducts and alveoli if it is grown in vivo within mammary mesenchyme [12]. Thus mesenchymal cues can influence both the branching architecture *and* the functional differentiation of the epithelium, regardless of its origin. Indeed, even non-mammalian chick and duck epidermis, which normally forms skin and feathers, instead forms branched mammary glandular tissue if it is grown within rabbit mammary mesenchyme; a finding that not only highlights the instructive importance of the mesenchyme, but one that also gives new meaning to the term 'chicken breast' [13].

Like embryonic mesenchyme, adult stroma can also be developmentally instructive. For instance, transplant studies show that when mammary epithelial tissues from a mouse strain that normally has highly branched glands are recombined with the adult fat pads of a strain that tends to have sparse branching, or *vice versa*, the branching patterns of the recombined glands tend to reflect the genetic background of the mammary stroma in which they were grown rather than that of the donor epithelium *or* that of the immune-compromised host [14]. Thus stromal rather than epithelial or systemic factors dictate the side-branching patterns that characterize different strains of mice. And, as is true in mice, stromal factors are also apt to influence human breast development. Indeed, human breast epithelial cells will only form functional glandular structures if they are transplanted to 'humanized' mouse mammary fat pads that contain human breast fibroblasts [15] or if they are transplanted within collagen matrix gels that also contain normal human or mouse mammary fibroblasts [16]. Thus, stromal cell- and matrix-derived signals appear to play a critical role in mammary ductal development, which raises obvious questions concerning what stromal factors are involved and how they influence the developing epithelium.

Stromal EGFR is Required for Mammary Development

The epidermal growth factor receptor (EGFR/ErbB1) is a transmembrane tyrosine kinase that elicits its intracellular and downstream signaling effects upon binding one of seven possible ligands: epidermal growth factor (EGF), transforming growth factor-α (TGFα), amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin or epigen, each of

which is initially expressed as a transmembrane precursor that is proteolytically shed from the cell surface [17]. Once occupied, EGFR dimerizes with another EGFR monomer or with one of three related receptors—ErbB2, ErbB3 or ErbB4—in order to exert its downstream effects.

An early suggestion that an unidentified EGF receptor might be involved in mammary development dates back to the late 1960s when it was shown that the recently isolated growth factor EGF supported mammary epithelial growth in mouse mammary explants that contained both epithelial and stromal constituents [18]. Similar observations were later extended to whole mammary gland organ cultures and isolated mammary TEBs [19, 20]. And since then, numerous studies have shown that, despite the implications associated with their name, EGFR ligands as a class are mitogenic for both epithelial *and* stromal cells alike [17].

Direct evidence of EGFR involvement was not obtained, however, until after the identification of EGFR itself. Notably, in vivo analyses revealed that mammary development was impaired in *waved-2* mice that harbored a mutant (kinase-deficient) EGFR on all cells [21, 22] and similarly impaired in transgenic mice that expressed a dominant-negative EGFR on their mammary epithelial cells alone [23], suggesting that epithelial EGFR played a key role. However, EGFR is also abundant within the mammary stroma [24, 25]. Indeed, exogenous EGF has been shown to induce EGFR phosphorylation in gland-free fat pads without any epithelium whatsoever [22]. In addition, the mammary stroma appears to be relatively enriched for EGFR by immunohistochemistry [24] and significantly more ^{125}I-EGF has been shown to bind to the stromal cells immediately surrounding developing TEBs than to any other part of the developing gland [26]. Most notably, however, whole mammary glands from EGFR-null mice revealed impaired ductal outgrowth when they were grown under the renal capsules of immuno-compromised host mice [27]. Moreover, when wild-type or EGFR-null epithelial ducts were surgically recombined with fat pads of either genotype and then transplanted, the ducts grew regardless of their own genotype if the stroma contained EGFR, but not if the stroma lacked EGFR, thus indicating that stromal rather than epithelial EGFR was essential for ductal development. Nevertheless, the under-developed EGFR-null transplants still formed alveoli in response to prolactin from adjacent pituitary isografts, suggesting that EGFR was essential for ductal but not alveolar development. Similar recombination studies were later performed in the presence of slow-release estradiol pellets in order to also assess the role of EGFR in estrogen-induced alveolar differentiation, and once again, wild-type and EGFR-null epithelium grew in fat pads that contained EGFR, but not in EGFR-null fat pads [28]. And, as was true for pituitary prolactin, EGFR was not required for estrogen-induced alveolar expansion. Thus stromal EGFR is required for mammary ductal development but not for alveolar development, and epithelial EGFR is unnecessary for either process (Fig. 1).

Epithelial AREG is Essential for Mammary Development

Clearly, the importance of EGFR in mammary development implies that one or more of its ligands must also be involved. Although at least six EGFR ligands are expressed at one stage or another during mammary development, only AREG is strongly upregulated at puberty and then dramatically repressed during and after pregnancy; kinetics that are consistent with the importance of EGFR in ductal rather than alveolar development [24, 29]. And indeed, instead of forming a competent ductal tree that fills the entire mammary fat pad, AREG-deficient mice form what could be called an inadequate bush—a small mammary shrub of insufficient size to nourish pups [25]. Moreover, AREG is the *only* EGFR agonist that is absolutely required, since ductal outgrowth was only impaired in AREG-null mice, but not in mice lacking either EGF, TGFα, HB-EGF or BTC alone or in various combinations [25, 30].

Although the above genetic evidence indicates that AREG is uniquely required for normal mammary morphogenesis [25], other studies had shown that several EGFR ligands could rescue ductal development in ovariectomized and ERα-deficient mice [26, 31–33] and that all EGFR ligands had comparable effects in culture [17, 28]. So why were other EGFR agonists unable to compensate for the absence of AREG in vivo? Notably, array-based expression profiling of manually microdissected mammary glands has revealed that, of the seven EGFR agonists, AREG is the only one that can be readily detected during post-pubertal mammary development and its transcripts are substantially and significantly enriched in the developing TEBs and ducts as compared to the epithelium-free stroma [28]. Otherwise, the only other ErbB ligand that was readily detected in the post-pubertal mammary gland was the ErbB3/ErbB4 ligand neuregulin-4, which was more highly expressed in the stroma than in TEBs or ducts. Although these array data leave open the possibility that AREG is weakly expressed in the stroma or locally induced in the immediate peri-epithelial stroma, in situ hybridization data specifically confirm that AREG is substantially and *exclusively* expressed in the epithelium of the developing ducts and TEBs [25, 29]. Thus although other EGFR ligands *can* support ductal growth in culture and in vivo if provided exogenously, only AREG is expressed at significant levels in epithelial ducts during their actual outgrowth.

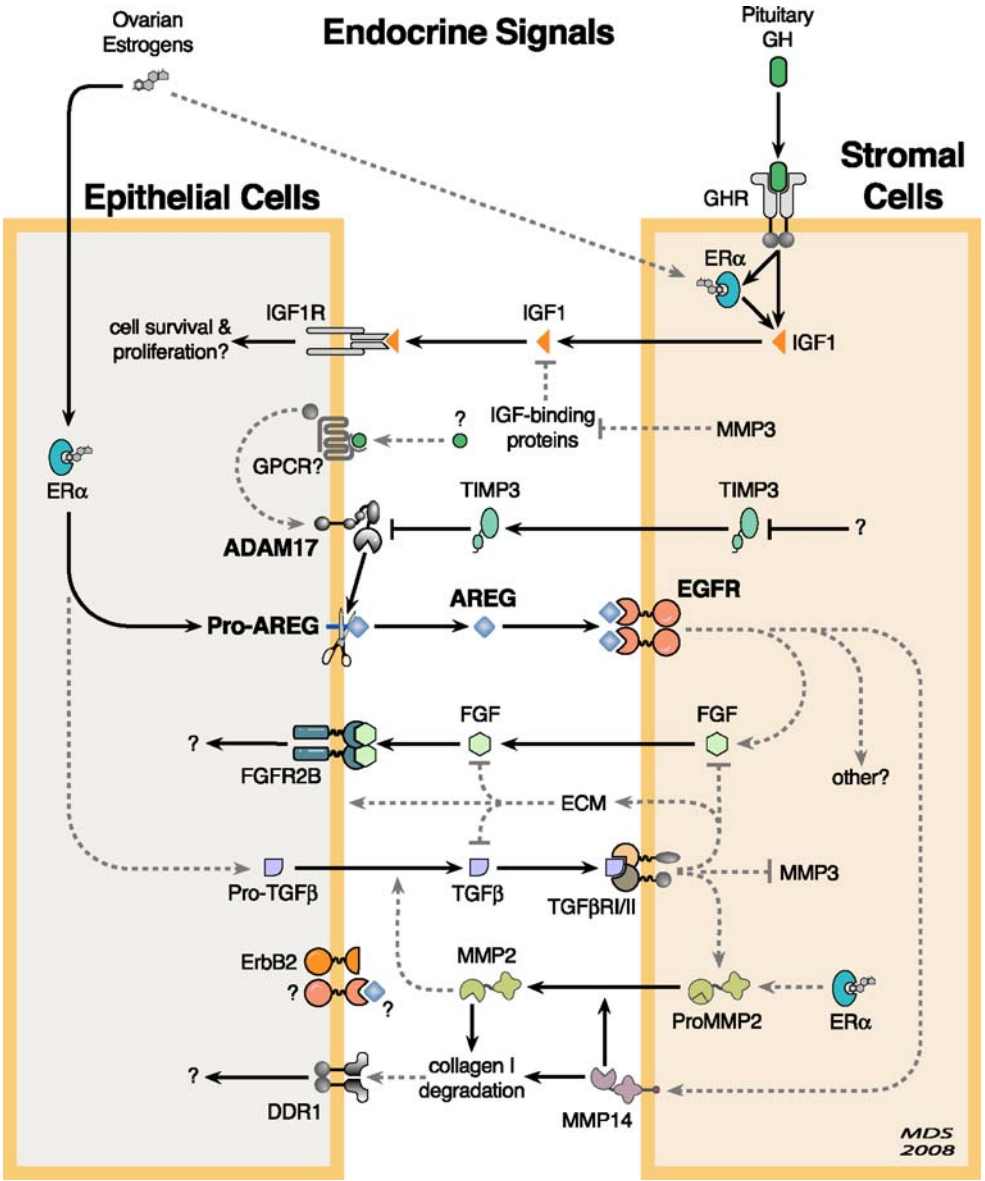

Figure 1 A working model depicting some of the critical endocrine and paracrine pathways that are thought to lie upstream and downstream of the ADAM17–EGFR axis in mammary ductal development. *Solid lines* indicate direct interactions that are supported by multiple lines of evidence, whereas *dashed lines* indicate putative and/or indirect interactions that contain unidentified signaling intermediates.

Since AREG is uniquely required for ductal development and lactational competence in whole animals and since it is only expressed in the mammary epithelial compartment, it was not surprising that when wild-type and AREG-deficient mammary tissues were recombined and transplanted under the kidney capsules of host mice, the wild-type epithelium grew regardless of the stromal genotype, whereas the null epithelium failed to grow in either type of stroma [28]. Thus, consistent with array and in situ hybridization data, only epithelial AREG was required for ductal development. And, like EGFR, AREG was not required for alveolar development in response to estradiol pellets, since alveolar formation was qualitatively and quantitatively normal regardless of the presence or absence of AREG in any tissue compartment. Thus AREG and EGFR both appear to regulate the size of the shrub rather than its ability to grow leaves—i.e., ductal rather than alveolar development.

The above findings therefore indicate that EGFR is required for mammary branching, but only within the stroma, whereas its essential ligand, AREG, is exclusively expressed and solely required in the epithelium [22, 25, 26, 28]. In other words, AREG is only expressed on mammary epithelial cells, yet it has to bind and activate EGFR on nearby—but physically separate—stromal cells. Moreover, like all EGFR ligands, AREG is expressed as a transmembrane precursor. So for AREG to activate adjacent stromal cells, it has to be shed from the epithelial cell surface; which raises the obvious question as to what enzyme or enzymes are responsible for its release.

Epithelial ADAM17 is Required for Mammary Development

Extensive data show that various members of the ADAM (a disintegrin and metalloproteinase) family of zinc-dependent cell surface enzymes that includes ADAM17 (TNFα converting enzyme, TACE), are responsible for the release of all EGFR ligands, including AREG, in culture [34–37]. Moreover, even juxtacrine activation of EGFR via direct cell-to-cell contact may require the ADAM17-mediated processing of EGFR agonists [38], although such contact-dependent activation is almost certainly irrelevant during mammary development, since epithelial and stromal cells are generally separated by a basement membrane.

Notably, ADAM17-null mice [39, 40] display the same aberrant eyelid, hair and whisker phenotypes as TGFα-null mice [41, 42], the same altered cardiac valve development as HB-EGF knockout and cleavage-resistant HB-EGF knockin mice [30, 43, 44], and the diverse epithelial defects and perinatal lethality of EGFR-deficient mice [45–47]. Moreover, studies using single-, triple- and quadruple-gene knockout mice lacking ADAMs 9, 12, 15 and/or 17 indicate that only ADAM17 is responsible for the open eyelid and cardiac valve phenotypes of EGFR, TGFα and HB-EGF deficient mice [36]. Thus ADAM17 alone is required for the efficient processing of TGFα and HB-EGF in these embryonic settings. Nevertheless, even though ADAM17 had been shown to process AREG in culture and even though it was clearly a key regulator of EGFR signaling in some developmental circumstances, it was still possible that other ADAMs (or other proteinases altogether) might play a role in the activation of EGFR during mammary development.

For epithelial AREG to activate EGFR within the mammary stroma, not only would AREG have to be released by a proteinase, but that enzyme would also need to be present in the same location as its substrate—i.e., within the epithelial compartment. Expression profiling, however, revealed that *all* catalytically active ADAMs were highly expressed in the developing mammary gland, and that most, including ADAM17, were expressed at similar levels in the epithelial and stromal compartments (the only notable exception being ADAM9, which was enriched in the stroma where it would not be expected to promote the release of epithelial AREG) [28]. Interestingly, array profiling also revealed that tissue inhibitor of metalloproteinases 1 (TIMP1) was substantially enriched in TEBs, whereas TIMP3 was strongly enriched in the stromal compartment. Moreover, TIMP1 transcript levels were significantly higher in TEBs than in ducts, whereas TIMP3 levels were significantly lower in TEBs than in ducts, further suggesting that TIMP1 is specifically upregulated in actively developing TEBs, while TIMP3 is specifically downregulated. Because TIMP3 is the sole endogenous inhibitor of ADAM17 [48], this inverse regulation of TIMP1 and TIMP3 in TEBs would tend to de-constrain ADAM17 and increase its net proteolytic activity in an area of active ductal invasion and branching, while at the same time limiting the activity of other TIMP1-inhibitable ADAMs. Thus, given its ability to process AREG in culture and its documented importance in other EGFR-dependent processes, ADAM17 seemed the most likely candidate to be responsible for AREG-mediated EGFR activation in mammary development.

Like EGFR-null neonates, ADAM17-deficient pups were found to have fewer mammary branches and shorter ductal trees than their wild-type littermates shortly before and after birth [28]. Moreover, when ADAM17-null glands were transplanted underneath the kidney capsules of host mice, they lacked normal TEBs and were ~90% smaller than contralaterally transplanted wild-type glands after 2 weeks without exogenous estradiol. Likewise, when ADAM17-null mammary glands were transplanted to surgically cleared (gland-free) host mammary fat pads, they displayed little or no growth in the absence of exogenous estradiol, and even after 5 weeks, they were still no larger than the rudiments of newborn wild-type mice. When estradiol pellets were added, the wild-type transplants tended to fill their own fat pads by 3 weeks, whereas the ADAM17-null epithelium occupied only 20–30% of the area of wild-type transplants at all time points up to 6 weeks. Indeed, the overall growth curve for the ADAM17-null glands was essentially a flat line, indicating that the null glands were not catching up to their wild-type counterparts over time. This, of course, meant that other enzymes were unable to compensate for the absence of ADAM17 in vivo. Moreover, ADAM17-null epithelium consistently failed to grow in wild-type stroma in tissue recombination and cleared fat pad experiments, whereas wild-type epithelium grew normally in ADAM17-null stroma and cleared contralateral fat pads. Thus, like AREG, ADAM17 was only required in the epithelium. This, of course, makes sense, since one would expect that a cell surface enzyme and its cell surface substrate would be required in the same place. Furthermore, like AREG and EGFR, the absence of ADAM17 had no apparent effect on estrogen-induced lobuloalveolar development. Thus ADAM17-, AREG- and EGFR-null mammary glands all tended to phenocopy one another in their developmental responses, except that EGFR was required in the stromal rather than epithelial compartment.

It was also reasoned that if ADAM17-null glands failed to develop due to a lack of proAREG processing, then exogenous slow-release AREG pellets should rescue their development. And indeed, AREG pellets provided substantial and significant increases in the epithelial areas of ADAM17-null transplants as compared to paired contralat-

eral transplants harboring placebo pellets, but only if the AREG-releasing pellets were within ~0.75 mm of the epithelium [28]. This failure of the more distant pellets to rescue the ADAM17-null transplants was probably because the exogenous AREG had become sequestered before it ever reached the critical peri-epithelial stroma; captured by EGFR-positive stromal cells and/or heparan sulfate proteoglycans that would naturally be abundant between the pellets and the epithelium [49].

In addition, if ADAM17 were responsible for the release of epithelial AREG and subsequent activation of stromal EGFR, then EGFR activation should only occur if each protein was expressed in its appropriate compartment. And 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 [28]. Thus, as was true for development, epithelial ADAM17, epithelial AREG and stromal EGFR were required for EGFR phosphorylation in vivo, thus lending further credence to the notion that ADAM17-mediated release of epithelial AREG is required for the activation of stromal EGFR and ductal development.

It was also reasoned that if EGFR regulates mammary development downstream of ADAM17 and AREG, then any EGFR ligand should be able to foster the growth and branching of ADAM17-null and AREG-null mammary epithelium in culture. Indeed, when embryonic and neonatal mammary organoids were grown in three-dimensional culture, wild-type, ADAM17-null and AREG-null organoids underwent considerable growth and branching in the presence of either EGF, TGFα, HB-EGF or AREG, whereas absolutely no growth was observed when EGFR-null organoids were cultured in the presence of any EGFR agonist or when insulin was the only growth or survival factor present [28]. In addition, when AREG-saturated heparin-acrylic beads were embedded in the 3-D matrix to mimic the presence of the AREG pellets in vivo, ~2/3 of the organoids within 400 μm of the pellets grew, whereas all organoids that were >1 mm away failed to grow; again probably due to sequestration of the AREG.

Because ADAM17 can cleave multiple substrates, it was also feasible that it might influence mammary development *via* other targets. One such candidate, TNFα, i.e., the substrate for which ADAM17/TACE had originally been named, had previously been shown to stimulate the growth and branching of cultured mammary epithelial cells in an EGFR-independent, but metalloproteinase-dependent manner, thus suggesting the involvement of its namesake [50, 51]. Nevertheless, exogenous TNFα failed to support mammary organoid growth in multiple independent experiments and its transcripts were undetectable in developing mammary glands [28]. Moreover, mice that lack TNFα or either of its receptors (which can also be shed by ADAM17 [39]) have no overt phenotype [52–54] and are lactationally competent (J. Peschon, L. Old and G. Kollias, personal communications). Thus, TNFα is not required for mammary gland development or function and is not an important substrate for ADAM17 in this setting.

Genetically defined organoids were also used to identify molecules that might act upstream or downstream of ADAM17 and EGFR, respectively. That is, factors that supported the growth of EGFR-null organoids might act downstream or independently of EGFR, whereas those that fostered the growth of wild-type, but not ADAM17-null organoids might act upstream. Although EGFR-null organoids were refractory to EGFR agonists, approximately half of the EGFR-null organoids did grow in response to the ErbB3/ErbB4 ligand neuregulin-1-β1 (NRG1β1), as did wild-type, ADAM17-null and AREG-null organoids. However, unlike the multiple glandular sprouts that formed in response to EGFR agonists, the NRG1β1-treated organoids formed large expanding mounds and folds. In addition, the fibroblast growth factors (FGF)2/bFGF and FGF7/KGF stimulated branching in all organoids, including EGFR-null organoids, whereas FGF1 and FGF10 elicited minimal growth in only a handful 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 that often possessed solid club-like ends that resembled TEBs. Thus, since FGF2 and FGF7 supported the growth of EGFR-null organoids, it was concluded that they might (1) act downstream of EGFR, (2) regulate other aspects of ductal morphogenesis independent of EGFR, or (3) provide compensatory effects when administered pharmacologically.

What Cues Act Upstream of the ADAM17–AREG–EGFR Axis?

The above data demonstrate that the ADAM17–EGFR axis is an essential paracrine pathway whereby ADAM17 releases epithelial AREG, which then activates stromal EGFR, thus eliciting reciprocal responses that further orchestrate mammary epithelial development. However, they also raise new questions and possibilities concerning the cues that act upstream and downstream of this particular interaction. It has long been recognized that the over-riding signals that regulate all aspects of mammary development are hormonal in origin. Indeed, ovariectomy and hypophysectomy studies show that ovarian and pituitary hormones are absolutely essential for post-pubertal mammary development. In addition, exogenous estrogens can rescue mammary development in ovariectomized mice [55], suggesting that ovarian estrogens act as an essential 'on-switch', thereby initiating the process of ductal develop-

ment; a suggestion that is consistent with the fact that true ductal development begins at puberty. However, just as an electrical switch has no effect in the absence of electricity, estrogens alone cannot rescue mammary development in hypophysectomized animals [56]. Estrogens can, however, restore TEB and duct development in rats that have been both hypophysectomized and ovariectomized if GH or insulin-like growth factor-1 (IGF1) are also provided [56]. On the other hand, the administration of both estrogen and pituitary prolactin does not rescue their development, suggesting that GH is the master pituitary hormone—or electricity—that is missing in hypophysectomized rats, and that its downstream effects are elicited via IGF1. Moreover, mammary gland development is impaired in mice lacking the GH receptor [57], IGF1 [56], ERα [8] or the aromatase responsible for estrogen biosynthesis [58], but occurs normally in mice lacking ERβ, PR or the prolactin receptor [8], thus confirming the importance of GH, IGF1, ovarian estrogens and their respective receptors in post-pubertal mammary development.

Although IGF1 rescues ductal development in hypophysectomized (GH-deficient) animals, exogenous GH and estrogen fail to rescue the development of IGF1-null glands, suggesting that locally produced IGF1 acts downstream of GH and/or ovarian estrogens [56]. The importance of locally produced rather than systemic IGF1 is also supported by the observation that mammary branching proceeds normally in mice with a liver-specific IGF1 deletion that results in a 75% decrease in circulating IGF1 levels without affecting mammary-specific IGF1 production [15]. On the other hand, mammary branching *is* impeded in global IGF1 knockout mice lacking both systemic *and* local IGF1 production. Furthermore, IGF1 receptor (IGF1R) deficient mammary transplants exhibit reduced growth in surgically cleared wild-type fat pads [59], suggesting that IGF1R is required in the epithelium, since it was otherwise expressed in the host fat pads. By comparison, similar experiments show that the GH receptor is required in the stroma [57]. Moreover, (1) GH induces IGF1 and ER expression in cleared epithelium-free fat pads, (2) the induction of IGF1 by GH is enhanced by estrogen, and (3) only GH-treated glands express stromal ER, further indicating that GH regulates mammary development via the stroma [56]. Thus it appears that pituitary GH, which is present prior to the pubertal surge in ovarian estrogens, acts *via* its stromal receptor to elicit stromal IGF1 expression, which in turn interacts with its receptor on mammary epithelial cells to stimulate TEB formation and epithelial branching in a paracrine manner.

The above data also indicate that ovarian estrogens act in concert with GH and IGF1 to stimulate mammary branching, but fail to pin-point precisely where ERα is required. Initial embryonic tissue recombination studies suggested that ERα was solely required within the stromal compartment [60], whereas adult tissue transplants suggested that it was needed in both the stromal *and* epithelial compartments [61]. More recent results, however, indicate that these former studies were hampered by incomplete ERα inactivation and instead suggest that ERα is only required within the mammary epithelium [62]. Nevertheless, ERα-null mammary epithelial cells can still contribute to all portions of the developing mammary tree if they are rescued by nearby wild-type cells, thus indicating that ERα affects mammary development in a non-cell-autonomous manner. This, of course, could be accomplished by eliciting the release of a paracrine factor that had a comparable effect on both ERα expressing and non-expressing cells. Notably, EGFR ligands can rescue ductal development in ERα deficient mice [32] and exogenous estradiol stimulates EGFR and ErbB2 phosphorylation in ovariectomized mice [22], suggesting that the EGFR axis acts downstream of ERα and may influence mammary development in concert with ErbB2. Indeed, AREG—the only EGFR ligand that is adequately expressed and enriched in the developing mammary epithelium—is strongly induced by estrogens [63]. Thus a key action of ovarian estrogens may be their induction of epithelial AREG expression at the onset of puberty, without which normal mammary development fails to occur. That said, estrogen-induced ERα signaling may also affect mammary development through a whole host of other avenues, including the induction of transforming growth factor β, the matrix metalloproteinase (MMP)2 (gelatinase A), or factors that foster the effects of IGF1 in stromal cells.

Clearly, many inputs influence the expression and activity of ADAM17, AREG and EGFR during mammary development. But whereas only one EGFR ligand (AREG) appears to be expressed in the right abundance, place and time during post-pubertal mammary development, several ADAMs are expressed at this stage, at least two of which (ADAMs 15 and 17) can process AREG [64]. Nevertheless, only ADAM17 appears to be required, as other ADAMs are unable to compensate for its absence and since triple-null mice lacking ADAMs 9, 12 and 15 are fully able to nurse their pups (C. Blobel, personal communication). What this means then, is that either ADAM17 is the only physiologic sheddase for AREG, that it is regulated independently of other available ADAMs, or that it is both independently regulated *and* the only enzyme that can process Areg in the mammary gland.

Several potential avenues are available for the differential regulation of ADAM17. As regards its activation, furin-like proprotein convertases remove the latency-maintaining propeptide domain of ADAM17 during its transit through the trans-Golgi network, thus rendering ADAM17 fully active at the cell surface [65]. As already noted, the only

known inhibitor of ADAM17—TIMP3 [48]—is specifically downregulated in and around invading TEBs (but not trailing ducts). Thus, even though ADAM17 is ubiquitously expressed and activated, the local downregulation of its sole inhibitor would tend to enhance its net activity in an apt location. And this, of course, would tend to augment the local release of its only readily available substrate, AREG, thereby enhancing the paracrine activation of EGFR on nearby stromal cells. Conversely, the coincident upregulation of TIMP1 in TEBs would tend to offset the loss of TIMP3 as far as other TIMP1-inhibitable enzymes are concerned, while having no direct effect on ADAM17 itself. It is also likely that other inputs influence the local activity of ADAM17. For instance, ADAMs 10 and 17 are differentially affected by phorbol esters and calcium influx [66]. In addition, G-protein coupled receptors (GPCRs) can induce the ADAM17-mediated release of AREG and the resulting transactivation of EGFR in culture [67–69], however it remains unclear exactly how they do so, whether they play a role in mammary development itself, and if so, which receptor-agonist pairs are involved. Phosphorylation of the cytoplasmic domain of ADAM17 also appears to regulate the processing of some ADAM17 substrates [70, 71], whereas the ADAM17 cytoplasmic domain appears to be dispensable for the processing of other substrates [72]. In addition, evidence suggests that integrin $\alpha_5\beta_1$ may affect ADAM17 activity [73], and the Adam and Eve inspired EVE-1/Sh3d19/PACSIN3 protein, which binds to the cytoplasmic domain of various ADAMs and which has comparable expression to that of ADAM17 during mammary development [28], appears to promote the processing of various EGFR ligands, including AREG [74]. Perhaps most interestingly, the prolonged activation of EGFR itself in human breast and epidermoid carcinoma cells causes substantial increases in processed (i.e., active) ADAM17 levels not by increasing its transcription or translation, but by enhancing its stability [75]. Therefore, positive feedback mechanisms may also affect ADAM17 function, though it is unclear whether this could happen in a non-cell autonomous fashion. Thus, although several possibilities exist, the precise physiologic cues that regulate ADAM17 activity during mammary development—or any in vivo process for that matter—are not yet known.

Does EGFR Affect Ductal Development Alone or in Concert with Other ErbB Receptors?

It is also unclear whether EGFR forms homodimers during mammary development or whether it forms heterodimers with other ErbB receptors. One observation that favors the formation of homodimers as opposed to heterodimers is that EGFR is enriched in the mammary stroma, whereas other ErbB receptors are either enriched in the epithelium (ErbB2), absent until mammary glands mature (ErbB3), or only expressed during pregnancy and lactation (ErbB4) [24]. As has been noted, stromal EGFR regulates mammary development, yet ductal development is also impaired in transgenic mice that express dominant-negative EGFR in the epithelium alone [23]. Although this could reflect downregulation of epithelial ErbB2 signaling, the mammary-targeted expression of a dominant-negative ErbB2 transgene causes alveolar defects that only become apparent in dams at parturition [76]. Nevertheless, genetically rescued (cardiac ErbB2 transgenic) ErbB2-null mammary glands *do* exhibit delayed ductal penetration and TEB defects when they are transplanted to cleared wild-type mammary fat pads, though they do eventually catch up and undergo lactational differentiation [77]. In this instance, only epithelial ErbB2 is required, since the host fat pads contain ErbB2 (and EGFR). Indeed, the selective ablation of ErbB2 in mammary epithelial cells yields a comparable phenotype [78], further indicating that epithelial ErbB2 is required. However, since ErbB2 has no known ligand, it has to act in concert with another ligand-bound ErbB coreceptor, which raises the question of which other ErbB receptor interacts with ErbB2 during mammary development. The answer is unclear, however, because ErbB3 and ErbB4 are in short supply during ductal development and because ErbB4 and epithelial EGFR are apparently expendable [22, 28, 79]. Nevertheless, even though epithelial EGFR–ErbB2 interactions are not absolutely necessary, they may still influence the *rate* of ductal development, a parameter that was not specifically addressed in EGFR-null transplantation studies [27, 28]. Although organotypic 3-D culture experiments show that the EGFR-null epithelium is still able to grow and branch in response to possible downstream agonists, the fact that wild-type epithelium responds to EGFR ligands in culture may also mean that epithelial EGFR signaling contributes to normal ductal development in a non-essential way. Nevertheless, >12% of the cells in mammary organoid cultures are stromal in origin [80], such that stromal EGFR activation could still be responsible for some of the changes in epithelial cell behavior in this setting. Thus it remains unclear precisely how ErbB2 influences ductal development or whether epithelial EGFR–ErbB2 heterodimers are even involved.

EGFR could also potentially interact with ErbB3 or ErbB4, however it is still unclear whether ErbB3 even participates in mammary development, and unlike EGFR, ErbB4 only appears to influence lobuloalveolar development. For instance, ErbB4-deficient mice that have been genetically rescued from lethality by a cardiac myosin promoter-driven ErbB4 transgene develop alveolar defects during pregnancy and lactation, yet their rate of ductal development often *surpasses* that of their

wild-type littermates [79]. One possible explanation for their accelerated ductal development is that in the absence of ErbB4, otherwise heterodimerized EGFR monomers may be liberated to better promote ductal development. Likewise, mice that express a mammary-targeted, dominant-negative ErbB4 or that lack the ErbB4 ligand NRG1α exhibit impaired alveolar differentiation but normal ductal development [81, 82]. Although these alveolar effects could also involve EGFR, genetic transplantation studies suggest that EGFR is not needed for alveolar development in response to estradiol or prolactin [27, 28]. Still, EGFR ligand-deficient dams do display more compact alveoli than wild-type dams during true pregnancy and lactation, when other important stimuli, such as placental lactogens, are also abundant [25]. Although this alveolar compaction could very well reflect alveolar crowding as a consequence of impaired ductal outgrowth, some EGFR ligands may still affect lactational differentiation. Indeed, EGF is strongly upregulated during late pregnancy and lactation [24, 29]. Moreover, if EGF *were* involved in lactational differentiation, then its participation would help to explain why triple EGF/AREG/TGFα-deficient mothers had an even more severe lactational insufficiency than did dams that were deficient in AREG alone [25]. Nevertheless, any effects that EGF might have could still be independent of EGFR, since ErbB4 has a high affinity for EGF in the presence of ErbB2 [83]. Clearly, the unique expansile growth of cultured mammary organoids in response to the ErbB3/ErbB4 ligand NRG1β1 does not require EGFR, since it also occurs in EGFR-null 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 interact during ductal development, since ErbB4 is neither necessary nor expressed during post-pubertal branching.

How Does Stromal EGFR Activation Regulate Mammary Epithelial Development?

Because mammary epithelial development requires stromal EGFR, reciprocal stromal-to-epithelial responses must also contribute. So what lies downstream of EGFR? Since TIMP1 inhibits mammary branching in culture and in vivo, even though it does not inhibit ADAM17 [84, 85], at least one other metalloproteinase must be involved. And since broad-spectrum metalloproteinase inhibitors block branching in culture in response to EGFR agonists and KGF [80, 85], they must be blocking enzymes that act downstream of EGFR. By comparison, the absence of ADAM17 alone does not block branching in response to EGFR agonists either in culture or in vivo [28], because it acts upstream of EGFR. Notably, AREG administration induces expression of the matrix metalloproteinase inducer (EMMPRIN), MMP2 and MMP9 (gelatinase B) in cultured breast epithelial cells [86]. Although MMP9 does not appear to affect mammary ductal development in vivo, MMP2 promotes mammary ductal elongation, while MMP3 (stromelysin-1), which may or may not be linked to EGFR signaling, promotes ductal side-branching [85]. Notably, the physiologic activator of latent MMP2, MMP14 (membrane type 1-MMP), is strongly induced by EGFR activation in neonatal lung and cultured embryonic fibroblasts [87] and is highly enriched in the stromal cells immediately surrounding invading mammary TEBs [85]. And indeed, in vivo data indicate that MMP14 promotes ductal development by activating latent MMP2 and by collaborating with it to degrade interstitial type I collagen (M. Egeblad, M.D. Sternlicht, B.S. Wiseman and Z. Werb, unpublished results). MMP14 is also the only collagenolytic MMP that is not inhibited by TIMP1, which is, again, specifically upregulated in and around TEBs, yet it is inhibited by TIMP3, which is specifically downregulated in the same location. Thus, not only is MMP14 physically enriched in the stroma surrounding TEBs, but its activity, like that of ADAM17, would be locally enhanced by the specific downregulation of TIMP3 at the same site. Moreover, consistent with the specific induction of MMP14 in the presumably activated stromal cells that surround invading TEBs, tissue recombination studies suggest that MMP14 is only required in the stromal rather than epithelial compartment (M.D. Sternlicht and Z. Werb, unpublished results), thus lending further support to the notion that one of the critical consequences of EGFR activation in mammary development is the induction of stromal MMP14. However, because MMP14 is membrane-bound, it can only influence epithelial behavior indirectly. Moreover, the collagen accumulation that characterizes MMP14-null mammary glands is apparently absent in Adam17-, AREG- and EGFR-null glands (M.D. Sternlicht and Z. Werb, unpublished results). Thus either EGFR activation does not actually induce MMP14 expression during mammary development or collagen remodeling and accumulation is relatively limited in the absence of ductal development itself.

In addition, FGFs may regulate mammary branching in response to or in parallel with EGFR signals. This scenario is supported by the observation that FGFs 2 and 7 support the growth and branching of cultured EGFR-null mammary organoids [28], while EGFR agonists and FGFs fail to support the growth of organoids lacking FGF receptor 2 (FGFR2) [88]. Notably, FGFR2b is expressed on mammary epithelial cells and is required for forming embryonic mammary placodes, as is stromal FGF10 [5]. Moreover, the conditional genetic ablation of FGFR2 on mammary epithelial cells causes a severe delay in adolescent ductal

development and, as a result of incomplete recombination (genetic mosaicism), those epithelia without FGFR2 are eliminated from the ducts that do develop [88]. This latter observation suggests that the FGFR2-null epithelial cells are at a selective disadvantage as compared to wild-type cells and/or that FGFR2 is required in a cell-autonomous manner. On the other hand, no mammary phenotype has been described in FGF7-deficient mice, possibly due to the compensatory effects of other FGFs, and it remains unclear whether other FGF receptors or specific receptor isoforms are involved. Nevertheless, contrary to the implications associated with their name, stromal FGFs and their epithelial receptors play key roles in *Drosophila* tracheal branching and in mammalian lung, salivary gland and kidney branching, suggesting that similar mechanisms may also affect mammary branching [3, 89, 90]. Indeed, considerable data suggest that FGF signaling can affect cell migration through chemotaxis as well as cell proliferation. For instance, FGF10-soaked beads promote the proliferation of embryonic lung epithelial cells and their migration toward the source of FGF—effects that are blocked by the TGFβ superfamily member bone morphogenetic protein 4 [91], thus suggesting an interplay between such growth factors in establishing the branching pattern.

Thus it seems likely that stromal EGFR activation initiates multiple independent responses, including FGF-mediated guidance cues, MMP14-mediated path clearing and signaling cues, and other cues that promote cell division, movement and survival. However, not only are such interactions still speculative, but it remains unclear what intracellular pathways EGFR and FGFR activate to carry out their effects or even what their specific effects in the mammary gland are. Not surprisingly, similar predicaments exist in other branched systems. For instance, inhibition of intracellular phosphoinositide-3 kinase signaling blocks renal tubular branching and elongation in kidney organ cultures [92], whereas mitogen-activated protein kinase (MAPK) inhibition affects branching but not elongation, suggesting that the two processes are differentially regulated [93]. Given its central importance in multiple receptor-mediated pathways, extracellular signal-regulated kinase (ERK)/MAPK signaling is almost certainly involved in mammary branching in one way or another, and yet the combined absence of AREG, EGF and TGFα has no apparent effect on either proliferation, apoptosis or ERK activation within mammary TEBs in vivo, even though their absence clearly inhibits mammary development [25]. However, unraveling the precise way in which a universal signaling component such as MAPK contributes to branching or any other in vivo process is clearly daunting when that component has so many critical roles.

Implications Regarding Malignant Disease

The ADAM17–EGFR axis is clearly part of a larger cascade of signals that pass back and forth between neighboring cells of the developing mammary gland. Indeed, the proper development and function of any multicellular tissue requires constant two-way communication between the disparate cell types that make up that tissue. And, as is true in society and elsewhere, breakdowns in communication can have untoward, even malignant consequences. Indeed, normal developmental pathways are often corrupted, hijacked or circumvented during the development and progression of cancer. Thus many of the factors that influence mammary development—ERα, ErbB2, and MMPs 2, 3 and 14, for instance—have been shown to play a causal or promotional role in the onset and/or progression of breast cancer. And so it is not surprising that each of the participants in the ADAM17–EGFR axis have also been implicated in breast cancer.

ADAM17, AREG, TGFα and EGFR are often upregulated in human breast cancers, with the co-expression of TGFα and EGFR tending to forecast a worse overall prognosis [38, 94–98]. Moreover, experimental data show that these molecules can actively contribute to the development and progression of cancer. For instance, mammary-targeted TGFα transgenic mice develop mammary cancers and, interestingly, the lesions in these mice and in other transgenic breast cancer models exhibit elevated AREG expression during their evolution [99]. In other experiments, the over-expression of proTGFα in CHO cells elicited robust tumor formation, whereas the expression of sheddase-resistant proTGFα mutants essentially abolished their tumorigenicity [38]. Moreover, those small tumors that did form contained revertant sheddase-sensitive cells and exhibited coincident activation of otherwise latent ADAM17 as well as EGFR phosphorylation, further suggesting that the ADAM17-mediated processing of TGFα and subsequent activation of EGFR played a critical role in the growth of these tumors. As regards AREG, its antisense suppression has been shown to vastly reduce the in vivo tumorigenicity of a cell line that was selected from immortalized normal human breast epithelial cells and in which AREG–EGFR signaling had previously been implicated [100]. And as regards EGFR, the over-expression of a human EGFR transgene driven by either of two mammary epithelial targeting promoters resulted in impaired mammary gland development and the formation of premalignant and malignant mammary lesions [101]. In addition, the ADAM17–EGFR axis can affect many of the normal cellular processes that are also considered hallmarks of malignant disease when they occur in an inappropriate way. For instance, the

siRNA-mediated knockdown of ADAM17 (but not ADAM12) suppresses the motility of squamous carcinoma cells in response to GPCR activation, but has no effect on their motility in response to exogenous AREG, thus suggesting that their GPCR-induced motility depends on the ADAM17-mediated release of AREG [68].

In a particularly pertinent effort, Kenny and Bissell [102] used a series of related cells—spontaneously immortalized, EGF-dependent, normal human breast epithelial S1 cells; premalignant, EGF-independent S2 derivatives; and tumorigenic T4-2 derivatives—to investigate their escape from EGF dependence as often occurs during malignant conversion. The malignant T4-2 cells had not acquired activating EGFR mutations, but had apparently upregulated their expression of both AREG and TGFα. Their incubation with either an EGFR inhibitor or a broad-spectrum ADAM/MMP inhibitor (TNF-α protease inhibitor, TAPI-2) elicited a malignant-to-nonmalignant phenotypic conversion in 3D culture. TAPI-2 administration also suppressed the levels of T4-2 proliferation and kinase activity downstream of EGFR, and these effects were overcome by the administration of EGF, suggesting the existence of an autocrine metalloproteinase-dependent EGFR activation pathway in these cells. Indeed, siRNA-mediated knockdown of ADAM17 expression suppressed the release of both AREG and TGFα in T4-2 cells and resulted in their malignant-to-nonmalignant reversion in 3D culture. Moreover, stably infected T4-2 cells that expressed constitutively secreted (i.e., transmembrane and cytosolic domain-deleted) forms of AREG and TGFα failed to undergo reversion in the presence of TAPI-2, but were still susceptible to direct EGFR inhibition. The authors also showed that ADAM17 inhibition suppressed AREG release and downstream kinase activity in other human breast cancer lines and found that elevated ADAM17 and TGFα expression were significantly associated with shorter overall survival in a publicly available breast cancer dataset. Conversely, elevated levels of ERα and its downstream target AREG (both of which participate in normal mammary development) were associated with prolonged survival. Thus the upregulation of ADAM17 and its generally absent substrate TGFα may permit cells to escape their dependence on otherwise limited growth signals. Although the above data suggest an autocrine escape mechanism, it is unclear whether stromal EGFR signaling also influences cancer progression as it does normal mammary development. Either way, the data suggest that the inhibition of ADAM17 may provide benefit in EGFR-dependent breast cancers. Thus a better understanding of the normal physiologic mechanisms that regulate ADAM17–AREG–EGFR signaling can only help in overcoming such mechanisms when they go awry.

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