INTRODUCTION

Mammary gland development consists of distinct stages, which include initial hormone-independent morphogenesis of the mammary buds and ducts during embryonic and perinatal life, hormonally regulated ductal development during puberty and lobulo-alveolar development during pregnancy. Each stage has distinct patterns of gene expression and specific hormonal requirements that influence the cross-talk between epithelium and mesenchyme to regulate development (Sakakura, 1987; Daniel and Silberstein, 1987). Mesenchymal-epithelial interactions are crucial during development of the embryonic mammary bud, when mammary mesenchyme induces the overlying ectoderm to form epithelial buds at day 12 of gestation (see Cunha and Hom, 1996 for review). Following formation of the mammary epithelial bud, reciprocal mesenchymal-epithelial interactions are critical for ductal growth and morphogenesis (Sakakura, 1987; Kratochwil, 1987). A number of growth factors are implicated as autocrine and paracrine mediators of mesenchymal-epithelial interactions in the mammary gland (Wysolmerski et al., 1995; Yang et al., 1995; Niranjan et al., 1995; Soriano et al., 1995; Imagawa et al., 1994; Vonderhaar, 1987; Coleman et al., 1988). Ligands of the epidermal growth factor receptor (EGFR) are believed to be particularly important downstream mediators of steroid hormone action in the mammary gland, acting locally to regulate mammary gland growth and development via stromal-epithelial interactions.

EGFR, a member of the ErbB/type 1 family of receptor tyrosine kinases, can form homodimers or heterodimers with the other family members: ErbB2, ErbB3 and ErbB4 (Earp et al., 1995). Multiple ligands bind to the EGFR including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF (HB-EGF) (Pinkas-Kramarski et al., 1997), and epiregulin (Komurasaki et al., 1997). These growth factors are synthesized as membrane-bound precursors that are cleaved by

SUMMARY

Stromal-epithelial interactions are critical in determining patterns of growth, development and ductal morphogenesis in the mammary gland, and their perturbations are significant components of tumorigenesis. Growth factors such as epidermal growth factor (EGF) contribute to these reciprocal stromal-epithelial interactions. To determine the role of signaling through the EGF receptor (EGFR) in mammary ductal growth and branching, we used mice with a targeted null mutation in the Egfr. Because Egfr−/− mice die perinatally, transplantation methods were used to study these processes. When we transplanted neonatal mammary glands under the renal capsule of immuno-compromised female mice, we found that EGFR is essential for mammary ductal growth and branching morphogenesis, but not for mammary lobulo-alveolar development. Ductal growth and development was normal in transplants of mammary epithelium from Egfr−/− mice into wild-type (WT) gland-free fat pads and in tissue recombinants prepared with WT stroma, irrespective of the source of epithelium (StromaWT/Epi−/−, StromaWT/EpiWT). However, ductal growth and branching was impaired in tissue recombinants prepared with Egfr−/− stroma (Stroma−/−/EpiWT, Stroma−/−/Epi−/−). Thus, for ductal morphogenesis, signaling through the EGFR is required only in the stromal component, the mammary fat pad. These data indicate that the EGFR pathway plays a key role in the stromal-epithelial interactions required for mammary ductal growth and branching morphogenesis. In contrast, signaling through the EGFR is not essential for lobulo-alveolar development. Stimulation of lobulo-alveolar development in the mammary gland grafts by inclusion of a pituitary isograft under the renal capsule as a source of prolactin resulted in normal alveolar development in both Egfr−/− and wild-type transplants. Through the use of tissue recombinants and transplantation, we have gained new insights into the nature of stromal-epithelial interactions in the mammary gland, and how they regulate ductal growth and branching morphogenesis.

Key words: EGFR, Mammary gland, Stromal-epithelial interaction, Ductal morphogenesis, Mouse, Growth factor

INTRODUCTION

Mammary gland development consists of distinct stages, which include initial hormone-independent morphogenesis of the mammary buds and ducts during embryonic and perinatal life, hormonally regulated ductal development during puberty and lobulo-alveolar development during pregnancy. Each stage has distinct patterns of gene expression and specific hormonal requirements that influence the cross-talk between epithelium and mesenchyme to regulate development (Sakakura, 1987; Daniel and Silberstein, 1987). Mesenchymal-epithelial interactions are crucial during development of the embryonic mammary bud, when mammary mesenchyme induces the overlying ectoderm to form epithelial buds at day 12 of gestation (see Cunha and Hom, 1996 for review). Following formation of the mammary epithelial bud, reciprocal mesenchymal-epithelial interactions are critical for ductal growth and morphogenesis (Sakakura, 1987; Kratochwil, 1987). A number of growth factors
proteases to release the active ligands. In the mouse mammary gland, the EGFR is expressed in the stromal cells surrounding the terminal end buds, in cap cells of end buds, and in adipocytes, myoepithelial cells and luminal epithelial cells (Coleman et al., 1988; Coleman and Daniel, 1990; DiAugustine et al., 1997). EGF stimulates growth of primary mammary epithelial cells from virgin or pregnant mice in vitro (Richards et al., 1982; Imagawa et al., 1985; Taketani and Oka, 1983). When slow-release implants containing EGF are introduced into growth-arrested mammary glands of ovariectomized mice, terminal end buds reappear in the zone around the implant (Coleman et al., 1988). To evaluate the role of signaling through the EGF receptor during ductal growth and branching morphogenesis in vivo, we used mice in which Egfr was inactivated by targeting exon 2 by homologous recombination (Miettinen et al., 1995). In this report, we have elucidated how signaling through the EGFR influences mammary ductal morphogenesis and lobulo-alveolar development. Moreover, we have determined that the stromal EGFR is a necessary component of the stromal-epithelial signaling interactions required for ductal growth and branching morphogenesis.

MATERIALS AND METHODS

Animals and determination of genotypes
Egfr<sup>−/−</sup> mice on a 129SvJxSwiss Black background (Miettinen et al., 1995) were produced by breeding pairs heterozygous for the targeted Egfr allele. Heterozygotes were genotyped by PCR (5′ primer: AGTAAACGGCTCACCACCTGG, 3′ primer: CTACCCGCTTCCA TTGCTCAGC). Amplification conditions were: denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes for 35 cycles. Egfr<sup>−/−</sup> mice were identified at birth by their open eyelids and short curly whiskers. There were no discernible differences between the heterozygotes and the wild-type homozygotes. Therefore, these animals are referred to as wild types throughout the grafting studies described. Intact female athymic nude mice (Balb/C) were purchased from Harlan (Indianapolis, IN).

Mammary gland dissection and transplantation
The #4 (inguinal) mammary glands (main duct, ductal branches and the entire fat pad) were removed from wild-type or Egfr<sup>−/−</sup> littermates on postnatal days 1-3. The glands were transplanted under the renal capsules of virgin female athymic mice (wild-type and Egfr<sup>−/−</sup> under contralateral renal capsules in the same animal) and grown for one month (n=14). To induce lobulo-alveolar development, some of the nude mouse hosts received a transplant of an adult pituitary gland under the renal capsule (Adler, 1986; Cunha et al., 1982; Imagawa et al., 1985; Taketani and Oka, 1983). EGF stimulates growth of primary mammary adipocytes, myoepithelial cells and luminal epithelial cells (Coleman et al., 1988; Coleman and Daniel, 1990; DiAugustine et al., 1997). EGF<sup>−/−</sup> mice were produced by breeding pairs of wild-type (WT) and Egfr<sup>−/−</sup> mice (wild-type and Egfr<sup>−/−</sup> stroma + WT + Egfr<sup>−/−</sup> epi (n=26), WT stroma + Egfr<sup>−/−</sup> epi (n=31), and Egfr<sup>−/−</sup> stroma + Egfr<sup>−/−</sup> epi (n=22). Tissue recombinants were placed under the renal capsules of nude mice and permitted to grow for 1 month. Animals were injected with BrdU 2 hours before the grafts were harvested.

Morphologic analysis
For histology, the harvested grafts were placed in 4% parafomaldehyde overnight at 4°C, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Fig. 1. (A) Whole-mount preparations of mammary glands from 11-day-old female mice. The epithelial ducts (arrows) infiltrate the Egfr<sup>−/−</sup> fat pad much less than in the wild-type gland. (B-D) Whole-mount preparations of mammary glands grown for one month under the renal capsule of a virgin athymic female mouse. The epithelial ducts seen in the outlined green box in C. Bar, (A) 1 mm, (B,C) 2 mm, (D) 0.7 mm.
by diaminobenzidine. The slides were counterstained with hematoxylin.

To analyze apoptosis, paraffin sections of the grafts were stained using the Apoptag Fluorescein kit (Oncor) and cells counterstained with propidium iodide (Oncor). Cells undergoing apoptosis were visualized by fluorescence microscopy.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

β-casein gene expression was detected by isolating RNA from 6 paraffin sections of the mammary graft tissue according to the procedure of Weizsäcker et al. (1991). The reverse transcription reaction proceeded for 45 minutes at 48°C and PCR was carried out in the same tube using the Access RT-PCR system (Promega). PCR was performed with primers specific for the mouse β-casein gene (primer #1: AAGACCTTCTGCAGTACCTAGA; primer #2: CCTGTAATATAACTGAGAACCA) using the following conditions: denaturation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute and 68°C for 2 minutes for amplification, and final extension at 68°C for 7 minutes. PCR products were run through a 1% agarose gel and visualized with ethidium bromide staining.

**Morphometric analysis**

Determination of total mammary gland and epithelial area was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Whole-mount preparations were imaged using a Leaf Lumina camera scanner (Leaf Systems, Scitex America Corp., Bedford, MA). The image files were opened in NIH Image. The total area of the gland defined by the perimeter of the fat pad was chosen by the 'Threshold' or 'Density Slice' command and a binary image was made. The area of this binary image was measured in NIH Image. The same commands were used to select epithelial area, but some of the selection had to be done manually due to contrast irregularities within the specimens. For each tissue recombinant type, at least 3-5 specimens were measured. Statistical differences in the values for each tissue recombinant type were assessed by an unpaired T-test done in the program Statview.

**RESULTS**

**Initial development of the mammary anlage occurs in the Egfr<sup>-/-</sup> mouse**

Although some Egfr<sup>-/-</sup> pups are born at a size similar to that of their wild-type littermates, the smallest mice (about 50% of the Egfr<sup>-/-</sup> mice) die within the first 24 hours. As a result of severe developmental defects in pulmonary and gastrointestinal epithelia (Miettinen et al., 1995), the surviving mice are growth retarded and, within a few days, typically have a body weight about one-third that of their wild-type littermates. The majority of Egfr<sup>-/-</sup> mice die within 3 days of birth. Based upon analysis of whole-mount preparations of #4 (inguinal) glands (data not shown), mammary gland development was equivalent at birth in both Egfr<sup>-/-</sup> mice and wild-type littermates suggesting that prenatal mammary development is normal in Egfr<sup>-/-</sup> mice. A few exceptional Egfr<sup>-/-</sup> mice survived until the age of puberty, when mammary ductal growth begins. By postnatal day 11 (Fig. 1A), the epithelial ducts have begun to migrate into the mammary fat pad in the wild-type gland, while very little ductal development has occurred in the Egfr<sup>-/-</sup> gland. These data indicate that, while mammary gland development is normal in Egfr<sup>-/-</sup> mice during the prenatal period, mammary ductal development is

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**Fig. 2.** Cell morphology, proliferation and apoptosis in wild-type and Egfr<sup>-/-</sup> mammary gland grafts. (A,B) The epithelial ducts (d) infiltrate through a mammary fat pad composed of adipose tissue (a). Very little ductal development is seen in the Egfr<sup>-/-</sup> grafts (B) compared to the wild-type grafts (A), and the few ducts present in the Egfr<sup>-/-</sup> gland have abnormally large, distended lumens. (C,D) Cell proliferation in mammary gland grafts as determined by BrdU incorporation. The proliferating cells are stained brown (arrows). There are fewer proliferating cells in both the stroma and the ductal epithelial cells in the Egfr<sup>-/-</sup> gland (D), compared to the wild-type gland (C). (E,F) Apoptosis in mammary gland grafts. Cells undergoing apoptosis are green (arrows). Since there are few ducts that have developed in the Egfr<sup>-/-</sup> gland (F), very little apoptosis is seen compared to the wild-type gland (E). The periductal fibroblasts are reduced in number around the few ducts present in the Egfr<sup>-/-</sup> gland (D,F). Bar, (A,B) 400 μm, (C-F) 100 μm.
impaired in surviving Egfr−/− pups during the prepubertal period.

**Signaling through the EGFR is required for mammary ductal morphogenesis**

To assess the full potential for mammary ductal morphogenesis that normally occurs during puberty, inguinal glands from Egfr−/− mice at postnatal days 1-3 \( (n=14) \) and glands from their wild-type littermates \( (n=14) \), were transplanted under contralateral renal capsules of athymic virgin mice. After 1 month, the epithelial ducts grew and filled the mammary fat pad in grafts of wild-type glands, while very little ductal development occurred in grafts of Egfr−/− glands (Fig. 1B-D). The few ducts in the Egfr−/− glands had abnormally large, distended lumens (Fig. 1D). Microscopically, the lack of ductal development in grafts of Egfr−/− mammary glands and the abnormally large lumens in the few existing ducts were clearly seen in H&E-stained sections (Fig. 2A,B). The Egfr−/− glands had very few proliferating cells (37% that of the wild-type glands) in either the stroma or the epithelial ducts, as determined by the labeling index with BrdU, after 1 month of growth in the nude mouse hosts (Fig. 2C,D). Wild-type glands had a few apoptotic ductal epithelial cells that were shed into the lumen, and both wild-type and Egfr−/− glands contained a small number of apoptotic cells in the stroma (1-2%) (Fig. 2E,F), when assessed after 1 month of growth in the nude mouse hosts. However, periductal fibroblasts were reduced in number around the few abnormal ducts that were present in the Egfr−/− glands compared to the wild-type glands (Fig. 2C-F). From these grafting experiments, we conclude that the Egfr−/− gland has a profound defect in ductal growth and morphogenesis, as indicated by the reduced number of ducts, the abnormally wide ducts, the decrease in epithelial and stromal proliferation, and the reduction in periductal fibroblasts.

The Egfr−/− epithelium is competent to undergo ductal development

Normal mammary epithelium contains stem cells that are competent to form ducts and secondary alveoli when transplanted into a cleared fat pad (DeOme et al., 1959). To determine whether the defect in ductal development in the Egfr−/− gland is due to the lack of EGFR signaling in the stroma, the epithelium, or both, we transplanted wild-type or Egfr−/− epithelium \( (n=4) \) into cleared fat pads of virgin female athymic mice and examined the ductal tree after 2 months. As seen in whole-mount preparations, the Egfr−/− epithelium grew and filled the fat pad to the same extent as the wild-type epithelium (Fig. 3). These experiments demonstrate that the Egfr−/− epithelium is competent to grow and undergo ductal morphogenesis, if given the correct developmental cues from the stroma, and suggest that the defect in ductal development seen in grafts of whole Egfr−/− glands may reside in the mammary fat pad.

**Signaling through the stromal EGFR is necessary for ductal morphogenesis**

To determine the contribution of the stroma in regulating epithelial proliferation and development in the Egfr−/− mice, neonatal mammary glands were surgically separated into fat pad and main duct and then recombined as described in Materials and Methods (Fig. 4). When the tissue recombinant contained wild-type stroma (Fig. 4A,D), ductal development proceeded regardless of the source of the epithelium (WT = 33% of the fat pad filled) versus Egfr−/− (23% filling) (group A, Fig. 5). This outcome is consistent with the results of transplantation of epithelium into cleared fat pads. However, when the tissue recombinants contained Egfr−/− stroma (Fig. 4B,C,E), there was very little ductal development regardless of the source of the epithelium (WT = 7% versus Egfr−/− = 5%) (group B, Fig. 5). The tissue recombinants that contained wild-type stroma (A) filled the pad to a greater extent \( (P≤0.02, n=3-5) \) than those prepared with Egfr−/− stroma (B) (Fig. 5). However, there was not a significant difference between the tissue recombinants within group A (StromaWT/EpiWT and StromaWT/Epi−/−), or within group B (Stroma−/−/EpiWT and Stroma−/−/Epi−/−) (Fig. 5). These data indicate that signaling through the stromal EGFR is essential for normal ductal growth and branching.

The number of proliferating cells was minimal in the tissue recombinants containing Egfr−/− stroma (0.5%), compared to the tissue recombinants prepared with wild-type stroma (5%) (Fig. 6A-D), when assessed after 1 month of in vivo growth. Moreover, apoptosis was prominent in the periductal fibroblasts of tissue recombinants prepared with Egfr−/− stroma (Fig. 6G), when assessed after 1 month of growth in the nude mouse host. Indeed, the condensed layer of periductal fibroblasts was entirely missing in the tissue recombinants prepared with both Egfr−/− stroma and Egfr−/− epithelium (Fig. 6H). Taken together, these results indicate that signaling through the EGFR must occur in the stroma surrounding the...
epithelial ducts to induce normal ductal proliferation and morphogenesis in the mammary gland.

**Lobulo-alveolar units develop in the *Egfr*⁻/⁻ mammary gland**

Lobulo-alveolar development takes place during pregnancy to prepare the mammary gland for lactation. During this process, terminal alveolar units differentiate from presumptive stem cells. Hormonal requirements for lobulo-alveolar development are entirely different from those required for ductal growth. Estradiol (E2) stimulates ductal development during puberty, while progesterone and prolactin stimulate alveolar development during pregnancy (Daniel et al., 1987; Bocchinfuso and Korach, 1997; Haslam, 1988; Das and Vonderhaar, 1997; Lydon et al., 1995; Ormandy et al., 1997). The EGFR has been localized to the stroma surrounding the mammary ducts during puberty and in luminal epithelial cells during lactation (DiAugustine et al., 1997). Therefore, it was of interest to examine alveolar development in the *Egfr*⁻/⁻ mammary gland. To accomplish this, neonatal mammary glands from wild-type or *Egfr*⁻/⁻ mice were transplanted along with an adult pituitary under the renal capsule (*n*=8). The grafted pituitary secretes large amounts of prolactin, which stimulates alveolar development and β-casein expression in the mammary grafts (Adler, 1986; Cunha et al., 1992). Development of lobulo-alveolar units was nearly normal in *Egfr*⁻/⁻ glands grown in pituitary-grafted hosts (Fig. 7B, D). β-casein gene expression was induced by the pituitary graft in both the *Egfr*⁻/⁻ and WT glands as demonstrated by RT-PCR analysis (data not shown). These data indicate that the *Egfr*⁻/⁻ mammary glands are competent to respond to other hormonal signals (e.g. prolactin, progesterone) to produce a lactational phenotype. However, alveolar development was not as dense in the *Egfr*⁻/⁻ glands as in the wild-type glands (Fig. 7A-D),
owing to the underlying defect in ductal development in the 
Egfr$^{-/}$-glands (Fig. 7D). Both types of glands had similar 
numbers of proliferating (12%) (Fig. 7E,F) and apoptotic (2-
4%) cells (Fig. 7G,H) when grown in pituitary-grafted hosts. 
Alveolar development was also stimulated by a pituitary graft 
when either 
Egfr$^{-/}$-or wild-type epithelium was transplanted 
to wild-type cleared fat pads (data not shown).

DISCUSSION

Signaling through the EGFR is necessary for ductal 
morphogenesis

Our study using the grafting of neonatal mammary glands has 
demonstrated that signaling through the EGFR pathway is 
esential for mammary ductal development. The EGFR is 
normally present in the stromal fibroblasts that separate the 
ducts from the fatty stroma (DiAugustine et al., 1997; Cunha 
and Hom, 1996). Impaired ductal growth in 
Egfr$^{-/}$-mammary 
glands was associated with a marked reduction in the density 
of periductal fibroblasts. Thus, signaling through the EGFR 
may promote fibroblast survival, which in turn induces ductal 
epithelial cell proliferation. The reduction in periductal 
fibroblasts in the 
Egfr$^{-/}$-grafts due to reduced proliferation and 
increased apoptosis results in a profound impairment in ductal 
morphogenesis.

Six different ligands (EGF, TGF-α, AR, BTC, HB-EGF and 
epiregulin) have been reported to signal through the EGFR. 
EGFR ligands are expressed in multiple cell types of the 
mammary gland (DiAugustine et al., 1997; Snedeker et al., 
1992) and, apparently, have compensatory functions, because 
mice homozygous null for TGF-α display no overt mammary 
phenotype (Luetteke et al., 1993). However, overexpression of 
TGF-α in transgenic mice results in mammary hyperplasia, 
with an increased incidence and decreased latency of 
mammary tumorigenesis (Matsui et al., 1990; Jhappan et al., 
1990; Sandgren et al., 1990; Coffey et al., 1994; Halter et al., 
1992). Although the contributions of the various EGFR ligands 
in promoting ductal development is still under consideration, 
it is clear that signaling through the EGFR is absolutely 
necessary to optimally stimulate ductal growth and branching. 
Signaling through the EGFR has been shown to be important 
for the establishment of branching morphogenesis of the 
trachea in 
Drosophila (Wappner et al., 1997). Our results 
demonstrate not only that signaling through the EGFR is 
required for mammary ductal growth and development, but that 
other ErbB family members known to be present in the 
mammary gland (Pinkas-Kramarski et al., 1997) cannot 
compensate for the lack of the EGFR to promote ductal 
morphogenesis.

Other growth factors produced by mammary stroma can 
stimulate ductal growth. Hepatocyte growth factor (HGF) and 
keratinocyte growth factor (KGF) are stromal factors that 
stimulate ductal growth and development (Yang et al., 1995; 
Niranjan et al., 1995; Soriano et al., 1995; Soriano et al., 1995; 
Soriano et al., 1995; Imagawa et al., 1994; Yi et al., 1994; 
Ulich et al., 1994). Transgenic mice that 
express KGF under the control of the mouse mammary tumor 

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and Leder 1996). However, in the absence of Egfr, endogenous HGF and KGF cannot compensate to promote ductal morphogenesis, although it is unknown whether these growth factors are expressed normally in the Egfr⁻/⁻ mouse mammary gland.

Other molecules that may mediate cell-cell interactions in the mammary gland include the Wnt proteins (Gavin and McMahon, 1992; Bühler et al., 1993; Weber-Hall et al., 1994), homeobox-containing genes such as Msx-1, Msx-2, Bmp-2 and Bmp-4 (Friedmann and Daniel, 1996; Phippard et al., 1996; Pavlova et al., 1994), parathyroid hormone-related protein (PTHrP) (Wysolmerski et al., 1995; Wysolmerski et al., 1998) and the matrix metalloproteinases (Sympson et al., 1994; Wiesen and Werb, 1996; Werb et al., 1996). However, while many factors may influence mammary growth and development, our data indicate that the EGFR is an essential component of the stromal signaling cascade that controls growth and ductal branching morphogenesis in the mammary gland.

### Lobulo-alveolar development is not compromised in the Egfr⁻/⁻ mammary gland

In contrast to the lack of ductal development, alveolar development occurred in grafts of both Egfr⁻/⁻ and wild-type mammary glands in response to prolactin produced by the pituitary graft. Although the alveoli were morphologically normal in grafts of Egfr⁻/⁻ mammary glands, these structures did not penetrate entirely throughout the fat pad of the Egfr⁻/⁻ mammary glands compared to the wild-type glands, presumably due to the underlying defect in ductal development. This defect was not intrinsic to the epithelium, because Egfr⁻/⁻ epithelium transplanted into wild-type cleared pads showed normal ductal development and equivalent alveolar development in response to a pituitary graft. Therefore, signaling through the EGFR is dispensable for alveolar development, just as it is dispensable for ductal development. Similarly, ductal development is impaired, while lobulo-alveolar development remains normal in transgenic mice expressing a dominant negative, truncated version of EGFR in the mammary gland under the control of the MMTV-long terminal repeat (Xie et al., 1997). Interestingly, the waved-2 (Egfrwa-2/wa-2) mouse, which has a point mutation in the EGFR kinase domain resulting in diminished signaling, displays impaired lobulo-alveolar development and decreased lactation (Fowler et al., 1995). Whether this is due to an underlying defect in ductal development or a result of abnormal development of other endocrine organs, which are required to maintain the hormonal milieu necessary for lactation has not been determined. However, the viability of these mice and the greater degree of their mammary development suggests that the functional EGFR probably is the heterodimer with ErbB2.

Heregulin (neuregulin) is produced by mammary stromal cells during the lobulo-alveolar development during pregnancy, and binds to ErbB3 and ErbB4 (Carraway and Cantley, 1994). Transgenic mice that express heregulin in the mammary gland under the control of the MMTV promoter form adenocarcinomas, and ErbB3 was the only receptor phosphorylated (Krane and Leder, 1996). In culture, heregulin stimulates alveolar development (Yang et al., 1995). Thus, heregulin may compensate for the lack of EGFR in the Egfr⁻/⁻ mammary gland by directly binding to and signaling through ErbB3 and ErbB4.

Prolactin and progesterone are necessary for lobulo-alveolar development (Haslam, 1988; Das and Vanderhaar, 1997). Alveolar development is abolished in mice lacking either the progesterone receptor (Lydon et al., 1995) or the prolactin

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**Fig. 7.** Whole-mount preparations, cell morphology, proliferation and apoptosis in intact neonatal mammary glands grown in pituitary grafted female nude mouse hosts for one month. (A,B) Whole-mount preparations of mammary glands grafted with a pituitary gland. Alveolar development produced in response to the grafted pituitary is not compromised in the Egfr⁻/⁻ mammary gland (B), although the alveoli do not penetrate throughout the entire fat pad as seen with the wild-type graft (A). (C,D) Cell morphology of mammary glands grafted with a pituitary gland. In response to a pituitary graft, alveolar development (alv) occurs in both the Egfr⁻/⁻ (D) and wild-type (C) glands. The alveolar development is not as dense in the Egfr⁻/⁻ gland as in the wild-type gland due to the underlying defect in ductal morphogenesis. The large distended lumen seen in the Egfr⁻/⁻ gland is indicated (d). (E,F) Cell proliferation in mammary glands grafted with a pituitary gland as determined by BrdU incorporation. The proliferating cells are stained brown (arrows). Both wild-type (E) and Egfr⁻/⁻ (F) glands have a large percentage of proliferating cells during alveolar development. (G,H) Apoptosis in mammary glands grafted with a pituitary gland. Cells undergoing apoptosis are labeled with fluorescein (arrows). Wild-type (G) and Egfr⁻/⁻ (H) glands have similar levels of apoptosis during alveolar development. Bar, (A,B) 2 mm, (C,D) 200 μm, (E-H) 100 μm.
receptor (Ormandy et al., 1997). While ductal growth is unaffected in mice lacking the progesterone receptor, ductal growth is impaired in virgin mice lacking the prolactin receptor (Ormandy et al., 1997). Although ductal and alveolar development in the mammary gland share key regulatory molecules that transduce systemic hormone action such as the steroid receptor coactivator-1 (SRC-1) (Xu et al., 1998), we can conclude that ductal and alveolar development are also controlled by distinct pathways at the local level by growth factors via stromal-epithelial interactions.

**EGFR is a mediator of stromal-epithelial interactions during mammary ductal morphogenesis**

Understanding the nature of stromal-epithelial interactions that regulate mammary growth and function is crucial to understanding ductal morphogenesis and alveolar development. Members of the inhibin/activin family may be one component of the regulatory molecules that locally influence ductal morphogenesis and alveolar development via stromal-epithelial interactions (Hennighausen and Robinson, 1998). Interestingly, mice that lack the inhibin βb subunit (Inhbb−/−) needed for activin and inhibin signaling have a defect in both ductal and alveolar development. Transplantation of Inhbb−/− epithelium into wild-type cleared fat pads results in normal development suggesting that only stromal inhibin βb is necessary (Robinson and Hennighausen, 1997). Although inhibin βb is a stromally derived factor that controls epithelial growth, its action appears not to be restricted to ductal morphogenesis.

Ductal growth of the mammary gland is estrogen-dependent and is profoundly impaired in mice with a null mutation in the estrogen receptor-α (Estra−/−, ERKO) (Bocchinfuso and Korach, 1997). Tissue recombinant studies using the fat pads and mammary gland epithelia from Estra−/− (ERKO) and wild-type mice demonstrate that estrogen stimulates mammary ductal epithelial growth via a paracrine mechanism, acting through the stromal estrogen receptor. The epithelial estrogen receptor is neither necessary nor sufficient for ductal development (Cunha et al., 1997). EGFR signaling is thought to be a downstream effector of estrogen action in several target organs (Mukku and Stancel, 1985a,b; DiAugustine et al., 1988; Sakai et al., 1994; Nelson et al., 1994). Mammary gland epithelium expresses EGFR, and EGF and TGF-α are mitogens for mammary gland epithelial cells (DiAugustine et al., 1997). Despite this, our data clearly show that signaling through the EGFR is not essential in the epithelial component of the mammary gland in vivo. Instead, the EGFR is absolutely necessary for the stromal component, the fat pad, to induce estrogen-dependent ductal growth and branching morphogenesis as shown by the tissue recombination studies. These results suggest that, under estrogenic conditions, which stimulate the pubertal mammary gland, the stroma responds to estrogen action through an EGFR-mediated signaling event that is required for stimulation of epithelial growth and development. In contrast, the epithelial EGFR is neither necessary nor sufficient.

Transgenic mouse models and the development of tissue recombinant technology have allowed identification of several stromally derived regulatory molecules. Whether these factors are present in a single pathway or in parallel pathways awaits further epistatic experiments to order these components.

**Mammary tumorigenesis – a disease of altered stromal-epithelial interactions**

While most mammary tumors are derived from ductal epithelium, the surrounding stroma plays a crucial supporting role via reciprocal cell-cell interactions. The stromally derived signals that influence tumor formation and growth could be via growth factors, homeobox-containing genes that specify cell fate, changes in adhesion molecules, production of metalloproteinases, changes in the extracellular matrix, or regulation of cell proliferation and apoptosis (Krane and Leder, 1996; Kitserberg and Leder, 1996; Lundy et al., 1991; Friedmann and Daniel, 1996; Werb et al., 1996; Wiesen and Werb, 1996). The overexpression of ErbB family members and their ligands is frequently seen in cancers of the breast. This overexpression is correlated with poor prognosis, as these growth factors and receptors appear to be expressed to a greater degree in malignant than in normal breast tissue (Sainsbury et al., 1985; Fitzpatrick et al., 1984; Klijn et al., 1992). The majority of breast cancers that overexpress growth factors, also overexpress the EGFR, which may set up an autocrine loop to escape hormone dependence (Lundy et al., 1991; Umekita et al., 1992). In fact, the overexpression of the EGFR and the ErbB2 receptor are associated with progression to hormone-independence in human breast cancer (Sainsbury et al., 1985; Fitzpatrick et al., 1984; Klijn et al., 1992). It would be interesting to determine whether the stroma loses its normal capacity to regulate epithelial growth via reciprocal stromal-epithelial interaction when growth factors and their receptors are expressed in the epithelial tumors. If the mechanism through which stromal EGFR signaling regulates epithelial growth and development were identified, this could lead to strategies for intervention in cancer.

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