Dynamic Expression of Erbb Pathway Members during Early Mammary Gland Morphogenesis

Olivia Wansbury[1], Heena Panchal[1], Michelle James[1], Suzanne Parry[1], Alan Ashworth[1] and Beatrice Howard[1]

Similar to other epithelial appendages, mammary anlagen progress from stratified epithelium through placode and bud stages. Embryonic mammary morphogenesis is elicited by a combination of local cell migration, adhesion changes and proliferation, and these same developmental processes impact breast cancer etiology. The Erbb signaling network plays important roles in postnatal mammary gland morphogenesis and carcinogenesis. Neuregulin3 (Nrg3), an Erbb family ligand, has recently been shown to be involved in the specification of mammary glands in mice. To further examine the possible involvement of other Erbb family members and their ligands in early mammary morphogenesis, we have characterized their expression patterns during this process. We used whole mount *in situ* hybridization to analyze the expression patterns of these genes at stages prior to and during mammary placode formation. Immunohistochemistry was used to examine expression patterns at later bud stages. The Neuregulin ligands, Nrg1, Nrg2, Nrg3, Nrg4 and the receptors, Erbb1, Erbb2, Erbb3, Erbb4, were expressed either at stages prior to morphological appearance of the mammary placode or from the time that the placode is first morphologically distinct through to later bud stages. The expression patterns presented here suggest that multiple members of this signaling network are potential mediators of early mammary morphogenesis.

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INTRODUCTION

Neuregulins are a family of ligands containing Egf-like domains that signal through the Erbb receptor tyrosine kinases to activate downstream signaling pathways such as phosphatidyl inositol 3 kinase and mitogen-activated protein kinase. This network of ligands and receptors mediates a wide range of processes that have relevance to both embryonic development and cancer, including cell adhesion, differentiation, proliferation, migration and death (Yarden and Sliwkowski, 2001). The Erbb signaling network also plays significant roles in postnatal mammary gland development (Troyer and Lee, 2001; Stern, 2003). Studies of mammary tissue from *Erbb1* and *Erbb2*-null mouse models have shown that these genes have important roles during mammary ductal outgrowth and morphogenesis (Wiesen *et al.*, 1999; Jackson-Fisher *et al.*, 2004; Andrechek *et al.*, 2005; Sternlicht *et al.*, 2005). Furthermore, *Erbb4* is required for terminal differentiation of the mammary gland and lactation fails in its absence (Long *et al.*, 2003; Tidcombe *et al.*, 2003). Impaired mammary epithelial proliferation and lobuloalveolar defects are observed in *Nrg1α*-null mice but their mammary glands are capable of sustained lactation (Li *et al.*, 2002). *Amphiregulin*-null mice display severe ductal outgrowth defects at puberty but lactate effectively (Luetteke *et al.*, 1999). Although mice mutant for the genes encoding *Egf* and *Tgfα* display no apparent mammary defects when these genes are singly mutated, compound null mutations for *Amphiregulin, Egf,* and *Tgfα* display severe lactation defects (Luetteke *et al.*, 1999). These observations suggest that this network plays key roles during development and that compensatory functions are likely to exist so that in the absence of one ligand or receptor, another may substitute.

In addition to roles in mammary epithelial development, the Erbb receptors have profound relevance to breast cancer (Holbro *et al.*, 2003). Twenty to thirty percent of breast cancers display overexpression and/or amplification of HER2/Erbb2, which confers a poor prognosis (Berger *et al.*, 1988; Slamon *et al.*, 1989). The HER2-targeted therapeutic, trastuzumab (Herceptin), has shown a significant survival benefit in clinical trials (Baselga *et al.*, 1996; Cobleigh *et al.*, 1999; Smith, 2001; Harries and Smith, 2002). Furthermore, mouse models that overexpress Erbb2 effectively mimic human breast cancer (Hutchinson and Muller, 2000). Therefore, it appears that some features of the Erbb pathway act in a similar manner in both the human breast and mouse mammary gland.

Mammary placodes are thought to arise as a result of local cell migration (Propper, 1978) and early stages of placode formation are characterized by epithelial stratification (Balinsky, 1950), which requires a proliferative basal epithelium (Smart, 1970; Lechler and Fuchs, 2005). The

[1]*The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, UK*

Correspondence: Dr Beatrice Howard, Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK. E-mail: beatrice.howard@icr.ac.uk

Abbreviations: Nrg1, Neuregulin1; Nrg2, Neuregulin2; Nrg3, Neuregulin3; Nrg4, Neuregulin4; nt, nucleotide

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initiation of mammary morphogenesis is believed to be elicited by signals arising from the dermis (Sakakura, 1987; Cunha and Hom, 1996). After the mammary epithelial bud forms, it induces the surrounding mesenchymal cells to form specialized mammary mesenchyme (Sakakura, 1987). The mammary bud then forms a primary sprout and grows downward to the mammary fat pad, the secondary mammary mesenchyme (Sakakura, 1987), which sustains the growth of mammary epithelial cells, including outgrowths from single mammary epithelial stem cells (Shackleton *et al.*, 2006). By the end of gestation in the mouse, a rudimentary ductal tree has formed, embedded within the fat pad (Sakakura, 1987). Placode morphogenesis is governed by molecules that alter cell adhesion dynamics and, in most cases, proliferation of pluripotent epithelial cells (Blanpain and Fuchs, 2006; Mikkola and Millar, 2006). As with the development of all epidermal appendages, undifferentiated stem cells are committed to specific lineages, and a population of cells with a high capacity for proliferation is delimited, which subsequently differentiates (Olivera-Martinez *et al.*, 2004; Blanpain and Fuchs, 2006; Watt *et al.*, 2006).

Our previous studies indicated that Nrg3 promotes the differentiation of squamous epithelium into mammary epithelium (Howard *et al.*, 2005). In the mouse embryo, Nrg3 appears to regulate epithelial stratification and the formation of local epithelial aggregations at the sites that mammary placodes will form. Mice harboring the *ska* mutation, a hypomorphic allele of *Nrg3*, often fail to form placode 3, which can be restored after culture with recombinant Egf domain of Nrg3. Furthermore, we have recently found that mis-expressing Nrg3 throughout the developing epidermis using the K14 promoter in a transgenic mouse model results in the formation of ectopic mammary glands (Panchal *et al.*, in press). Nrg3 may promote mammary placode formation by influencing the balance between stem cell renewal, lineage selection and differentiation. As Nrg3 is a member of a complex signaling network, it seems plausible that signals from multiple ligands and receptors may be integrated to regulate epidermal homeostasis and cell fate. Therefore, we sought to investigate further expression of the Neuregulin and Erbb family members during early mammary morphogenesis.

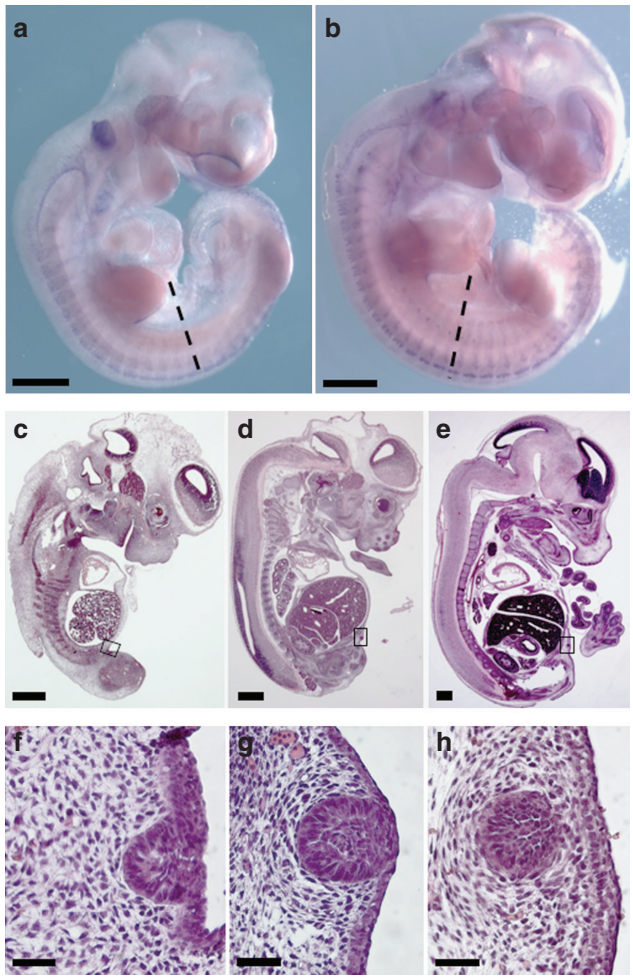

Figure 1. Stages of early mouse mammary gland morphogenesis. An example of a C57BL/6 (B6) embryo processed for whole mount *in situ* with *Erbb3* as probe at (**a**) E11.0 and (**b**) E11.5. The dotted line denotes the level of the vibratome sections shown in subsequent figures. Examples of sagittal sections used in the immunohistochemical study stained with hematoxylin and eosin at (**c**) E12.5, (**d**) E13.5, and (**e**) E14.5 are shown and the location of the mammary bud 4 is indicated with a black box. Magnified images of the mammary bud are shown at (**f**) E12.5, (**g**) E13.5, and (**h**) E14.5. Bars = 1 mm in (**a** and **b**); 500 μm in (**c**–**e**); 30 μm in (**f**); 45 μm in (**g**); 50 μm in (**h**).

RESULTS

Localization of the Erbb receptors during early mammary morphogenesis

To study expression patterns of the Erbb receptors and their ligands in the developing mammary gland (prior to and as it initially forms), we studied pre-placode and placode stages. We present data for placode 3 due to the ease of locating this site in vibratome sections and since it is the first of the five mammary placodes to become morphologically evident. However, we did not observe significant differences in expression among the other four mammary placodes that were also observed and assessed. In Figure 1, the approximate plane of the cross sections through the future site of mammary placode 3 is shown in E11.0 C57BL/6 (B6) embryos (Figure 1a) and at mammary placode 3 in E11.5 B6 embryos (Figure 1b). Later bud stages were analyzed from sagittal sections from E12.5, E13.5, and E14.5 B6 embryos (Figure 1c–h).

Prior to the formation of the mammary placode at E11.0, *Erbb1* is expressed throughout the surface ectoderm (Figure 2a and b). *Erbb1* transcripts are observed throughout the epidermis and expression is especially intense in the surface ectoderm along the flank above the Wolffian ridge at the time the mammary placode is first morphologically distinct at E11.5 through E12.0 (Figure 2c–e). Erbb1 is expressed in all surface epithelium at E12.0, including that of the mammary bud proper (Figure 2f). From E12.5 to E13.5, Erbb1 and *Erbb1* expression is weak in the mammary bud and the adjacent epithelium (Figure 2g–i). At E13.5, Erbb1 is expressed in mesenchyme directly adjacent to the mammary mesenchyme on both the dorsal and ventral edges of the mammary bud

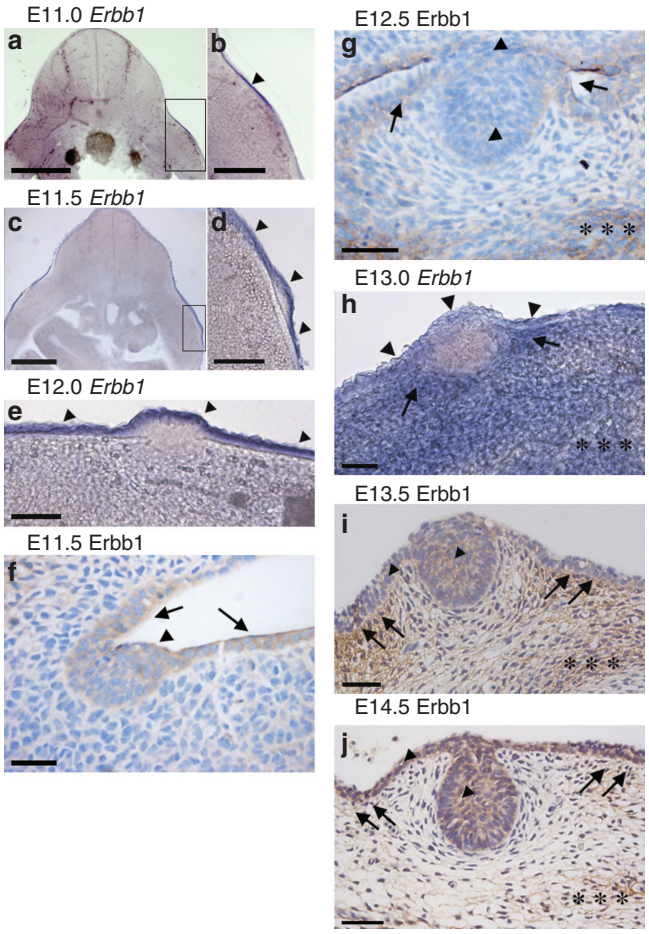

Figure 2. Erbb1 expression in the developing mammary gland. Expression patterns at the site of placode 3 from whole mount *in situ* with *Erbb1* as probe. (**a**) E11.0, vibratome cross section (70 μm) across level where number 3 mammary anlage will form. (**b**) The presumptive mammary region is boxed and shown magnified. *Erbb1* is expressed in the entire surface ectoderm of the embryo. Arrowhead denotes expression along presumptive mammary placode 3 region. (**c**) E11.5, vibratome cross section (70 μm) at the level of mammary placode number 3. (**d**) The mammary placode is boxed and shown magnified. Arrowheads denotes epithelial expression of *Erbb1* along and adjacent to mammary placode 3. (**e**) E12.0, vibratome cross section (70 μm) of mammary placode number 3 also shows epithelial expression of *Erbb1* along and adjacent to mammary placode 3 (arrowheads). (**h**) E13.0, vibratome cross section (70 μm) of mammary placode number 3. *Erbb1* is expressed in the dermal mesenchyme (arrows) flanking the mammary bud and at lower levels in the mammary bud and adjacent epithelium (arrowheads) and at the site of the future fat pad (asterisks) (**h**). Erbb1 expression at (**f**) E11.5, mammary bud 3, (**g**) E12.5 mammary bud 4, (**i**) E13.5 mammary bud 4, (**j**) E14.5 mammary bud 4. At E11.5, Erbb1 is expressed in the entire surface epithelium of the embryo (arrows), including the periderm of the mammary bud (arrowhead) (**f**). At E12.5, Erbb1 expression is lower in the mammary bud epithelium (arrowheads) than in the adjacent epithelium (arrows) (**g**). At E13.5, Erbb1 is expressed in the dermal mesenchyme (arrows) flanking the mammary bud and at low levels in the mammary bud and adjacent epithelium (arrowheads) and at the site of the future fat pad (asterisks) (**i**). At E14.5, Erbb1 is expressed throughout the surface epithelium and mammary bud epithelium (arrowheads) and in the dermal mesenchyme (arrows), but not in the mammary mesenchyme surrounding the mammary bud (**j**). The future fat pad is denoted by three asterisks in (**g**–**j**). Bars = 600 μm in (**a**); 210 μm in (**b**); 700 μm in (**c**); 350 μm in (**d**); 30 μm in (**e** and **f**); 40 μm in (**g**); 45 μm in (**h** and **i**); 50 μm in (**j**).

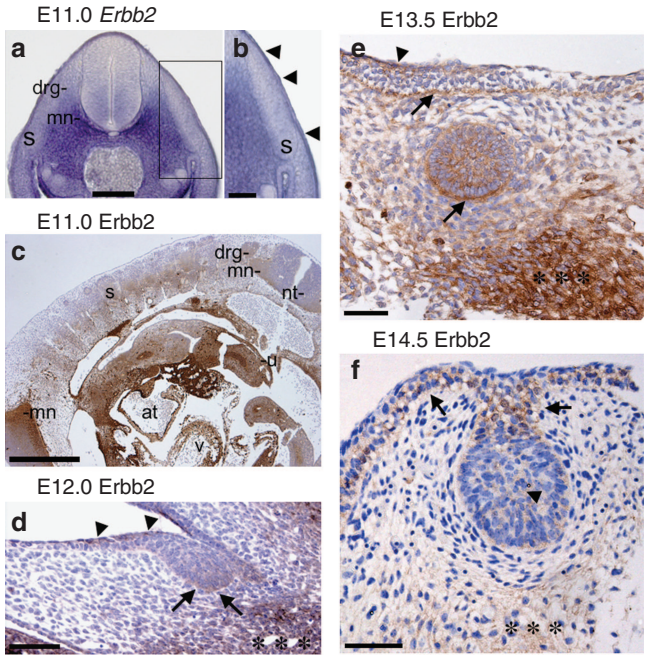

Figure 3. Erbb2 expression in the developing mammary gland. Expression patterns at the site of mammary placode 3 from whole mount *in situ* with *Erbb2* as probe. (**a**) E11.0, vibratome cross section (70 μm) at the level where number 3 mammary anlage will form. The presumptive mammary region is boxed and shown magnified in (**b**). (**b**) *Erbb2* is expressed in the epithelium (arrowheads) along the Wolffian ridge and in the underlying somite. Erbb2 expression at (**c**) E11.0, (**d**) E12.0 mammary bud 2, (**e**) E13.5 mammary bud 4, and (**f**) E14.5 mammary bud 4. Erbb2 is expressed broadly in the embryo at E11.0, including in the dorsal root ganglion, motor neurons, atrium, ventricle, urogenital ridge, somite, and mesenchyme (**c**). Erbb2 is expressed in the early mammary bud (arrows) and in the periderm (arrowheads) and at the site of the future fat pad (asterisks) at E12.0. (**d**) At E13.5, Erbb2 is expressed in the periderm (arrowhead) and the basal layer of the surface epithelium and of the mammary bud (arrows) and at the site of the future fat pad (asterisks) (**e**). At E14.5, Erbb2 is expressed at low levels in the mature mammary bud (arrowhead) and at the site of the future fat pad (asterisks) and at moderate levels in the neck of the mammary bud and in the adjacent surface epithelium (arrows) (**f**). at, atrium; drg, dorsal root ganglia; mn, motor neuron; nt, neural tube; s, somite; u, urogenital ridge; v, ventricle. Bars = 100 μm in (**a**); 50 μm in (**b**); 600 μm in (**c**); 25 μm in (**d**); 45 μm in (**e**); 50 μm in (**f**).

(Figure 2i). At E14.5, Erbb1 is expressed throughout the mammary bud and surface epithelium and is expressed at very low levels throughout the dermal mesenchyme except for the mammary mesenchyme, where no Erbb1 expression is detected (Figure 2j).

Erbb2 is expressed very broadly throughout the embryo at E11.0. *Erbb2* expression is detected in the surface ectoderm along the flank at the future sites of the mammary placodes and very broadly in the mesenchyme in embryos at stages prior to the formation of the mammary placode (Figure 3a and b). Broad expression of Erbb2 during embryogenesis at E11.0 was confirmed with immunohistochemistry (Figure 3c). We previously showed strong Erbb2 expression in the early mammary placode and slightly less intense expression in the adjacent mesenchyme (Howard *et al.*, 2005). As the placode matures, Erbb2 is expressed in the mammary bud and adjacent epithelium (Figure 3d). At the late bud stage

(E13.5), Erbb2 expression appears strongest at the basal junction of the epithelium and mesenchyme in both the surface epithelium and mammary bud (Figure 3e). By E14.5, Erbb2 is expressed at moderate levels in the surface epithelium and neck of the mammary bud and at lower levels within the mature mammary bud (Figure 3f). Erbb2 expression is observed in the mesenchyme in the vicinity of the future site of the fat pad from E12.0 through E14.5 (Figure 3d–f).

Erbb3 is expressed in the derivatives of the neural crest, the dorsal root ganglion and Schwann cells (Figure 4a). *Erbb3* is strongly expressed throughout the epithelium and in the myotome at the site where placode 3 will form (Figure 4a and b). At the early mammary placode stage, epithelial *Erbb3* expression is mainly confined to the placode and adjacent epithelium (Figure 4c and d). At E11.5, weak Erbb3 expression is observed in the surface epithelium, including the mammary placode epithelium (Figure 4e). By the E12.5 bud stage, Erbb3 expression is observed mostly in the surface epithelium and in the epithelium flanking the mammary bud and at lower levels within the bud proper (Figure 4f). At E13.5, Erbb3 is expressed more strongly in epithelium flanking the mammary bud and at very low levels in the mammary bud (Figure 4g). At E14.5, Erbb3 is expressed in the basal layer of the surface epithelium and at very low levels in the mature mammary bud (Figure 4h).

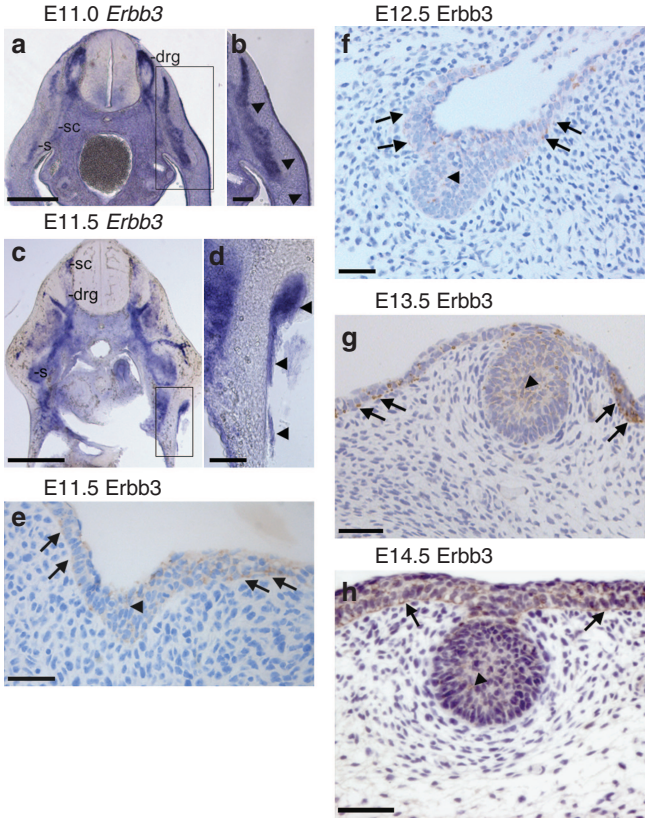

Figure 4. Erbb3 expression in the developing mammary gland. Expression patterns at the site of mammary placodes from whole mount *in situ* with *Erbb3* as probe. (**a**) E11.0, vibratome cross section (70 μm) across level where number 3 mammary anlage will form. (**b**) The presumptive mammary region is boxed and shown magnified in (**b**). *Erbb3* is expressed in the dorsal root ganglia, Schwann cells, surface epithelium overlying the Wolffian ridge (arrowheads) and in the somite. (**c**) E11.5, vibratome cross section (70 μm) across level of mammary placode number 1. The mammary region is boxed and shown magnified in (**d**). *Erbb3* is expressed in the mammary placode and adjacent ventral epithelium (arrowheads) and in the somite. Erbb3 expression at (**e**) E11.5 mammary placode 4, (**f**) E12.5 mammary bud 4, (**g**) E13.5 mammary bud 4, (**h**) E14.5 mammary bud 4. At E11.5 and E12.5, Erbb3 is expressed in the surface epithelium of the mammary placode or bud (arrowhead) and adjacent epithelium (arrows) (**e** and **f**). At E13.5, Erbb3 is weakly expressed in the mammary epithelial bud (arrowhead) and higher levels of Erbb3 expression are observed in the epithelia flanking the mammary bud (arrows) (**g**). At E14.5, very low levels of Erbb3 are expressed in the mammary epithelial bud (arrowhead) and low levels of Erbb3 expression are observed in the basal surface epithelium (arrows) (**h**). drg, dorsal root ganglia; sc, Schwann cells; s, somite. Bars = 600 μm in (**a**); 150 μm in (**b**); 700 μm in (**c**); 150 μm in (**d**); 25 μm in (**e**); 35 μm in (**f**); 45 μm in (**g**); 55 μm in (**h**).

Erbb4 is expressed in the anterior somites from E9 and in all of the somites by E10.5 (Golding *et al.*, 2004). At E11.0 to E11.25, Erbb4 is expressed in the epaxial domain of the somite and in the dermal mesenchyme above the somite (Figure 5a–d). *Erbb4* transcripts are present in the surface epithelium along the flank, including that of the mammary placode when it first appears and in the dermal mesenchyme above the somite (Figure 5e and f). By E12.0, the mammary placode and adjacent epithelium express *Erbb4* and no mesenchymal expression adjacent to the mammary placode is observed (Figure 5h). Erbb4 expression is confined to the bud proper and adjacent epithelium at the E12.0 to E12.5 bud stages (Figure 5g and i). By E13.5, Erbb4 expression is observed in the vicinity of the site of the future fat pad (data not shown) and by E14.5, Erbb4 is barely detectable in the mammary epithelium and expressed at moderate levels in the surface epithelium (Figure 5k).

Localization of Nrg1, Nrg2, Nrg3, and Nrg4 during early mammary morphogenesis

Nrg1 transcripts are present in the dermal mesenchyme underlying the flank and in the overlying surface ectoderm at the pre-mammary placode stage (Figure 6a and b). When the placode is morphologically distinct, *Nrg1* is highly expressed in the dermal mesenchyme except for that directly underlying the center of the placode, and is expressed in the dorsal and ventral edges of the placode and in the adjacent epithelium extending along the flank (Figure 6c and d). From E11.5 to E12.5, Nrg1α is expressed very weakly in the mammary bud epithelium and some expression is detected in the flanking mesenchyme (Figure 6e and g). At E13.5, Nrg1α is expressed at low levels in the surface and mammary bud epithelium and in the dermal mesenchyme (Figure 6i). At E14.5, Nrg1α is expressed in the surface epithelium and low levels of mesenchymal expression are detected but not in the mammary mesenchyme surrounding the mammary bud (Figure 6k). At E11.5, E12.5, E13.5, and E14.5, Nrg1β expression is detected in the surface epithelium flanking the mammary bud and weak Nrg1β expression is observed within the mammary epithelium; no mesenchymal Nrg1β expression is observed (Figure 6f, h, j and l).

Nrg2 is weakly expressed in the epithelium along the flank prior to formation of the mammary placode (Figure 7a and b).

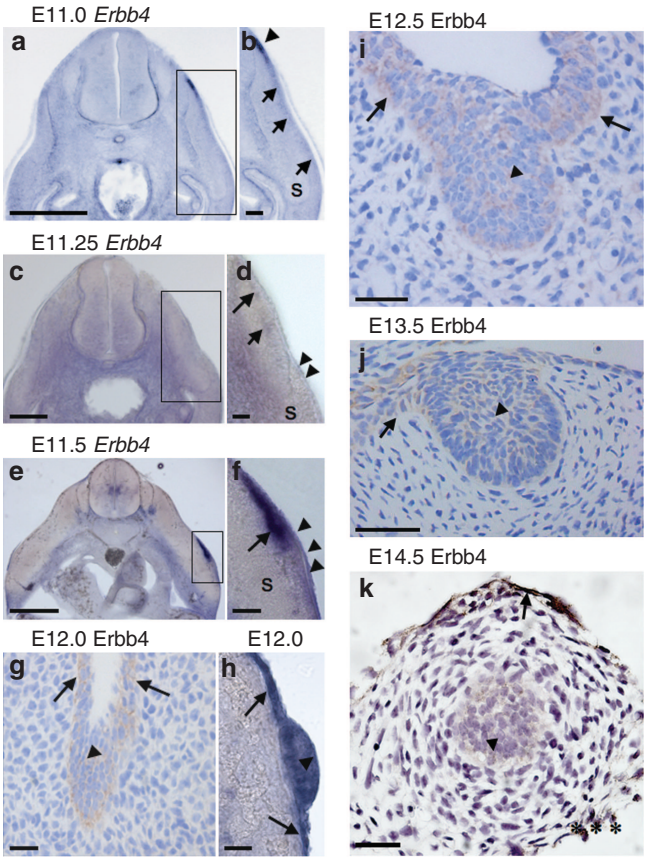

Figure 5. Erbb4 expression in the developing mammary gland. Expression patterns at the site of placode 3 from whole mount *in situ* with *Erbb4* as probe. (**a**) E11.0, vibratome cross section (70 μm) at the level where number 3 mammary anlage will form. The presumptive mammary region is boxed and shown magnified in (**b**). (**b**) *Erbb4* is expressed in the most ventral aspect of the somite (arrowhead) and in the dermal mesenchyme (arrows). E11.25, vibratome cross section (70 μm) at the level where mammary placode number 3 will form (**c**). The presumptive mammary region is boxed and shown magnified in (**d**). *Erbb4* is expressed in the pre-placode epithelium (arrowheads in (**d**)) and in the dermal mesenchyme (arrows in (**d**)). E11.5, vibratome cross section (70 μm) at the level where mammary placode number 3 is forming (**e**). The nascent mammary placode is boxed and shown magnified in (**f**). *Erbb4* is expressed in the surface epithelium of the developing mammary placode and adjacent epithelium (arrowheads) and in the dermal mesenchyme (arrow). Erbb4 expression at E12.0 mammary bud 4 (**g**), E12.5 mammary bud 4 (**i**), E13.5 mammary bud 4 (**j**), E14.5 mammary bud 2. Erbb4 expression at E12.0 and E12.5 shows weak expression in the mammary epithelial bud (arrowhead) and adjacent epithelium (arrows) in (**g** and **i**). (**h**) E12.0, vibratome cross section (70 μm) of mammary placode number 3 also shows epithelial expression of *Erbb4* in (arrowhead) and adjacent to the early mammary bud 3 (arrows). (**i**) At E13.5, Erbb4 is expressed in the mammary bud (arrowhead) and adjacent epithelium (arrow). (**j**) By E14.5, very weak Erbb4 expression is observed in the mammary bud (arrowhead) and moderate levels of Erbb4 are expressed in the surface epithelium (arrow and data not shown) and at the site of the future fat pad (asterisks). s, somite. Bars = 600 μm in (**a**); 150 μm in (**b**); 325 μm in (**c**); 100 μm in (**d**); 500 μm in (**e**); 100 μm in (**f**); 30 μm in (**g**); 40 μm in (**h**); 40 μm in (**i**); 45 μm in (**j**); 55 μm in (**k**).

At the early placode stage, *Nrg2* transcripts are present in the epithelium along the flank in the surface epithelium of the mammary placode (Figure 7c and d). Epithelial transcripts are observed in similar pattern through the early bud stage (Figure 7f). At E11.5, E12.5, and E13.5, very low levels of Nrg2 expression are detected in the mammary and adjacent epithelium (Figure 7e, g, and h). At E14.5, low levels of Nrg2 expression are detected in the mature mammary bud epithelium and strong surface epithelial expression is observed (Figure 7i).

Nrg3 is expressed in the dermal mesenchyme underlying the flank prior to formation of the mammary placode; *Nrg3* transcripts are more abundant at the ventral boundary of the future site of the placode (Figure 8a and b). At the very late pre-placode stage, just prior to when the placode first appears, *Nrg3* is expressed in the epithelium along the flank and in the dermal mesenchyme underlying the site of the placode (Figure 8c and d). We previously showed that *Nrg3* expression becomes confined to the edges of the epithelium of the mammary placode (Howard *et al.*, 2005). At the placode and early bud stage, Nrg3 is expressed only in the mammary and adjacent surface epithelium (Figure 8f and g). At the E13.5 bud stage, Nrg3 is expressed throughout the bud epithelium and at the site of the future fat pad (Figure 8h). At E14.5, Nrg3 is expressed moderately in the neck of the mammary bud and adjacent surface epithelium. Nrg3 is also expressed at the site of the future fat pad and at very low levels throughout the mammary bud epithelium (Figure 8i).

At E11.0, *Nrg4* is expressed in the epaxial domain of the somite, no *Nrg4* expression is detected in the epithelium and very low levels of transcripts are detected in the mesenchyme of the presumptive mammary region (Figure 9a and b). At very late pre-placode stages, just prior to when the mammary placode appears, *Nrg4* is expressed in the epithelium along the flank and in the dermal mesenchyme underlying the site of the placode and in the epaxial, central, and hypaxial domains of the somite (Figure 9c and d). At the placode stage, *Nrg4* is expressed in the dorsal and ventral edge of the placode epithelium and in the dermal mesenchyme spanning most of the flank except for directly under the placode (Figure 9e and f). Nrg4 expression is detected in a similar pattern in the E11.5 placode with slightly stronger mesenchymal expression flanking the placode (Figure 9g). At the E12.5 and E13.5 bud stages, Nrg4 expression is not detected in the mammary bud epithelium and low levels are detected in the surface epithelium (Figure 9h and i). Nrg4 expression is detected throughout the mammary mesenchyme adjacent to the mammary bud (Figure 9h and i). At E14.5, no Nrg4 expression is detected in the mature mammary bud epithelium and moderate levels of Nrg4 are detected in the mammary mesenchyme and in the surface epithelium (Figure 9j).

DISCUSSION

Previous studies of the expression patterns of the Erbb receptors during mammary gland development have focused primarily on postnatal stages (Schroeder and Lee, 1998; Darcy *et al.*, 2000). Since our interest is in the inductive events that lead up to the formation of the mammary placode, we sought to determine the gene expression patterns during embryogenesis. It seems highly likely that Erbb signaling

contributes to early mammary morphogenetic events since multiple ligands and receptors of the pathway are expressed at the sites of epithelial-mesenchymal interactions and are known mediators of early mammary morphogenetic events (Figure 10).

Nrg1α and Nrg1β are ligands for Erbb3 and Erbb4. Nrg2, Nrg3, and Nrg4 are ligands for Erbb4. Erbb2 has no known ligand but is the preferred heterodimerization partner for the other Erbb receptors. Erbb3 lacks intrinsic kinase activity and is only active when partnered with a heterodimer. Erbb4 signaling is further complicated by the existence of four distinct isoforms that demonstrate differential activation of downstream pathways and appear to mediate distinct biological functional processes (Elenius *et al.*, 1999; Rio *et al.*, 2000). The Erbb/Nrg network acts in an integrative manner (Citri and Yarden, 2006). Signaling pathways such as the phosphatidyl inositol 3-kinase pathway and mitogen-activated protein kinase pathway are activated upon Erbb receptor activation, which can then lead to a variety of biological outcomes (Citri and Yarden, 2006). The effects that occur upon Erbb receptor activation include cell proliferation, migration, differentiation, cell death and survival but knowledge of precisely how particular cellular responses are elicited is very limited. All of these cellular responses have been implicated in the morphogenetic processes needed for embryonic mammary development and have roles in breast carcinogenesis.

Tissue recombination experiments suggest that the mesenchyme signals mammary placode formation (Propper and Gomot, 1967; Sakakura, 1987; Cunha *et al.*, 1995; Cunha and Hom, 1996). Our functional studies have demonstrated that Nrg3 can elicit mammary placode formation in explanted embryos and suggest that Nrg3 functions as a mesenchymal paracrine signal (Howard *et al.*, 2005). It is, therefore, of note that prior to the morphological appearance of the placode, the receptors Erbb2, Erbb3, and Erbb4 and

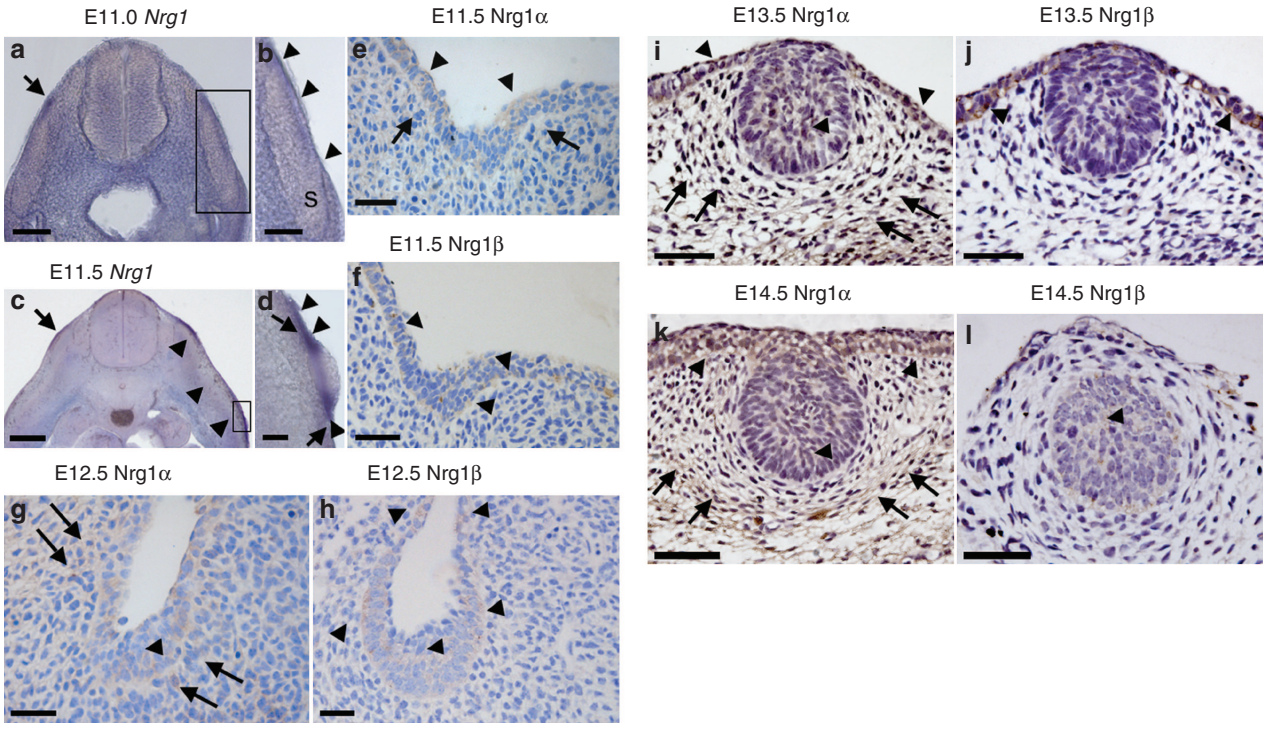

Figure 6. Nrg1 expression in the developing mammary gland. Expression patterns at the site of placode 3 from whole mount *in situ* with *Nrg1* as probe. (**a**) E11.0, vibratome cross section (70 μm) at the level where number 3 mammary anlage will form. The presumptive mammary region is boxed and shown magnified in (**b**). *Nrg1* is expressed in the epithelium (arrowheads in (**b**)) and in the epaxial domain of the somite (arrow in (**a**)) and adjacent dermal mesenchyme along the Wolffian ridge. (**c**) E11.5, vibratome cross section (70 μm) at the level of mammary placode number 3. The mammary placode is boxed and shown magnified in (**d**). *Nrg1* is expressed in the somite (arrowheads in (**c**); epaxial expression denoted by arrow in (**c**)), in the surface epithelium of the embryo and very intensely along the dorsal and ventral edges of the mammary placode (arrowheads in (**d**)) as well as in the dermal mesenchyme flanking the mammary placode (arrows in (**d**)). Nrg1α expression at (**e**) E11.5 mammary placode 4, (**g**) E12.5 mammary bud 2, (**i**) E13.5 mammary bud 4, (**k**) E14.5 mammary bud 4. Nrg1β expression at (**f**) E11.5 mammary placode 4, (**h**) E12.5 mammary bud 2, (**j**) E13.5 mammary bud 4, (**l**) E14.5 mammary bud 2. Both Nrg1α and Nrg1β are expressed at low levels in the E11.5 mammary placode and E12.5 mammary bud and adjacent surface epithelium (arrowheads in (**e**, **f**, **g** and **h**)); low levels of Nrg1α expression are detected in the flanking mesenchyme (arrows in (**e** and **g**)). Nrg1α is expressed at low levels throughout most of the mesenchyme (arrows) and in the mammary epithelium and flanking surface epithelium (arrowheads) at E13.5 (**i**). Nrg1β is expressed at low levels in surface epithelium flanking the mammary bud (arrowheads) at E13.5 (**j**). At E14.5, Nrg1α is expressed at low levels throughout most of the mesenchyme (arrows) with the exception of the mammary mesenchyme surrounding the mammary bud where no expression is detected; low levels of Nrg1α expression are detected in the mammary bud epithelium and flanking surface epithelium (arrowheads) (**k**). At E14.5, low levels of Nrg1β expression are detected in the mammary bud (arrowhead) and moderate levels of Nrg1β expression are detected in the surface epithelium (data not shown) (**l**). Bars = 300 μm in (**a**); 300 μm in (**b**); 350 μm in (**c**); 60 μm in (**d**); 25 μm in (**e** and **f**); 37.5 μm in (**g** and **h**); 45 μm in (**i** and **j**); 55 μm in (**k** and **l**).

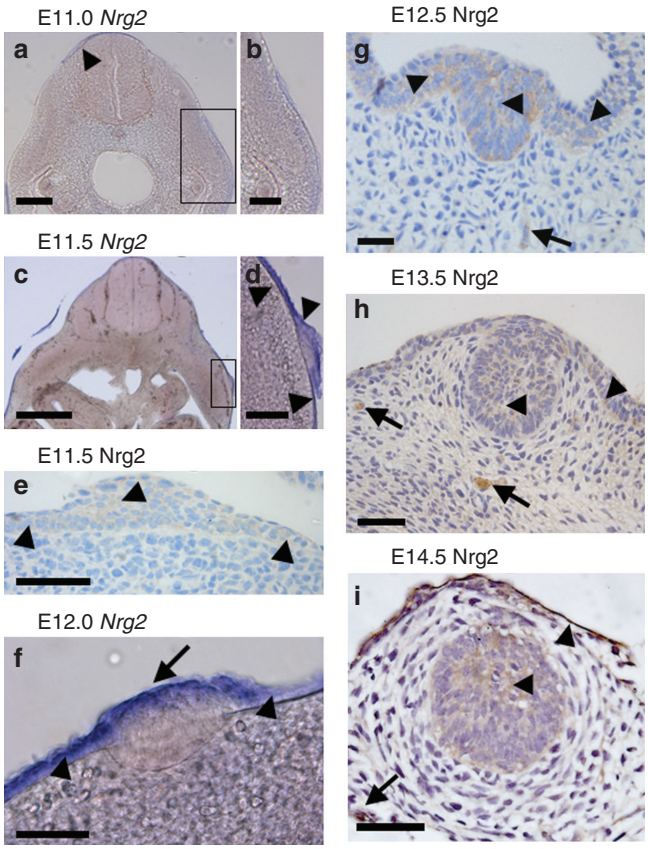

Figure 7. Nrg2 expression in the developing mammary gland. Expression patterns at the site of placode 3 from whole mount *in situ* with *Nrg2* as probe. (**a**) E11.0, vibratome cross section (70 μm) at the level where number 3 mammary anlage will form. *Nrg2* is expressed in some of the surface epithelium (arrowhead) (**a**). No *Nrg2* expression is observed in the presumptive mammary region, which is boxed and shown magnified in (**b**). (**c**) E11.5, vibratome cross section (70 μm) across level of mammary placode number 3. The mammary region is boxed and shown magnified in (**d**). (**d**) *Nrg2* is expressed in the periderm of mammary placode and adjacent epithelium (arrowheads). (**f**) E12.0, vibratome cross section (70 μm) at the level of mammary bud number 3. *Nrg2* is expressed in the surface epithelium of the mammary bud (arrow) and in the adjacent epithelium (arrowheads) (**f**). Nrg2 expression at (**e**) E11.5 mammary placode 3, (**g**) E12.5 mammary bud 4, (**h**) E13.5 mammary bud 4, (**i**) E14.5 mammary bud 2. Nrg2 is expressed at very low levels in the mammary bud and adjacent epithelium (arrowheads) at E11.5, E12.5, and E13.5 (**h**). Nrg2 expression is detected in nerves (arrows) (**g**–**i**). At E14.5, Nrg2 is expressed at moderate to high levels in the mammary bud and surface epithelium (arrowheads) and in nerves (arrow) (**i**). Bars = 300 μm in (**a**); 150 μm in (**b**) 700 μm in (**c**); 150 μm in (**d**); 60 μm in (**e**); 25 μm in (**f**); 30 μm in (**g**): 45 μm in (**h**); 55 μm in (**i**).

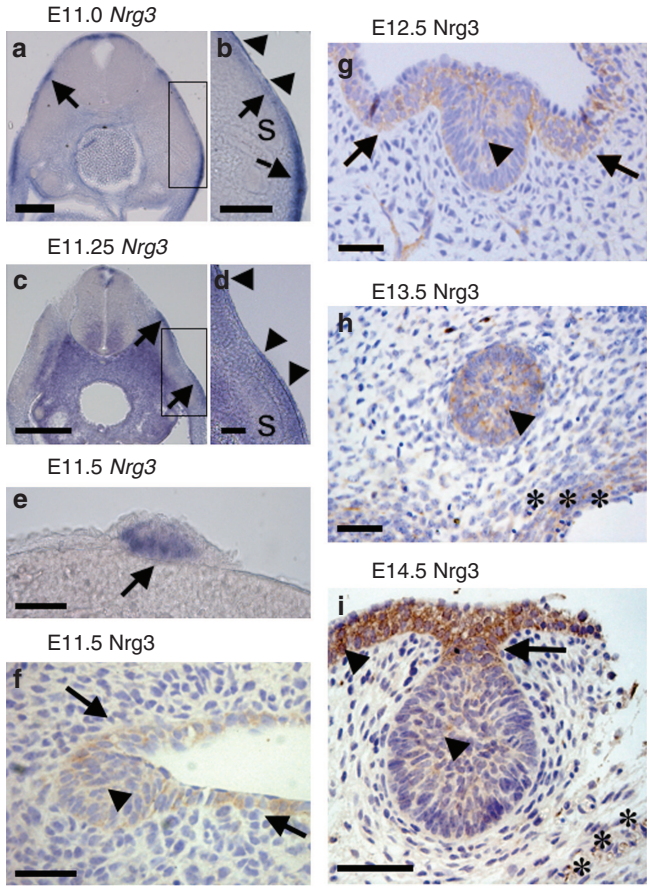

Figure 8. Nrg3 expression in the developing mammary gland. (**a**) Expression patterns at the site of placode 3 from whole mount *in situ* with *Nrg3* as probe. E11.0, vibratome cross section (70 μm) at the level where number 3 mammary anlage will form. The presumptive mammary region is boxed and shown magnified in (**b**). *Nrg3* is expressed in the dermal mesenchyme along and ventral to the Wolffian ridge (arrows in (**a** and **b**)). A few epithelial cells located dorsally to the future site of the mammary placode express *Nrg3* (arrowheads in (**b**)). (**c**) E11.25, vibratome cross section (70 μm) across level of mammary placode number 3 (arrow). *Nrg3* is expressed in the dermal mesenchyme above both the epaxial and hypaxial domains of the somite (arrows). The mammary region is boxed and shown magnified in (**d**). At the late pre-placode stage, *Nrg3* is expressed in the epithelium along the site the mammary placode will soon form and adjacent epithelium (arrowheads) along the Wolffian ridge. (**e**) E11.5, vibratome cross section (70 μm) of mammary placode number 3 shows epithelial expression of *Nrg3* in the nascent mammary placode 3. Nrg3 expression at (**f**) 11.5 mammary placode 3, (**g**) E12.5 mammary bud 4, (**h**) E13.5 mammary bud 4, (**i**) E14.5 mammary bud 4. At E11.5 and E12.5, Nrg3 is expressed in the mammary epithelial placode or bud (arrowheads in **f** and **g**) and adjacent epithelium (arrows in **f** and **g**). At E13.5, Nrg3 is expressed at low to moderate levels in the mammary bud (arrowhead) and at the site of the future fat pad (asterisks) (**g**). By E14.5, Nrg3 is expressed at very low levels in the mammary bud (arrowhead) and at higher levels in the neck of the bud (arrow) and the adjacent epithelium (arrowhead); Nrg3 expression is detected in the future fat pad site (asterisks) (**i**). Bars = 275 μm in (**a**); 150 μm in (**b**); 650 μm in (**c**); 150 μm in (**d**); 50 μm in (**e**); 37.5 μm in (**f**); 40 μm in (**g**); 45 μm in (**h**); 55 μm in (**i**).

their ligands, Nrg1, Nrg3, and Nrg4 are all expressed in either the somite, the dermal mesenchyme or in both tissues. Nrg1, Nrg3, and Nrg4 could, therefore, contribute to mediating the activation of Erbb 2:3, 2:4, 4:4, and 3:4 dimers within the somites and Erbb 2:4 and 4:4 dimers within the dermal mesenchyme. Nrg1, Nrg3, and Nrg4 would also be poised to mediate signaling from the mesenchyme to the overlying epithelium, which expresses all four Erbb receptors. Since multiple isoforms of *Nrg1*, *Nrg3, Nrg4* and *Erbb4* also exist, it is possible that distinct signals (that is, from the mesenchyme to epithelium) are mediated by particular profiles of isoforms (Falls, 2003; Hayes *et al.*, 2007), Both membrane-bound and secreted forms of the Erbb ligands exist. In some cases, cleavage and release of the ligand from the membrane is

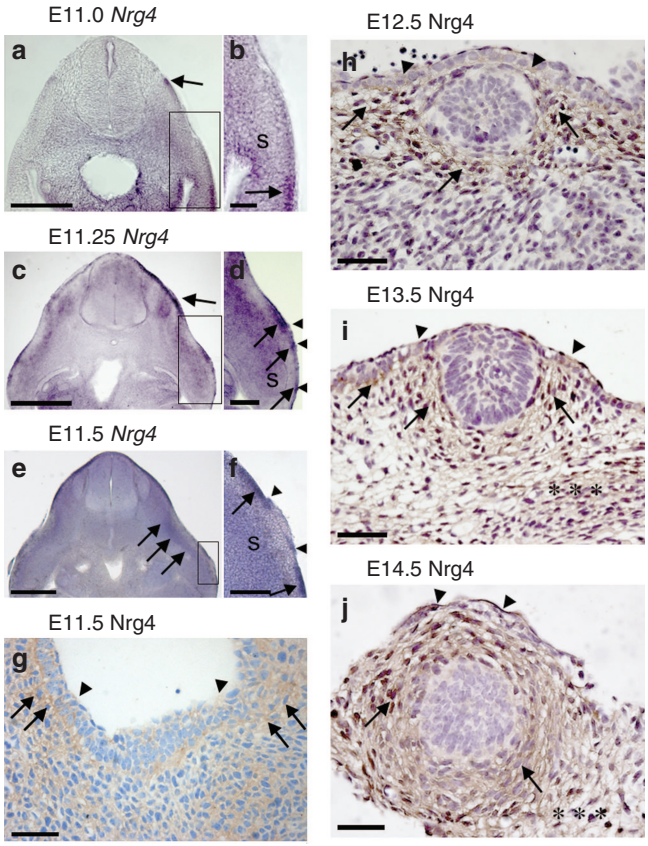

Figure 9. Nrg4 expression in the developing mammary gland. Expression patterns at the site of placode 3 from whole mount *in situ* with *Nrg4* as probe. (**a**) E11.0, vibratome cross section (70 μm) at the level where number 3 mammary anlage will form. *Nrg4* transcripts are detected in the epaxial domain of the somite (arrow in (**a**)). The presumptive mammary region is boxed and shown magnified in (**b**). No *Nrg4* expression is detected in the epithelium and weak expression in the lateral plate mesoderm is detected ventral to the future site of the mammary placode (arrow in (**b**)). (**c**) E11.25, vibratome cross section (70 μm) at the level of mammary placode number 3. The epaxial somitic expression is denoted by the arrow in (**c**) and *Nrg4* expression extends the length of the somite (**c**). The mammary region is boxed and shown in magnification in (**d**). (**d**) At the late pre-placode stage, *Nrg4* is expressed in the epithelium at the future site of the mammary placode (arrowheads) and in the underlying dermal mesenchyme (arrows) and in the somite. At the placode stage, E11.5, *Nrg4* is expressed in the somite (arrows in (**e**)) and in the epithelium at the dorsal and ventral edges of the mammary placode (arrowheads in (**f**)) and in the dermal mesenchyme underlying the epithelium directly adjacent to but not under the mammary placode (arrows in (**f**)). Nrg4 expression at (**g**) E11.5 mammary placode 4, (**h**) E12.5 mammary bud 4, (**i**) E13.5 mammary bud 4, (**j**) E14.5 mammary bud 2. At E11.5, Nrg4 is expressed in the mesenchyme adjacent to the mammary placode (arrows) and diffusely throughout the dermal mesenchyme and at low levels in the surface and mammary epithelium (arrowheads) (**g**). At E12.5, Nrg4 is expressed in the mesenchyme surrounding the mammary bud (arrows) and at very low levels in the surface epithelium (arrowheads) (**h**). At E13.5, Nrg4 is expressed in the mammary mesenchyme surrounding the mammary bud (arrows) and some expression is detected at the site of the future fat pad (asterisks); weak Nrg4 expression is detected in the surface epithelium adjacent to the mammary bud (arrowheads), and no expression is detected in the mammary bud epithelium (**i**). At E14.5, Nrg4 is expressed in the mammary mesenchyme surrounding the mammary bud (arrows) and at the site of the future fat pad (asterisks); moderate Nrg4 expression is detected in the surface epithelium (arrowheads and data not shown), and no expression is detected in the mammary bud epithelium (**j**). Bars = 600 μm in (**a**); 150 μm in (**b**); 650 μm in (**c**); 150 μm in (**d**); 700 μm in (**e**); 150 μm in (**f**); 35 μm in (**g**); 40 μm in (**h**); 45 μm in (**i**); 55 μm in (**j**).

necessary for functional activity (Sternlicht *et al.*, 2005). A wide spectrum of signaling possibilities exists since it is thought that specificity of response to Erbb ligands is dependent on ligand concentration and the availability of heterodimerization partners as well as ligand/receptor affinities (Riese and Stern, 1998; Hobbs *et al.*, 2002).

Nrg1, Nrg2, Nrg3, Erbb1, Erbb2, Erbb3, and Erbb4 expression within the mammary anlagen persists from the time the placode forms to the later bud stages. Once the placode has formed, Erbb1 and Erbb2 are the only Erbb receptors expressed in the mesenchyme adjacent to the mammary epithelium. None of the Erbb receptors are detected in the primary mammary mesenchyme. Nrg4 is the only ligand expressed in the mammary mesenchyme and could therefore mediate mesenchymal-epithelial communication from the very earliest stages through to later mammary development stages. Once the mammary placode has formed, changes in the adhesive profile of the anlage epithelium are thought to allow rearrangement of epithelial cells during the mammary bud stages (Nanba *et al.*, 2001). Survival signals are also necessary to maintain the mammary bud (Boras-Granic *et al.*, 2006). Both cell proliferation and apoptosis occur as the mammary bud develops (Michno *et al.*, 2003; Boras-Granic *et al.*, 2006). Erbb signaling has been linked to all of these cellular processes; based on the expression of its components, it could potentially contribute to any of them. Nrg1α acts as a motility factor released by keratinocytes during wound healing and in Paget's disease of the nipple, and could possibly act to elicit local migration of Erbb-expressing cells during mammary morphogenesis (Schelfhout *et al.*, 2000, 2002). Nrg1α, Nrg3, and Nrg4 are expressed in the secondary mammary mesenchyme, the future site of the fat pad, at later bud stages. Erbb1, Erbb2, and Erbb4 are also detected and therefore could be responsible for downstream signaling from these ligands at this stage. During postnatal mammary development, *Nrg1* is expressed in the mammary stroma but not in mammary fat pads that have been cleared of mammary epithelia (Yang *et al.*, 1995). This suggests that signals from the mammary epithelia are required to sustain *Nrg1* expression in the mammary stroma. Our results suggest the dialog between the mammary epithelium and future mammary stroma could begin during embryogenesis.

Mammary buds form in the viable *Erbb1* and *Erbb3*-null models and in the genetically rescued *Erbb2* and *Erbb4*-null mice (in which heart-specific myosin promoters are used to provide expression of the ablated genes to overcome lethality), so it appears that signaling through any of the four Erbb receptor homodimers is not required for inductive events (Wiesen *et al.*, 1999; Tidcombe *et al.*, 2003; Jackson-Fisher *et al.*, 2004). However, embryonic mammary bud phenotypes have not been analyzed, so it is unclear if they develop normally in absence of particular Erbb homodimers. Mammary ductal outgrowth is impeded when mammary buds or tissue are isolated from either *Erbb1* or *Erbb2*-null mice and transplanted into cleared fat pads to analyze epithelial outgrowths (Wiesen *et al.*, 1999; Jackson-Fisher *et al.*, 2004). It is possible that mammary placode formation is

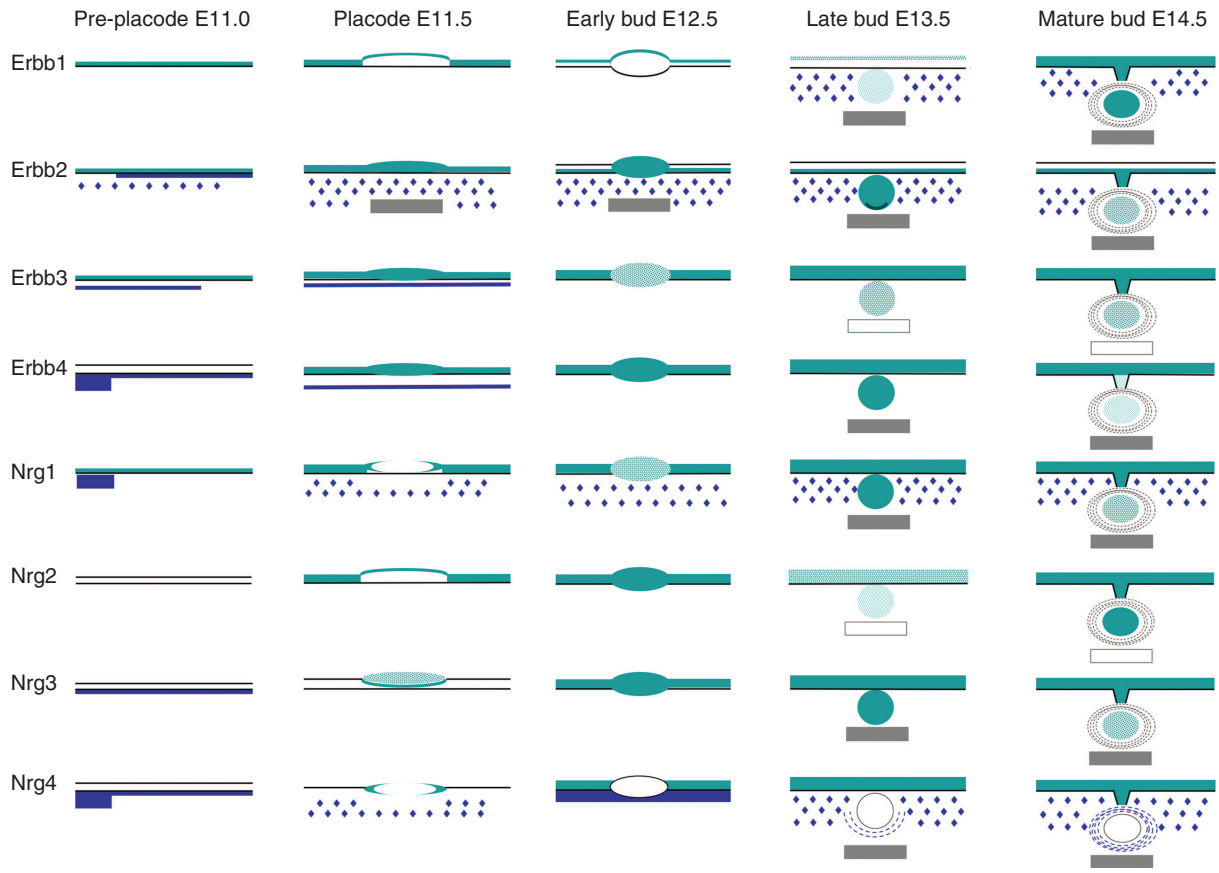

Figure 10. Summary of the distribution of Erbb signaling molecules during early mammary morphogenesis. Transcript localization is determined by whole-mount *in situ* hybridization for mammary pre-placode and placode stages. Expression in mammary bud stages is determined by immunohistochemistry. Moderate and high levels of epithelial expression are represented by solid green. Low levels or diffuse expression are denoted by a patterned green. Solid dark blue represents high levels of mesenchymal expression. Dark blue diamonds denote low or moderate levels of dispersed mesenchymal expression. Solid gray denotes expression at the future fat pad site. The basement membrane is denoted by black line; the site of the future fat pad is denoted by a rectangle; the mammary mesenchyme is denoted by the concentric rings around the mammary bud; the developing mammary anlagen are shown so that dorsal is left and ventral is right.

usually elicited through heterodimers and when one member is not available, another compensates. Since members of the Erbb signaling network may perform redundant or partially redundant functions, the analysis of the effects of the total loss of either Erbb or Neuregulin functions is likely to require the generation of mice bearing multiple mutations. Further functional studies, such as the very elegant analysis by Sternlicht *et al.* (2005) that demonstrated the requirement during mammary branching morphogenesis for paracrine activation of stromal Egfr by epithelial Amphiregulin, will be required to assign roles to Erbb pathway members.

Regulation of Erbb signaling components at both the expression level and through other mechanisms, such as feedback loops and receptor compartmentalization, are implemented so that overexpression and overactivation of the pathway are usually avoided (Citri and Yarden, 2006). Erbb receptors are overexpressed and/or amplified and activated frequently in human cancers and have been used in developing targeted therapeutics that are now used as clinical therapeutic agents (Zahnow, 2006; Bublil and Yarden, 2007). Herceptin/trastuzumab, a humanized monoclonal antibody against Erbb2/HER2, has been used with impressive clinical results in the treatment of breast cancers (Baselga *et al.*, 1996; Cobleigh *et al.*, 1999; Smith, 2001; Harries and Smith, 2002). Recently, it has become apparent that the expression of Erbb/HER ligands also impacts the clinical outcome of breast cancer patients treated with Herceptin (de Alava *et al.*, 2007). In this retrospective study, patients with breast cancer tumors that expressed high levels of transmembrane Nrg1 and low HER2 expression showed greater time to disease progression and better overall survival when treated with Herceptin when compared to those with HER2-expressing tumors (de Alava *et al.*, 2007). Other studies have also suggested that some patients with breast cancers that do not express HER2 also benefit from Herceptin treatment (Vogel *et al.*, 2002). These finding underscores the importance of understanding the roles of both ligand and receptor in the normal tissue, as it is possible that this may serve as a paradigm for other Erbb-based therapeutics.

We have recently found that mis-expressing Nrg3 throughout the developing epidermis using the K14 promoter in a transgenic mouse model results in the formation of ectopic

mammary glands (Panchal *et al.*, in press). Other epidermal phenotypes are also observed in this mouse model, which are similar to those found in transgenic mouse models that deregulate c-Myc expression in the developing epidermis using the K14 promoter (Arnold and Watt, 2001; Waikel *et al.*, 2001; Frye *et al.*, 2003). In addition to increased c-Myc expression in the transgenic epidermis, we observe decreased expression of the adhesion receptors, α6-integrin and β1-integrin. Cell-cell and cell-matrix interactions regulate epithelial organization. Desmosomal and hemidesmosomal expression profiles are downregulated during early mammary bud formation and are thought to mediate differences in cell adhesive properties within the forming bud and are subsequently restored during later stages of mammary gland formation (Nanba *et al.*, 2001). Downregulation of E-cadherin mediated by a Wnt signal and Noggin also results in decreased cell adhesion in the epidermis and has been proposed as a general mechanism that mediates morphogenesis of all budding organs (Jamora *et al.*, 2003). It is possible that epithelial cells that will form the mammary placode respond to the inductive signals from the dermal mesenchyme by changing their cell adhesion properties to effect early morphogenesis, although this remains to be demonstrated.

Deregulated cell organization and disrupted cell polarity are features of most epithelial cancers, including breast cancers. Integrins are structural proteins that interact with the extracellular matrix and play key roles in regulating epithelial adhesion, growth and differentiation (Watt, 2002). α6β4 integrin is concentrated at hemidesmosomes, which mediate attachment of epithelial cells to the basement membrane (Sonnenberg *et al.*, 1991). Most breast tumors lack hemidesmosomes and exhibit diffuse and elevated expression of α6β4 integrin rather than the basal expression that is observed in normal epithelial cells (Mercurio *et al.*, 2001; Wilhelmsen *et al.*, 2006). Altered α6β4 integrin expression is associated with poor prognosis in breast cancer patients (Tagliabue *et al.*, 1998). α6β4 integrin is thought to promote cell migration by stabilizing dynamic structures such as lamellipodia (Rabinovitz *et al.*, 1999; Gagnoux-Palacios *et al.*, 2003). Integrins can initiate and modulate signals by forming a cooperative signaling network with receptor tyrosine kinases to regulate a variety of biological processes (Hynes, 2002; Miranti and Brugge, 2002; Gagnoux-Palacios *et al.*, 2003). Recent studies of the Erbb2 mouse model of tumorigenesis have led to advancements in our understanding in how aberrant Erbb receptor signaling alters mammary epithelium so that tumorigenesis is promoted. The α6β4 integrin cooperates with Erbb2 in the formation of mammary tumors (Guo *et al.*, 2006). β4 integrin is thought to form a complex with Erbb2 and promotes tumor progression by amplifying Erbb2 signaling, which results in disrupted epithelial polarity and hyperproliferation tumors (Guo *et al.*, 2006). Many other studies have also shown that significant cross-talk exists between Erbb members and integrins, which can contribute to breast cancer progression (Falcioni *et al.*, 1997; Siegel *et al.*, 1999; Gambaletta *et al.*, 2000; Yoon *et al.*, 2006a, b; Folgiero *et al.*, 2007). Further elucidation of the link between integrins and receptor tyrosine kinases will lead to a deeper understanding that is relevant to both developmental and cancer biology.

In summary, Nrg1, Nrg2, Nrg3, and Nrg4 are expressed in patterns during early mammary morphogenesis that suggest they could mediate intra-epithelial or intra-mesenchymal signals within tissues and epithelial-mesenchymal interactions with adjacent tissues expressing Erbb2, Erbb3 and Erbb4. Egf and Egf-related ligands (Tgfα, Amphiregulin, Betacellulin, and HB-EGF) are ligands for Erbb1, which have not been examined in this study. Therefore it is not clear what type of signals they might mediate. The expression patterns observed in this study suggest that an Erbb juxtacrine signaling network may mediate epithelial and mesenchymal interactions during early mammary morphogenetic events and that autocrine/paracrine loops may also exist. Somitic Nrg/Erbb signals may also be involved in early mammary morphogenesis as has recently been elegantly demonstrated with Fgf10 (Veltmaat *et al.*, 2006). Both embryonic and postnatal mammary gland development rely on intercellular communication within the epithelium and between epithelial and mesenchymal cells. On the basis of the expression patterns reported here and in conjunction with their known roles in postnatal mammary development, the Erbb network members are candidates for some of the signals that operate during early mammary morphogenetic events.

Conclusions

This molecular histology study defines the spatial and temporal expression pattern of key members of the Erbb signaling network during mammary placode and bud morphogenesis. The expression patterns suggest that multiple members of the Erbb network could mediate and modulate early mammary morphogenetic events.

MATERIALS AND METHODS

Whole-mount *in situ* hybridization

All mouse studies have been approved by the Institute of Cancer Research's Ethics Board. Mouse embryos were from timed C57BL/6 (B6) matings. *In situ* hybridization protocols were as described (Wilkinson and Nieto, 1993). Two to four embryos were analyzed for each probe for each of the shown stages. Digoxigenin-labelled sense and anti-sense RNA was synthesized following standard protocols (Roche, Mannheim, Germany). Anti-sense and sense probes were generated from plasmids containing mouse DNA: *Erbb1* probe corresponding to nucleotides (nt) 1146–2028 of Genbank accession no. NM_007912; *Erbb2* probes corresponding to nt 2483–3453 or 2722–3453 of Genbank accession no. NM_001003817; *Erbb3* probe corresponding to nt 1342–2455 of Genbank accession no. NM_010153; *Erbb4* probe corresponding to nt 558–1527 of Genbank accession no. XM_136682; the *Nrg1* probe detects transcripts corresponding to nt 667–1242 of Genbank accession no. NM_178591 (*Hrgα*) and 126–1077 of AY648976 (*Hrg1β*); *Nrg2* probe corresponding to nt 1253-2068 of Genbank accession no. XM_001063076; *Nrg3* probes corresponding to nt 1242–1922 of Genbank accession no. NM_008734; *Nrg4* probe corresponding to nt 63–621 of Genbank accession no. NM_032002. A sense probe for each probe gave no specific signal (Figure S1). Whole mount *in situ* hybridization was performed as described

previously (Howard *et al.*, 2005). Embryos were sectioned by embedding in agarose and cutting 70-μm sections using a vibratome.

Immunohistochemistry

Time-mated B6 mouse embryos were fixed overnight in 4% paraformaldehyde, formalin, or Carnoy's and then paraffin embedded. At least three embryos were analyzed for each of the shown stages. Sections (5 μm) were rehydrated and processed with microwave antigen retrieval if required. After incubation in 3% H_2O_2 in methanol to block endogenous peroxidases, sections were rinsed and incubated in Dako blocking buffer for 10 minutes to block non-specific binding. Primary antibodies were diluted in 5% BSA/0.5% Tween 20 and sections were incubated with primary antibodies overnight at room temperature.

The primary antibodies at the indicated dilutions were: goat polyclonals: Nrg3 (1:500; R&D Systems, Minneapolis, MN), Nrg3 (1:500; Santa Cruz, Santa Cruz, CA, P-18), Nrg3 (1:500; Santa Cruz, D19) Nrg4 (1:50 Santa Cruz, L-16), rabbit polyclonals; Erbb2 (1:50; Santa Cruz, C-18), Erbb3 (1:100; Santa Cruz, C-17), Erbb4 (1:300; Santa Cruz, C-18), Nrg1α (1:75 LabVision, Fremont, CA, Ab-3), Nrg1β; 1:25; Santa Cruz, C-20) Nrg2 (1:25, Abgent, San Diego, CA), Nrg3 (1:50; Abgent), sheep polyclonal; EGFR (1:50; Upstate, Lake Placid, NY). Control experiments were performed in which primary antibodies were preincubated with blocking peptide or were left out of the procedure (Figure S2). Expression at other sites within the embryo was used for a positive control. Since no blocking peptide is available for the EGFR antibody, we have provided both *in situ* and immunohistochemical stain at E11.5, E12.5, and E13.5. For the other markers, a vibratome section of the mammary placode used in the whole-mount *in situ* hybridization and a section at a similar stage used in the immunochemistry study is included to demonstrate the validity of the reagents.

Peroxidase-labeled polymer (Envision rabbit, Dako, Glostrup, Denmark) was used for detection of primary antibodies raised in rabbits. Biotin-labeled donkey anti-goat IgG antibody (Invitrogen, Eugene, OR) was used for detection of the goat antibodies using Vector ABC kit (Vector Labs, Burlingame, CA) or Goat Histofine Simple Stain MAX PO (Nicherei). Biotin-labeled rabbit anti-sheep IgG antibody (Vector Labs) was used for detection of the sheep antibodies. 3,3′-diaminobenzidine was used as chromagen and sections were counterstained with hematoxylin.

CONFLICT OF INTEREST

The authors state no conflict of interests.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Figure S1. Vibratome sections of E11.0 and E11.5 B6 embryos hybridized with sense probes.

Figure S2. Peptide blocking experiments with E12.5 B6 mammary buds.

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