Sequential Requirement of Hepatocyte Growth Factor and Neuregulin in the Morphogenesis and Differentiation of the Mammary Gland

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Abstract. We have examined the role of two mesenchymal ligands of epithelial tyrosine kinase receptors in mouse mammary gland morphogenesis. In organ cultures of mammary glands, hepatocyte growth factor (HGF, scatter factor) promoted branching of the ductal trees but inhibited the production of secretory proteins. Neuregulin (NRG, neu differentiation factor) stimulated lobulo-alveolar budding and the production of milk proteins. These functional effects are paralleled by the expression of the two factors in vivo: HGF is produced in mesenchymal cells during ductal branching in the virgin animal; NRG is expressed in the mesenchyme during lobulo-alveolar development at pregnancy. The receptors of HGF and NRG (c-met, c-erbB3, and c-erbB4), which are expressed in the epithelial cells, are not regulated. In organ culture, branching morphogenesis and lobulo-alveolar differentiation of the mammary gland could be abolished by blocking expression of endogenous HGF and NRG by the respective antisense oligonucleotides; in antisense oligonucleotide-treated glands, morphogenesis could again be induced by the addition of recombinant HGF and NRG. We thus show that two major postnatal morphogenic periods of mammary gland development are dependent on sequential mesenchymal-epithelial interactions mediated by HGF and NRG.

GROWTH and morphogenesis of epithelia represent essential steps in the development of many eukaryotic organs. A series of developmental studies have demonstrated that growth and morphogenesis of epithelia are closely regulated by mesenchymal-epithelial interactions. These studies include transplantation and organ culture experiments, which have been carried out with various systems such as the salivary gland, the lung, the kidney, or the breast (Grobstein, 1953; Spooner and Wessells, 1970; Saxén, 1987; Sakakura, 1991). The signaling molecules involved in mesenchymal-epithelial interactions during development are still largely unknown. Recent evidence suggests, however, that epithelial tyrosine kinase receptors and their mesenchymal ligands can take over these functions (see Birchmeier and Birchmeier, 1993; for a review).

Several tyrosine kinase receptors with prevalent expression on epithelial cells have recently been characterized. The corresponding ligands are frequently synthesized by mesenchymal cells, i.e., could function in a paracrine manner (Stoker et al., 1987; Miki et al., 1991; Sonnenberg et al., 1991, 1993; Peles et al., 1992; Weidner et al., 1993; Schuchardt et al., 1994). Since certain tyrosine kinase receptors were first discovered because of their transforming potential when mutated, they have commonly been associated with mediating mitogenic signals. However, it has recently become evident that tyrosine kinase receptors can also regulate differentiation, cell movement, or morphogenesis, i.e., these receptors and their ligands may direct decisive events in epithelial development (see Birchmeier and Birchmeier, 1993). For instance, hepatocyte growth factor (HGF)[1] has been shown to induce branching morphogenesis of kidney epithelial cells in vitro (Montesano et al., 1991). Cell biological and developmental studies have indicated that HGF is synthesized by mesenchymal (and neuronal) cells whereas its receptor, the tyrosine kinase c-met, is located in cells that potentially interact with mesenchymal cells, e.g., epithelial cells (Stoker et al., 1987; Sonnenberg et al., 1993). Similarly, neuregulin (NRG) has been reported to affect growth and differentiation of epithelial (carcinoma) cells and is expressed in embryonic mesenchyme and neuronal cells (Peles et al., 1992; Meyer and Birchmeier, 1994). Thus, HGF or NRG and the corresponding receptors represent ligand-receptor pairs that could me-

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1. *Abbreviations used in this paper.* ECM, extracellular matrix; HGF, hepatocyte growth factor; NRG, neuregulin; WAP, whey acidic protein.

diate important mesenchymal-epithelial interactions in vivo.

The importance of tyrosine kinase receptors in mesenchymal-epithelial interactions during development of kidney, lung, and liver has recently been supported by genetic experiments. A targeted mutation was introduced into the murine c-ret gene, which is expressed during embryogenesis at the tips of the branching ureter buds in the kidney. Animals carrying two mutant alleles show severe hypoplasia or aplasia of the kidneys (Schuchardt et al., 1994). The ligand for c-ret is as yet unknown, but was suggested to derive from the mesenchyme. A dominant-negative keratinocyte growth factor receptor under the control of a lung surfactant promoter was used to target expression to the embryonal lung buds in transgenic mice. Newborn mice that carry this transgene show severe defects in lung development, i.e., branching morphogenesis and differentiation of the lung epithelium is completely abolished (Peters et al., 1994). Furthermore, a targeted mutation of the HGF gene in mice led to embryonal lethality caused by severe liver and placenta defects (Schmidt et al., 1995; Uehara et al., 1995). The development of both these organs is regulated by mesenchymal-epithelial interactions.

To study molecular aspects of mesenchymal-epithelial interactions, we used the mammary gland of female mice as an experimental system. The mammary gland is one of the few mammalian organs that undergoes major morphological and functional changes after birth (for reviews see Daniel and Silberstein, 1987; Sakakura, 1991). At birth, a primitive epithelial duct with few side-branches exists, which is embedded in the mesenchymal fat pad. During puberty, rapid elongation and branching of this primary epithelium proceeds until the ductal tree reaches the border of the fat pad at 12 wk. At pregnancy, a second morphogenic phase begins with the budding of multiple alveoli from the ducts, which are assembled into lobules. These lobulo-alveolar structures are the functional units of milk production at lactation. After weaning, the glands undergo massive remodeling, which results in the regression of the lobulo-alveolar structures and the return to the stage of the ductal tree. Branching morphogenesis and lobulo-alveolar differentiation of the mammary gland are regulated by a complex interplay of systemic hormonal signals and local factors (for reviews see Daniel and Silberstein, 1987; Imagawa et al., 1990). In these processes, mesenchymal-epithelial as well as epithelial-mesenchymal interactions between the cells of the fat pad and of the ducts and alveoli play a role (DeOme et al., 1958; for review see Sakakura, 1991). Three modes of signal transduction between mesenchyme and epithelium have been suggested: (a) interactions mediated by direct cell–cell contact; (b) interactions mediated by the extracellular matrix; and (c) diffusion of soluble factors (Levine and Stockdale, 1985; Sakakura, 1991; Howlett and Bissell, 1993). The identity of soluble factors involved in mesenchymal-epithelial interactions during mammary gland development were previously not known.

We have here identified two mesenchymal factors, hepatocyte growth factor and neuregulin, that play important roles in the development of the mammary epithelium. HGF, which is identical to scatter factor, is the prototype of an emerging family of motility and growth factors that resemble in their domain structure and mechanism of acti-

vation the blood proteinase plasminogen (Miyazawa et al., 1989; Nakamura et al., 1989; Weidner et al., 1991; Hartmann et al., 1992). The pleiotropic responses of HGF on cells are mediated by the receptor tyrosine kinase c-met (Bottaro et al., 1991; Naldini et al., 1991; Weidner et al., 1993). During mouse embryogenesis, various epithelia that form branching tubular structures, i.e., the lung, pancreas, or salivary gland, express c-met, while the surrounding mesenchyme produces HGF (Sonnenberg et al., 1993). Neuregulin, also called neu differentiation factor, heregulin, ARIA, glial growth factor, is a novel factor that has several functions on diverse cell types in culture: (a) induction of growth and differentiation of mammary carcinoma cells; (b) induction of growth of glial cells and glial cell fate from neuronal precursor cells; and (c) induction of terminal differentiation of myotubes (Holmes et al., 1992; Peles et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Shah et al., 1994). Various transcript isoforms (α-, β-, and isoforms containing a kringle-like domain) are all derived from one NRG gene by alternative splicing and probably, the use of two promoters (Falls et al., 1993; Marchionni et al., 1993; Peles and Yarden, 1993; Wen et al., 1994). An epidermal growth factor (EGF)-like motif was found to act as the essential functional domain in all NRG isoforms. Recently, it was shown that the interaction of NRG with the target cells is mediated by c-erb B4, which binds NRG directly and with high affinity (Plowman et al., 1993). Furthermore, c-erb B3 can act, alone or in combination with c-erbB2/neu or c-erb B4, as a further receptor for NRG (Sliwkowski et al., 1994; Carraway and Cantley, 1994). NRG is expressed in various neuronal and mesenchymal tissues during mouse embryogenesis (Orr Urtreger et al., 1993; Meyer and Birchmeier, 1994), while c-erbB3 and c-erbB4 are frequently found in cells that interact with neuronal or mesenchymal cells such as glia, muscle, or epithelial cells (Kraus et al., 1989; Press et al., 1990; Prigent et al., 1992; Plowman et al., 1993).

Materials and Methods

Mammary Gland Organ Culture

Whole mammary glands of hormonally primed mice were cultured in serum-free medium according to Banerjee et al. (1976): 4-wk-old BALB/c mice were injected subcutaneously for nine consecutive days with 1 mg progesterone and 1 μg estradiol (Sigma Chemical Co., St. Louis, MO) dissolved in gummi arabicum/0.9% NaCl. The second pair of thoracic glands was excised and cultivated for 5 d on cyclopore membranes (Falcon Plastics, Cockeysville, MD) in 2 ml medium 199 with Hanks' salts (GIBCO BRL, Gaithersburg, MD), gentamycin (40 μg/ml), aldosterone, bovine prolactin, insulin (each at 5 μg/ml; Sigma), and hydrocortisone (1 μg/ml; Merck, Darmstadt, Germany) (referred to as "APIH standard medium"). For experiments with NRG, prolactin concentration was reduced to 3 μg/ml (modified APIH medium). Medium was changed every 2 d. Three pairs of contralateral glands were routinely examined, one member of each pair was the test sample, the other the control. Formalin-fixed whole glands were stained with hematoxylin as described (Rivera, 1971). Stained glands were also embedded in paraffin, cut at 5 μm, and stained with hematoxylin/eosin. For the analysis of milk production, 500 alveolar structures on paraffine sections were examined in each experimental group for the presence of luminal secretory products and intraepithelial fat droplets (counted positive when >50% of epithelial cells contained droplets).

The 17 mer phosphorothioate oligonucleotides used in the organ culture were synthesized and purified by high-performance liquid chromatography (BioTez Berlin-Buch GmbH). Sequences were taken from the mouse HGF and NRG cDNAs, respectively, beginning with the translation start (Sonnenberg et al., 1993; Meyer and Birchmeier, 1994): HGF an-

tisense, 5'-TTGGTCCCCCACATCAT-3'; sense, 5'-ATGATGTGGGGG-ACCAA-3'; NRG antisense, 5'-TCTTTGCGCTCAGACAT-3', sense, 5'-ATGTCTGAGCGCAAAGA-3'. The oligonucleotides were added to the medium at day 1 of the culture period and supplied with each medium change.

Recombinant HGF and NRG

Recombinant HGF was produced in Sf9 insect cells by means of the baculovirus expression system as described (Weidner et al., 1993). In selected experiments, we also used the αβ-heterodimeric form of HGF cleaved by urokinase–Sepharose (Higgins and Vehar, 1987; Naldini et al., 1992). The EGF-like domain of the β-isoform of NRG was prepared as described (Holmes et al. 1992; Meyer and Birchmeier, 1993). Alternatively, a β_{2a}-subform of NRG was produced by the baculovirus expression system. The coding sequence (amino acids 20–239, Wen et al., 1994) was fused in frame to the signal sequence of expression vector pAcGP67 (Dianova, Hamburg, Germany). $NRG\beta_{2a}$ was purified on heparin–Sepharose using a NaCl gradient.

Expression Analyses

The second pairs of thoracic mammary glands from virgin, primiparous pregnant, lactating, or involuting BALB/c mice were prepared and snap frozen in liquid nitrogen. Total RNA was isolated according to Chomczynski and Sacchi (1987). RNase protection was performed as described by Birchmeier et al. (1987). Probes were synthesized as run-off transcripts from the corresponding cDNAs in the pBluescript vector with T7 RNA polymerase using $[\alpha^{32}P]$UTP (specific activity of 3,000 Ci/mmol; NEN Du-Pont). The probes were (a) mHGF1, a 449-nt transcript containing 126 nt of the murine HGF cDNA in antisense orientation (positions 467–593); (b) mMet1, a 471 nt transcript containing 137 nts of the murine c-met cDNA in antisense orientation (positions 3867–4004) and 334 nts of the plasmid derived sequence (Sonnenberg et al., 1993); (c) pNN, a 390-nt transcript composed of 290 nts of the murine NRG cDNA in antisense orientation (positions 391–681 of the cDNA sequence) and additional 100 nts from the plasmid-derived sequence; (d) pNα, a 346-nt transcript containing 259 nts of the murine NRG cDNA in antisense orientation (positions 899–1158 in the rat cDNA sequence) and additional 87 nts from the plasmid sequence (Meyer and Birchmeier, 1994); and (e) p-erb B4, a 223-nt transcript containing 141 nts of the murine c-erb B4 cDNA in antisense orientation (corresponding to positions 2486–2627 in the human sequence, Plowman et al., 1993) and additional 82 nts of plasmid sequence. A 304-nt antisense transcript derived from the murine β-actin cDNA containing 250 nts of coding sequence, was used as a control. For detection of HGF and c-met mRNA fragments, 40 μg of total RNA were hybridized to the corresponding ^{32}P-labeled transcripts (50,000 cpm of each probe). To analyze NRG and c-erbB-4 mRNA, 35 μg and 100 μg of total RNA were hybridized, respectively.

For in situ hybridization (Sonnenberg et al., 1991), cryostat sections of the second thoracic mouse mammary glands at various developmental stages were used. Antisense and sense RNA probes were synthesized from the cDNA fragments in the pBluescript vector with either T7 or T3 RNA polymerase and ^{35}S-labeled UTP and CTP (specific activities of >1,000 Ci/mmol). 10^5 cpm of the labeled probes were used for hybridization. Probes were as follows: mHGF2, obtained from a murine HGF cDNA (positions 1104–2634); mMet2, obtained from murine c-met cDNA (positions 2298–3974); pN1, obtained from murine NRG cDNA (corresponding to positions 391-1458); murine p-erbB4, corresponds to positions 834–2628. For Northern blot analysis, total RNA (15 μg) was electrophoresed in 1% agarose-formaldehyde gels, transferred to Hybond C-extra membranes (Amersham International, Amersham, UK), and hybridized to ^{32}P-labeled cDNA probes followed by autoradiography (Sambrook et al., 1989). Murine cDNA probes for β-casein (Binas et al., 1992), whey acidic protein (WAP, a kind gift of Dr. Nancy Hynes, Friedrich-Miescher-Institut, Basel) and β-actin were labeled by random priming (10^9 cpm/μg; Feinberg and Vogelstein, 1984). Hybridization signals were analyzed with a PhosphorImager.

Results

Effects of HGF and NRG in Mammary Gland Organ Culture

Mammary gland organ cultures were established from

hormonally primed mice at the age of four weeks (see Materials and Methods). At this stage, a sparsely developed epithelial tree with few end buds occupies ~40% of the mammary fat pad. Under optimal conditions for organ culture, ductal branching and alveolar morphogenesis take place within the next week leading to a glandular tree with lobulo-alveolar structures and the synthesis of milk constituents (Fig. 1, a, c, and e). This organ culture system thus allows testing the effects of exogenous and endogenous modulators on the various developmental processes, both in terms of kinetics and end points. Since contralateral pairs of glands exhibit virtually identical patterns of epithelial morphogenesis, the effect of factors on one member of the paired glands can be easily compared to the untreated control.

In organ culture, treatment of glands in standard APIH (serum-free) medium with recombinant HGF (at 20 ng/ml) for five days resulted in the appearance of unusually strongly branched glandular trees (compare Fig. 1, b and d with a and c). In the control, the primary ducts that originate from the nipple have few small side branches (Fig. 1, a and c, arrows). In the presence of HGF, branching is strongly intensified resulting in numerous main ducts that elongate toward the border of the fat pad (Fig. 1, b and d, arrows). Morphometric analysis revealed an average of two main side branches (range 1–3) in the absence and side side branches (range 3–8) in the presence of HGF (seven pairs of glands were examined). Concomitantly, HGF inhibited the secretory activity of the epithelial cells (Fig. 1, e and f): 82 ± 3% of the alveoli displayed secretory activity in the absence and 26 ± 2% in the presence of HGF (four pairs of glands were counted). Concentrations of HGF at 20 ng/ml were most effective for the stimulation of ductal branching; lower concentrations were less effective, 100 ng/ml slightly inhibited development of the glands.

In contrast, the addition of recombinant NRG to the culture medium for five days stimulated the production of lobulo-alveolar structures. This was particularly obvious at reduced prolactin concentration (i.e., in modified APIH medium), i.e., at conditions where differentiation is delayed. In the control glands, only single alveoli developed (Fig. 2, a and c). In the presence of NRG (3 nM), the number of alveoli was markedly increased and they were clustered into lobules (Fig. 2, b and d; arrows). Morphometric measurement (see Fig. 2 legend) revealed an average of three lobules per analyzed field (range 0–6) in the absence and 17 lobules (range 10–30) in the presence of NRG (five pairs of glands were examined). The concentration of 3 nM was found to be optimal for both the soluble β-form and the EGF-like domain of NRG. Histological analysis corroborated the more progressed lobular development in the presence of NRG and showed increased luminal protein secretion and intracellular fat droplet accumulation: 21 ± 9% of the alveoli displayed secretory activity in the absence and 97 ± 2% is the presence of NRG. Fat droplet-positive alveoli increased from 20 ± 8% to 99% (seven pairs of glands were examined).

We also analyzed in organ culture the effects of HGF and NRG on the expression of milk proteins β-casein and WAP. HGF added to single members of paired glands in standard APIH medium markedly reduced β-casein and
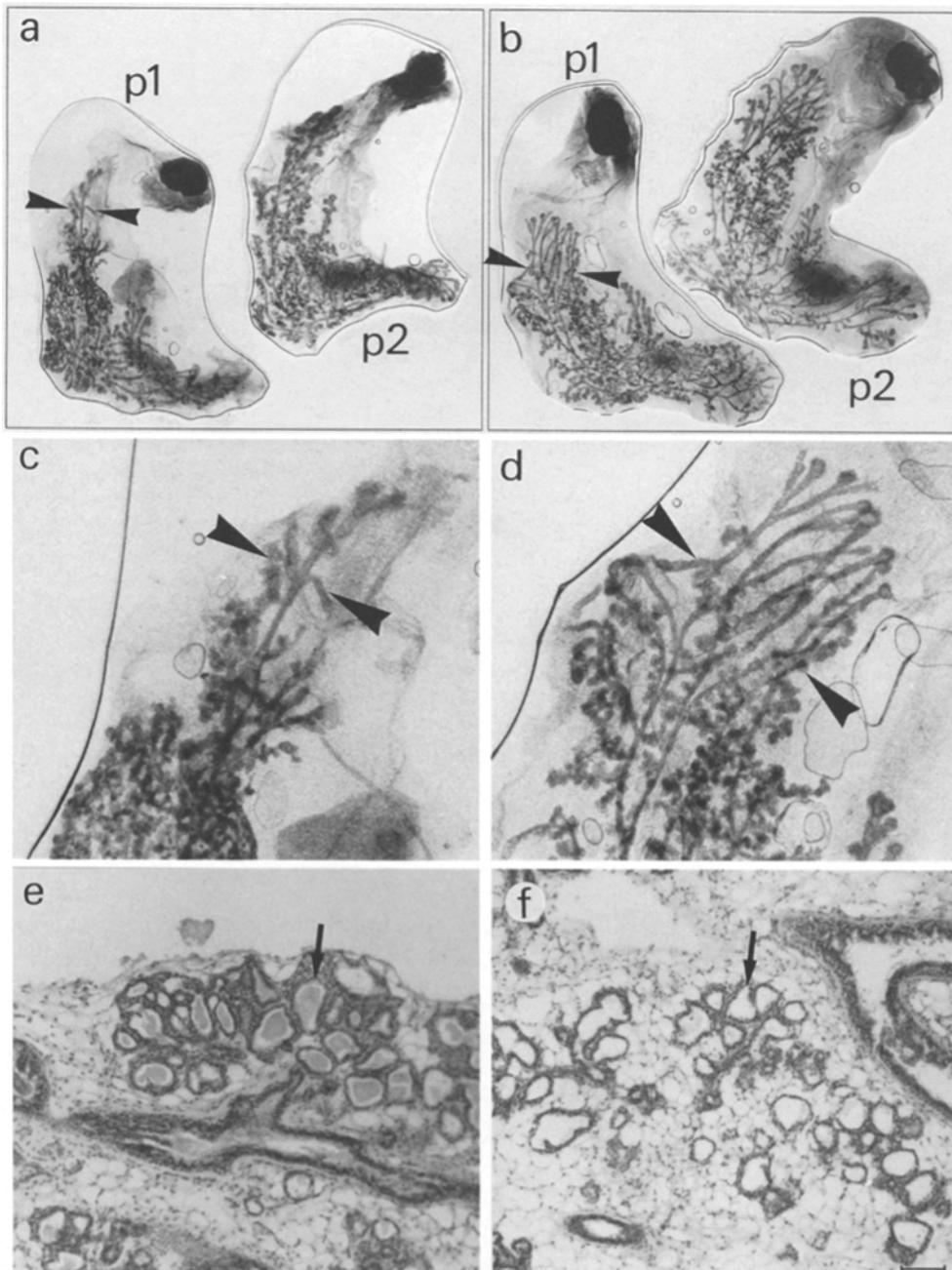
Figure 1. Effect of HGF on mammary gland morphology in organ culture. Whole mouse mammary glands were cultured for 5 d in APIH standard medium, (*a*, *c*, and *e*) in the absence or (*b*, *d*, and *f*) in the presence of 20 ng/ml recombinant HGF. (*a* and *b*) Whole mount staining with hematoxylin; p1 and p2 indicate pairs of contralateral glands. (*c* and *d*) Higher magnification of the areas indicated in *a* and *b* by arrowheads. (*e* and *f*) Histology of sections of cultured glands stained with hematoxylin/ eosin. Arrows indicate filled (*e*) or empty alveoli (*f*), respectively. Bars: (*e* and *f*) 100 μm. Morphometry of branching (see text) was performed in areas of newly formed structures (see Fig. 1, *c* and *d*).

abolished WAP expression (Fig. 3 *A*, compare lanes *2* and *4* with *3* and *5*). In contrast, NRG (at reduced prolactin concentration, i.e., modified APIH medium) induced the expression of WAP and β-casein (Fig. 3 *B*, compare lanes *2* and *4* with *3* and *5*). We also examined EGF for its effect in the organ culture system: EGF stimulated ductal proliferation but not branching. Furthermore, EGF inhibited alveolar maturation and milk production (data not shown).

Expression of HGF and NRG During Mammary Gland Development In Vivo

To examine whether HGF and NRG could possibly have the suggested functions in vivo, we examined by RNase protection analysis the temporal and spatial expression pattern of the two factors during the various stages of mammary gland development. We found that HGF expression is strongly regulated during mammary gland development, reaching the highest level during ductal morphogenesis in the virgin animal (Fig. 4, *A* and *B*). Expression levels decrease during pregnancy and lactation and increase again during involution. Quantitative analysis showed that HGF expression at 12 wk is upregulated by approximately one order of magnitude in comparison to the lactation stage. A single fragment, 126 nucleotides in length, was observed in the RNase protection analysis, indicating that the splice variant encoding HGF with additional five amino acids in kringle 1 (Weidner et al., 1991) is expressed in the mammary gland. In contrast, mRNA lev-
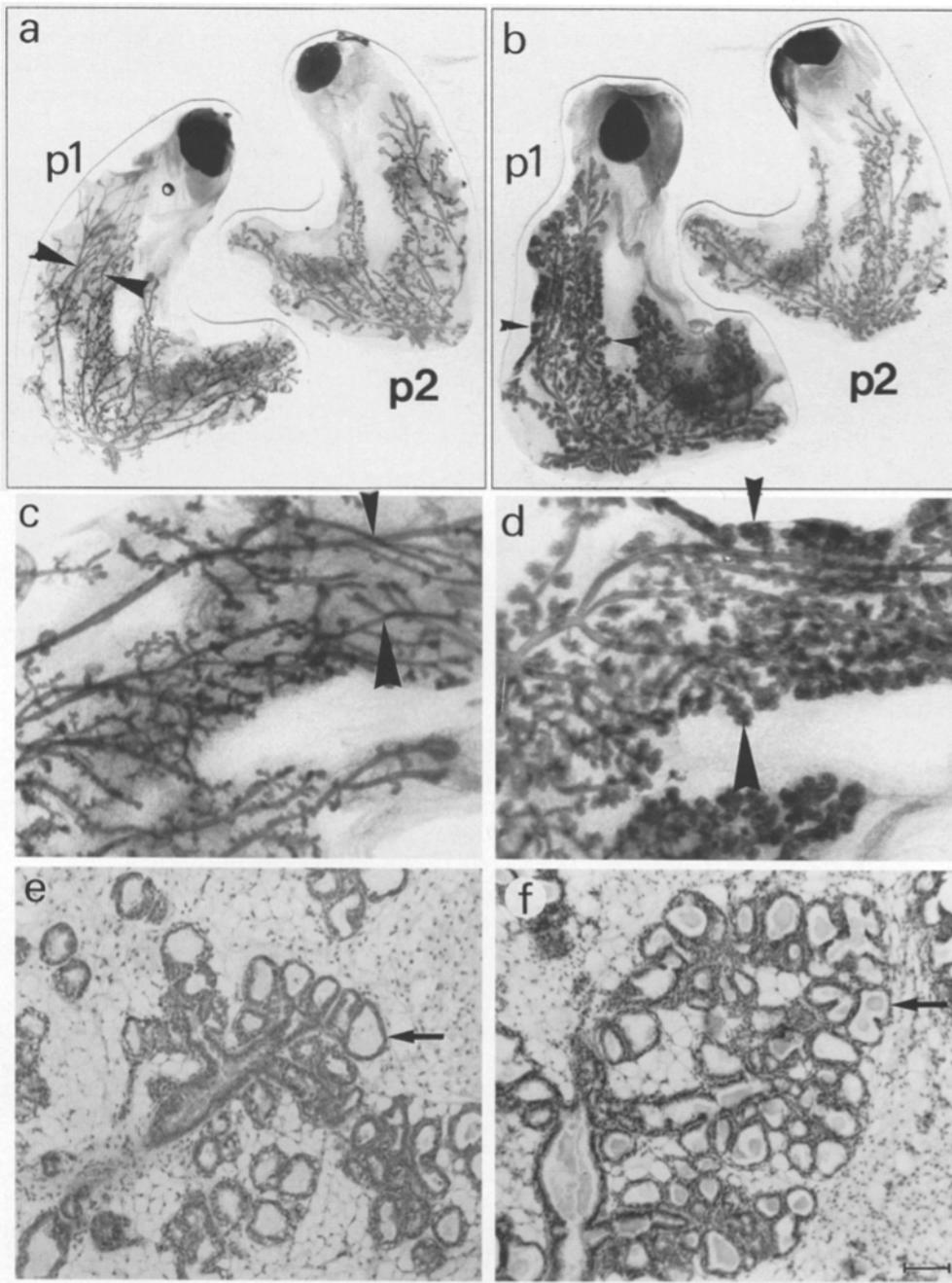
Figure 2. Effect of NRG on mammary gland morphology in organ culture. Mammary glands were cultured for 5 d in modified APIH medium, (*a*, *c*, and *e*) in the absence or (*b*, *d*, and *f*) the presence of 3 nM recombinant NRG. To decrease alveolar development, the concentration of prolactin in the medium was reduced to 3 μg/ml (modified APIH medium). (*a* and *b*) Whole mount staining; p1 and p2 indicate pairs of contralateral glands. (*c* and *d*) Higher magnification of the areas indicated in *a* and *b* by arrowheads demonstrates the increased density and thickness of lobular alveoli in the glands treated with NRG. (*e* and *f*) Histological analysis. Arrows indicate empty (*e*) and filled alveoli (*f*), respectively. Morphometry of lobule formation (see text) was performed in areas that were newly formed during organ culture (see field of Fig. 2, *c* and *d*). Bar, 100 μm.

els for the HGF receptor c-met (and for actin as a control) did not change significantly during mammary gland development.

NRG showed a completely different but again strongly regulated expression pattern: the highest level of transcript is observed during the process of lobulo-alveolar morphogenesis at pregnancy (Fig. 4, *A* and *C*). No NRG mRNA was measured during the virginal period (except for a weak signal in the adult animal at 12 wk), and expression again sharply decreased during lactation and was absent during involution. NRG expression at pregnancy was two orders of magnitude higher than in the adult stage. The presence of a protected band at 259 nt in length using probe pNα indicates that the α_2-subtype of NRG (see Pe-

les and Yarden, 1993; Meyer and Birchmeier, 1993) is expressed during pregnancy; no protected fragment of β variants (at 120 nt) was observed. RNase protection with probe pNN yielded no 240-nt fragment (as in brain, Fig. 4 *D*) and therefore, subforms containing the kringle-like domain are also not expressed in the mammary gland. The expression of the high affinity receptor of NRG was also examined; c-erbB4 is not expressed in the glands of prepubertal mice but is seen at approximately the same level in the virgin animals and in all further stages of development (Fig. 4 *C*).

HGF and NRG expression during mammary gland development was also examined by in situ hybridization: in the adult gland, HGF transcripts are detected in a thin

A   B
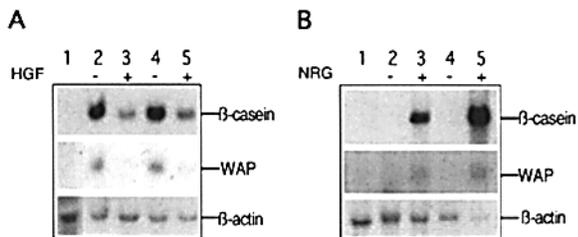
Figure 3. Effect of HGF and NRG on the expression of milk proteins in organ culture. The expression of β-casein and whey acidic protein (WAP) in two pairs of contralateral glands (one member of the pairs was treated, the other non-treated) was analyzed by Northern blotting. (*A*) Effect of 20 ng/ml HGF after 5 d of organ culture in standard APIH medium: (lanes *2* and *4*) RNA from control glands; (lanes *3* and *5*) RNA from treated contralateral glands. (*B*) Effect of 3 nM NRG after 5 d of organ culture in modified APIH medium (see legend to Fig. 2): (lanes *2* and *4*) RNA from control glands; (lanes *3* and *5*) RNA from treated contralateral glands. (lane *1* in *A* and *B*) RNA from noncultivated glands.

layer of mesenchymal cells adjacent to the ductal epithelium (Fig. 5 *A, a*). In contrast, the transcripts for the c-met receptor are localized within the basal epithelial cell layer of the ductal epithelium (Fig. 5 *A, b*). At pregnancy, NRG transcripts are widely detected in the connective tissue around both ductal and alveolar structures (Fig. 5 *B, a* and *b*). In contrast, the receptors c-erbB4 and c-erbB3 are expressed in the epithelium (shown for c-erbB4 in Fig. 5 *B, c*).

Effects of Treatment of Mammary Gland Organ Cultures with Antisense Oligonucleotides

Evidence for an obligatory function of HGF and NRG during branching and lobulo-alveolar morphogenesis of the mammary gland, respectively, is provided by interference with antisense phosphorothioate oligonucleotides. We first established the pattern of expression of both factors during our standard organ culture: HGF mRNA is expressed at the start of culture and slowly decreases, while NRG mRNA expression is initially absent but increases from day 3 on (data not shown, see also Fig. 6 *A*). Treatment of organ cultures with antisense HGF oligonucleotide (see Materials and Methods) abolished the expression of HGF mRNA, whereas sense oligonucleotide had no effect (Fig. 6 *A*, compare lanes *3* and *4*). Treatment of glands with antisense NRG strongly reduced expression of NRG mRNA (Fig. 6 *A*, compare lanes *7* and *8*). Microscopic inspection showed that antisense HGF oligonucleotide blocked ductal branching of the glands (Fig. 6 *B, b* and *d, arrows* and *arrowheads*), while the sense oligonucleotide had no effect (Fig. 6 *B, a* and *c*). Antisense NRG oligonucleotide quenched lobulo-alveolar development to a stage where mainly primary ducts are present (Fig. 6 *B, f* and *h, arrowheads*). Again, the corresponding sense oligonucleotide had no effect on lobulo-alveolar development (Fig. 6 *B, e* and *g*). NRG antisense oligonucleotide also markedly reduced milk protein expression (data not shown). Titration of biological activity of antisense oligonucleotides showed maximum effects at 10 (for HGF) and 3 μM (for NRG).

We also demonstrate that the effects of treatment of mammary glands with antisense oligonucleotides were compensated by addition of appropriate exogenous factors. Thus, HGF fully restores ductal branching in glands treated with HGF antisense oligonucleotides (Fig. 7 *A*, compare *a* and *b*). In the histological analysis, numerous developing side branches were seen after HGF treatment (arrows in Fig. 7 *A, d*, compare with the control in *c*). Similarly, exogenous NRG restores lobulo-alveolar development of the glands treated with NRG antisense oligonucleotide (Fig. 7 *B*, compare *a* and *b*). As shown by histological analysis, antisense NRG oligonucleotide fully quenched alveolar budding; only intraductal precursors were present (Fig. 7 *B, c, arrows*). Addition of exogenous NRG overcame the effect of antisense oligonucleotide, i.e., fully supported budding and maturation (Fig. 7 *B, d*).

Discussion

We demonstrate here that two mesenchymal factors, hepatocyte growth factor and neuregulin, control morphogenesis and differentiation of epithelia during mouse mammary gland development. In organ culture, exogenously added HGF induces intensive branching of mammary ducts, whereas exogenous NRG promotes the formation of lobular alveoli that is followed by terminal differentiation. The respective antisense oligonucleotides abolish morphogenesis and differentiation of the glands, apparently by interfering with expression of endogenous HGF and NRG. Furthermore, the in vivo expression of the two factors closely parallels their functional role, i.e., HGF is produced during the ductal branching period in virgin mice, whereas NRG is strongly induced during lobulo-alveolar development at pregnancy. Thus, two essential steps in mammary gland development can now be explained on a molecular level: the specific morphogens HGF and NRG are induced in the mesenchyme of the gland and then affect the nearby epithelium. Apparently, the expression of HGF and NRG is under tight hormonal control. We also show that the epithelial tyrosine kinase receptor c-met and the corresponding c-erbB receptors are expressed at appropriate times during the development of the mammary gland and therefore likely represent critical components in the response to the mesenchymal signals.

It has previously been found that HGF is an inducer of branching morphogenesis of MDCK (kidney) epithelial cells in collagen gels (Montesano et al., 1991). Similarly, HGF promoted the formation of branching duct-like structures of mammary gland and other epithelial cells in collagen gels (Soriano et al., 1995; V. Brinkmann and W. Birchmeier, manuscript submitted for publication). During mouse embryogenesis, the HGF receptor c-met is expressed in many tubular epithelia, for example, in branching epithelia of kidney, lung, salivary glands, and pancreas (Sonnenberg et al., 1993). In contrast, HGF expression is found in mesenchyme surrounding these tubular epithelia. HGF expression is also detected in primary fibroblasts isolated from human breasts, and c-met is located on ductal epithelial cells (Tsarfaty et al., 1992; Seslar et al., 1993). In the present study, we show that HGF is essential for induction of branching of mammary ducts in organ culture
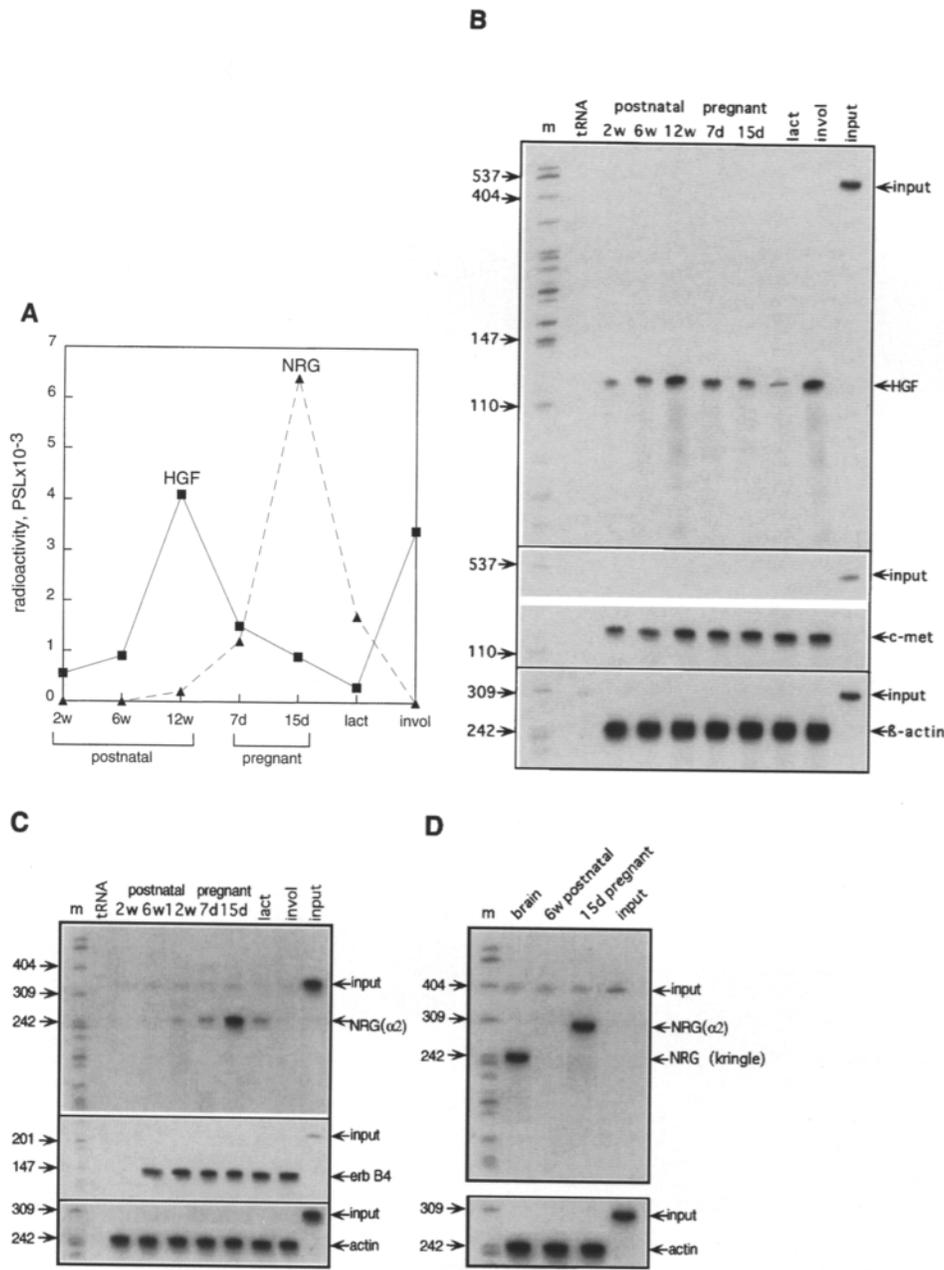
Figure 4. Differential expression of HGF and NRG during mammary gland development. (*A*) Quantitative patterns of expression levels for HGF (■) and the α_2-subtype of NRG (▲) were evaluated from the results of Fig. 4, *B* and *C* by phosphoimage analysis. Radioactive counts are expressed as PSL. (*B*) RNase protection analysis of HGF and c-met expression during the development of mouse mammary glands. Total RNA of whole mammary glands from postnatal (2, 6, and 12 wk old), from pregnant (at 7 and 15 d), and from lactating and involuting mice was subjected to RNase protection with a HGF transcript probe (mHGF1), with a c-met probe (mMet1), or an actin control probe (see Materials and Methods). *m*, Markers (MspI-digested pBR 322); t-RNA, control digest; input, nondigested probe. (*C* and *D*) RNase protection analysis of NRG and c-erb B4 expression in the mouse mammary gland. Total RNA from mammary glands of the different stages was subjected to RNase protection analysis using probe pNα for NRG and probe c-erbB4. In *D* probe pNN for NRG was used (see Meyer and Birchmeier, 1994). *m*, Markers; tRNA, control digest; input, nondigested probe.

(Figs. 1 *d* and 7 *A*). Specific antisense HGF oligonucleotides fully prevented endogenous branching that could be overcome by the addition of exogenous factor. Furthermore, HGF is expressed during virginhood exclusively in a thin layer of mesenchymal cells located around the ducts, whereas the c-met receptor is located in the basal layer of epithelial cells. This indicates that HGF functions in paracrine manner and provides a molecular basis for the local interactions between mesenchymal and epithelial cells. Interestingly, HGF in organ culture inhibited lobulo-alveolar development and milk production, indicating that branching morphogenesis must precede functional differentiation. This is in line with the fact that ductal growth in vivo occurs mainly during virginhood whereas functional differentiation is a subsequent event during pregnancy.

It has previously been shown that NRG can induce growth and differentiation of epithelial cells in vitro, e.g., breast cancer cell lines respond with the production of milk proteins (Wen et al., 1992; Bacus et al., 1993; Culouscou et al., 1993). NRG stimulated the expression of a 22-kD milk protein but inhibited DIP-induced expression of β-casein in HC11 mouse mammary epithelial cells (Marte et al., 1995). During embryogenesis of the mouse, a high concentration of NRG transcripts in the immediate vicinity of epithelia of many developing organs was observed (Meyer and Birchmeier, 1994). We demonstrate here that NRG plays an essential role during differentiation of mammary epithelia in vivo. In organ culture, NRG induces the production of alveoli that are assembled into lobules (Figs. 2 *d* and 7 *B*) and promotes the expression of milk proteins β-casein and WAP. Furthermore, NRG expression in the stroma of the mammary gland is strongly
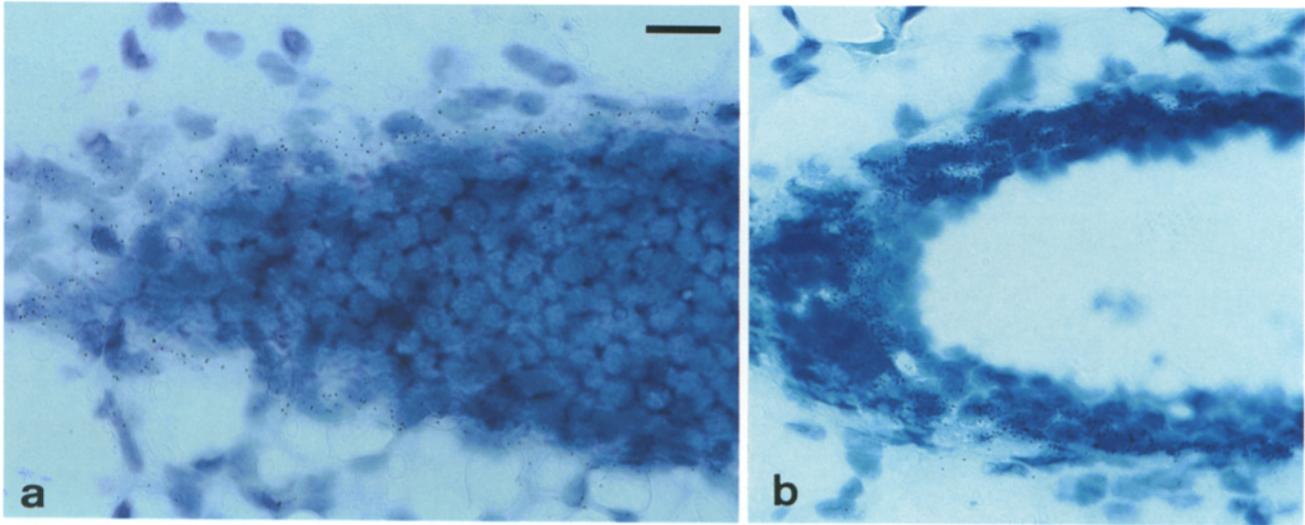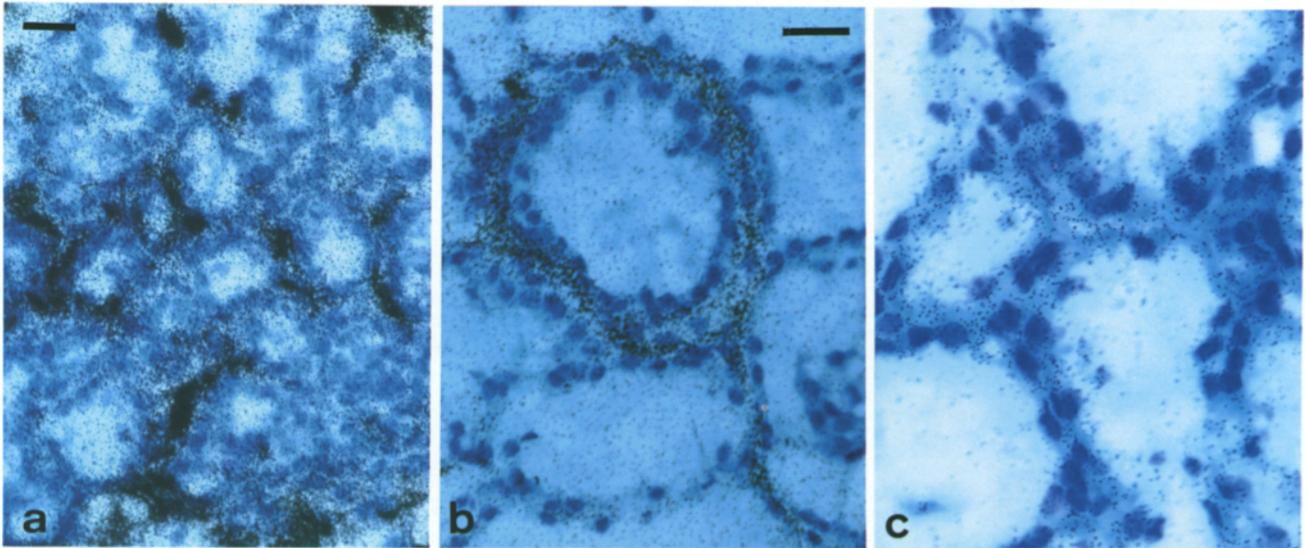
Figure 5. Cellular localization of HGF, c-met, NRG, and c-erbB4 expression in the mammary gland by in situ hybridization and bright field illumination. Sections of mammary glands of 12 wk adult (*A, a* and *b*) and 15 d pregnant mice (*B, a–c*) were hybridized with the HGF antisense probe mHGF2 (*A, a*), with the c-met antisense probe mMet2 (*A, b*), with the NRG antisense probe pN1 (*B, a* and *b*), or with the c-erbB4 antisense probe (*B, c*). Bars: (*A, a* and *b*) 20 μm; (*B, a*) 40 μm; (*B, b* and *c*) 20 μm.

induced at pregnancy, i.e., at times when lobulo-alveolar structures develop; specific antisense oligonucleotides inhibited lobule formation and production of milk proteins. We also show that an α-isoform of NRG, i.e., a mesenchymal form, is expressed during mammary gland morphogenesis. Thus, NRG is a further key player in the mesenchymal-epithelial interactions of the mammary gland which, in comparison to HGF, produces a qualitatively different response. Signal transduction by c-met and c-erbB receptors have recently been examined: HGF acts through a single receptor that activates characteristic downstream substrates (Hartmann et al., 1994; Ponzetto et al., 1994; Weidner et al., 1995). In contrast, NRG binding and signal transduction appears to involve complex hetero-oligomerization between three c-erbB receptors (Plowman et al., 1993; Sliwkowski et al., 1994) and therefore, multiple signal transduction pathways are involved (Ben Levy et al., 1994; Carraway and Cantley, 1994; Prigent and Gullick, 1994). Further understanding of the complex interplay of downstream targets of c-met and the c-erbBs is required to explain the different signaling capacities of the two receptor systems in mammary gland development.

Morphogenesis and regression of the mammary gland is accompanied by extensive extracellular matrix (ECM) remodeling that is mediated by ECM-degrading proteases (Talhouk et al., 1991; Sympson et al., 1994). This is in line
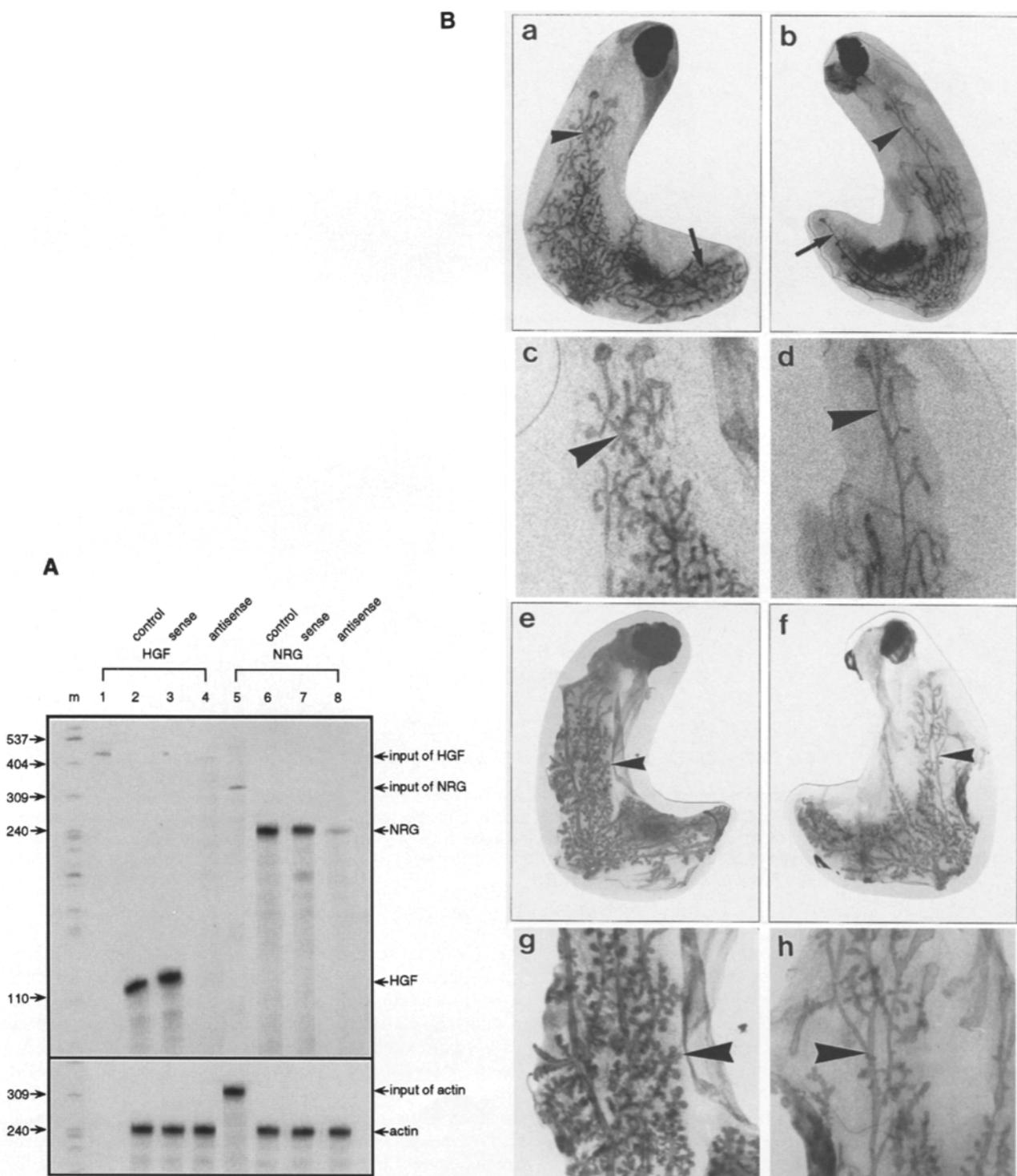
Figure 6. Effect of HGF and NRG antisense oligonucleotides in mammary gland organ culture. Mammary glands were cultured in standard APIH medium for 4 d (for HGF interference) and 6 d (for NRG interference) in the presence of the corresponding sense or antisense oligonucleotide phosphorothioates: (*A*) RNase protection analysis of HGF (lanes 2–4) and NRG expression (lanes 6–8). (lanes *1* and *5*) Input band; (lanes *2* and *6*) untreated gland; (lanes *3* and *7*) sense oligonucleotide; (lanes *4* and *8*) antisense oligonucleotide (10 and 3 μM for interference with HGF and NRG transcript formation, respectively). *m*, Marker. (*B*) Morphological evaluation of the effect of treatment with antisense oligonucleotides: (*a* and *c*) 10 μM HGF sense oligonucleotide; (*b* and *d*) 10 μM HGF antisense oligonucleotide; (*e* and *g*) 3 μM NRG sense oligonucleotide; (*f* and *h*) 3 μM NRG antisense oligonucleotide. *c*, *d*, *g*, and *h* show higher magnifications of the indicated areas in *a*, *b*, *e*, and *f* (see *arrows*).
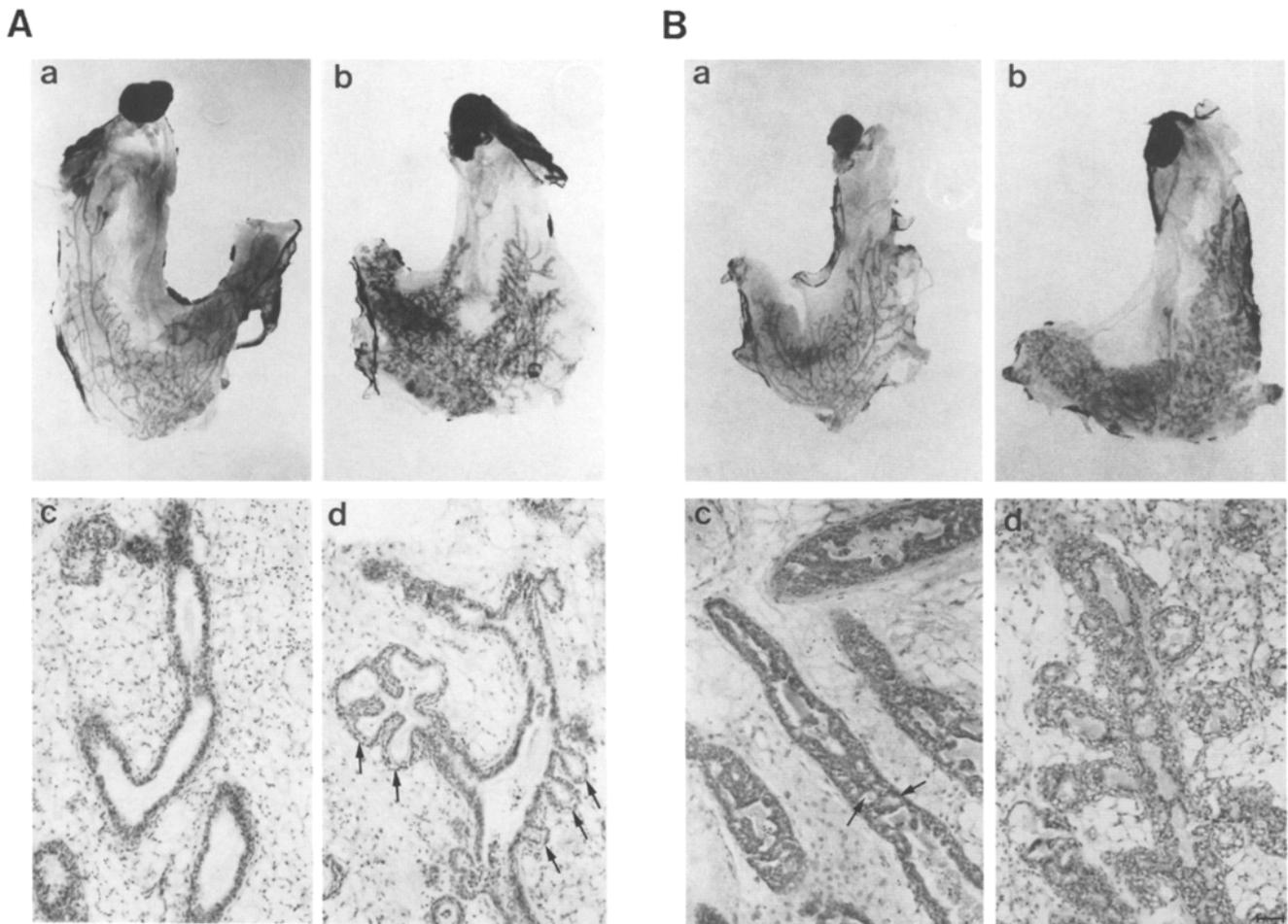
Figure 7. Restoration of branching morphogenesis and differentiation in antisense oligonucleotide-treated organ cultures by the addition of exogenous HGF and NRG. (*A*) Paired glands were cultured for 4 d in the presence of 10 μM HGF antisense oligonucleotide in the absence (*a* and *c*) and presence of 20 ng/ml exogenous HGF (*b* and *d*). Arrows in (*d*) indicate developing side branches. (*B*) Paired glands were cultured for 6 d in the presence of 3 μM NRG antisense oligonucleotide in the absence (*a* and *c*) and presence of 3 nM NRG (*b* and *d*). Arrows in *c* indicate intraductal alveolar precursors.

with the fact that HGF induces synthesis and secretion of proteases in epithelial cells (Pepper et al., 1992). Protease activities are also increased during involution (Talhouk et al., 1992), i.e., at times when HGF is highly expressed. In cell culture of mammary epithelial cells, NRG induces the expression of cell adhesion molecules such as ICAM-I and E-cadherin (Bacus et al., 1993; Staebler et al., 1994), which might be important for alveolar maturation and tissue-specific gene regulation. Various growth factors such as EGF, TGFα, and TGFβ1 are suppressors of HGF expression of fibroblasts in cell culture (Gohda et al., 1992; Seslar et al., 1993). These factors are produced in the epithelium of the mammary gland during postnatal development and influence ductal and alveolar development mainly in an autocrine manner (Vonderhaar, 1987; Coleman et al., 1988; Snedeker et al., 1991; Silberstein et al., 1992; Jhappan et al., 1993; Pierce et al., 1993; Spitzer et al., 1995). Other growth and differentiation factors are also expressed in the mammary gland, i.e., several members of the FGF and wnt families (Gavin and McMahon, 1992; Coleman-Krnacik and Rosen, 1994; Ulich et al., 1994). The regulation of HGF and NRG synthesis in the mesenchyme dur-

ing postnatal mammary gland development is obviously under control of systemic hormones. Mammary stromal cells in fact respond to hormonal signals (Haslam and Counterman, 1991), and glucocorticoids downregulate HGF expression in cell culture (Gohda et al., 1992). Increasing levels of glucocorticoids in the circulation might thus be responsible for decreasing HGF levels during pregnancy. In the mammary gland, reciprocal interactions of mesenchyme and epithelia are also apparent: our experiments show that mammary fat pads cleared of epithelia do not express HGF or NRG. The molecular nature of these epithelial signals is not yet known.

In conclusion, morphogenesis and differentiation of the mammary gland is regulated by a complex interplay of systemic hormones and local factors: mesenchymal cells of the fat pad appear to respond to hormonal stimuli and then produce short-range signals that control growth, morphogenesis, and differentiation of the epithelia. Our data suggest that puberty hormones, directly or indirectly, control the synthesis of HGF in the local mesenchyme, and demonstrate that this mesenchymal factor induces branching of epithelial ducts. At a later stage of development,

pregnancy hormones appear to control the synthesis of NRG in the local mesenchyme, and we demonstrate that this mesenchymal factor induces lobulo-alveolar development and milk production of the mammary gland epithelium. Thus, the molecular nature of two sequentially expressed factors has been elucidated, that control the postnatal development of the mammary gland. Many factors have been tested for functional effects on mammary gland epithelium. HGF and NRG are the only known factor that can induce branching and lobulo-alveolar morphogenesis in this system. Together with the high levels of HGF and NRG during puberty and pregnancy, respectively, and the tight regulation of gene expression in the local mesenchyme, this provides strong evidence for a crucial role of the two factors in morphogenesis and differentiation of the mammary gland.

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