Published October 19, 1998

Reconstitution of Mammary Gland Development In Vitro: Requirement of c-met and c-erbB2 Signaling for Branching and Alveolar Morphogenesis

Catherin Niemann, Volker Brinkmann, Eva Spitzer, Guido Hartmann, Martin Sachs, Helga Naundorf, and Walter Birchmeier

Max-Delbrück-Center for Molecular Medicine, D-13122 Berlin, Germany

Abstract. We have established a cell culture system that reproduces morphogenic processes in the developing mammary gland. EpH4 mouse mammary epithelial cells cultured in matrigel form branched tubules in the presence of hepatocyte growth factor/scatter factor (HGF/SF), the ligand of the c-met tyrosine kinase receptor. In contrast, alveolar structures are formed in the presence of neuregulin, a ligand of c-erbB tyrosine kinase receptors. These distinct morphogenic responses can also be observed with selected human mammary carcinoma tissue in explant culture. HGF/SF-induced branching was abrogated by the PI3 kinase inhibitors wortmannin and LY294002. In contrast, neuregulin-induced alveolar morphogenesis was inhibited by the MAPK kinase inhibitor PD98059. The c-met–mediated response could also be evoked by transfection of a c-met specific substrate, Gab1, which can activate the PI3 kinase pathway. An activated hybrid receptor that contained the intracellular domain of c-erbB2 receptor suffices to induce alveolar morphogenesis, and was observed in the presence of tyrosine residues Y1028, Y1144, Y1201, and Y1226/27 in the substrate-binding domain of c-erbB2. Our data demonstrate that c-met and c-erbB2 signaling elicit distinct morphogenic programs in mammary epithelial cells: formation of branched tubules relies on a pathway involving PI3 kinase, whereas alveolar morphogenesis requires MAPK kinase.

Key words: development of mammary gland • hepatocyte growth factor • neuregulin • PI3 kinase • MAPK kinase

Growth and morphogenesis of epithelia are essential processes in the development of many organs, which are driven not only by an intrinsic genetic program but also by signals provided by neighboring cells and tissues. Accordingly, studies performed in organ culture and transplantation experiments have demonstrated that growth and morphogenesis of epithelia from the salivary gland, kidney, lung, or the mammary gland are controlled by mesenchymal–epithelial interactions (Grobstein, 1953; Spooner and Wessells, 1970; Saxen 1987; Sakakura, 1991). The identification of molecules that provide the essential signals exchanged in mesenchymal–epithelial interactions is an area of active research. Recent evidence suggests that morphogenic programs of epithelia can be triggered by mesenchymal factors that signal via tyrosine kinase receptors (Montesano et al., 1991*a*,*b*; Schuchardt et al., 1994; Sutherland et al., 1996; for review see Birchmeier and Birchmeier, 1993). This is also supported by genetic experiments in mice that illustrate the importance of mesenchymal ligands of epithelial tyrosine kinases during development of the kidney, lung, and liver (Peters et al., 1994; Schuchardt et al., 1994; Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Similarly, branching of trachea in *Drosophila* is controlled by a fibroblast growth factor-like molecule produced in surrounding mesenchymal cells (Sutherland et al., 1996).

We have previously identified two mesenchymal ligands of epithelial receptor tyrosine kinases, hepatocyte growth factor/scatter factor (HGF/SF)[1] and neuregulin, to be im-

Address all correspondence to W. Birchmeier, Max-Delbrück-Center for Molecular Medicine, Robert-Rossle-Strasse 10, D-13122 Berlin, Germany. Tel.: (49) 30-9406-3800. Fax: (49) 30-9406-2656. E-mail: wbirch@mdc-berlin.de

V. Brinkmann's present address is Max-Planck-Institute for Infection Biology, Monbijoustrasse 2, 10117 Berlin, Germany.

E. Spitzer's present address is Institute for Medicine of Molecular Diagnostic, Frankfurter Allee 65, 10247 Berlin, Germany.

G. Hartmann's present address is Glaxo Wellcome Research and Development, Gunnelswood Road, Stevenage SG1 2NY, UK.

1. *Abbreviations used in this paper*: EGF, epidermal growth factor; HGF/SF, hepatocyte growth factor/scatter factor; KGF, keratinocyte growth factor; NGF, nerve growth factor.

Published October 19, 1998

portant for development of the mammary gland (Yang et al., 1995). In whole organ cultures of mouse mammary glands, HGF/SF promotes branching of ductal trees and inhibits terminal differentiation, as assessed by the expression of milk proteins. In contrast, neuregulin stimulates lobulo-alveolar differentiation and the production of milk proteins. Thus, different growth factors elicit distinct responses in organ culture of the mammary gland. This finding raises the question whether different cellular populations react to these factors, and how these different responses are evoked on a molecular level.

In collagen matrix, HGF/SF induces the formation of branched tubules from epithelial cells derived from the kidney (MDCK), mammary gland, and other organs (Montesano et al., 1991*a*,*b*; Berdichevsky et al., 1994; Brinkmann et al., 1995; Soriano et al., 1995). HGF/SF signals are mediated by the receptor tyrosine kinase c-met; in vivo, c-met is expressed by various epithelial cells, whereas HGF/SF transcripts are found in the mesenchymal compartment (Bottaro et al., 1991; Naldini et al., 1991; Sonnenberg et al., 1993; Weidner et al., 1993). The phosphorylated c-met receptor binds substrates such as PI3K, PLC-γ, Grb2, and others (Ponzetto et al., 1994; Fixman et al., 1997). A recently identified c-met-specific substrate, Gab1 (Weidner et al., 1996; Nguyen et al., 1997) suffices to elicit branching morphogenesis in kidney epithelial cells.

Neuregulin has been shown to affect growth and differentiation of epithelial and other cell types in vitro (Peles et al., 1992; Bacus et al., 1993; Falls et al., 1993; Marchionni et al., 1993; Shah et al., 1994; Staebler et al., 1994; Marte et al., 1995). Neuregulin signals are mediated by direct interaction with the high affinity receptors c-erbB4 or c-erbB3; in addition, c-erbB2 acts as an essential coreceptor for the transmission of neuregulin signals (Plowman et al. 1993; Carraway and Cantley, 1994; Carraway et al., 1994; Sliwkowski et al., 1994). Neuregulin is expressed in neuronal and mesenchymal cells during mouse development (Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994; Yang et al., 1995), whereas the receptors are found in epithelial and other cell types (Kraus et al., 1989; Press et al., 1990; Prigent et al., 1992; Plowman et al., 1993; Meyer et al., 1997).

Previously, analyses of HGF/SF and neuregulin-evoked responses in epithelial cells were mainly conducted with cells grown in monolayers; such culture conditions do not allow the formation of the complex three-dimensional structures observed during organ development. In contrast, organ culture systems provide a good model for the analysis of morphogenic events. However, they preclude a biochemical analysis of signaling cascades activated by morphogenic factors due to their complex cellular composition. Obviously, a lack of appropriate systems exists which allows the study of signaling cascades responsible for morphogenic responses, for instance in mammary epithelial cells.

Here we use EpH4 mammary epithelial cells grown on matrigel and observe that two growth factors that signal via tyrosine kinase receptors, HGF/SF and neuregulin, elicit fundamentally distinct morphogenic responses in these pluripotent cells: HGF/SF induces the formation of branched tubules, and neuregulin evokes the formation of alveolar structures. The complex structures generated in culture resembles the ones formed during mammary gland development. The HGF/SF-induced tubular structures were also observed after transfection of a c-met substrate, Gab1. The alveolar structures produced by neuregulin could also be induced by nerve growth factor in cells transfected with a trk/c-erbB2 hybrid receptor; this indicates that the signals provided by c-erbB2 suffice to induce alveoli. By the use of mutant receptors and specific inhibitors, we could show that PI3 kinase provides an essential signal for branching morphogenesis; in contrast, the MAP kinase pathway plays an essential role for the formation of alveolar structures. Thus, in this cell culture system one single cell type, EpH4, responds by two entirely different morphogenic programs upon stimulation of distinct tyrosine kinase receptors. The distinct responses can moreover be correlated with the activation of different essential signaling cascades.

Materials and Methods

Recombinant HGF/SF and Neuregulin

Recombinant HGF/SF was produced in Sf9 insect cells using the baculovirus expression system as described (Weidner et al., 1993; Brinkmann et al., 1995). For production of neuregulin, the coding sequence (corresponding to amino acids 20–239) of a mouse β_2 isoform (Wen et al., 1992; Meyer and Birchmeier, 1994) was fused in frame to the signal sequence of the baculovirus expression vector pAcGP67 (Dianova, Hamburg, Germany) followed by bulk transfection of Sf21 insect cells using calcium phosphate. Recombinant baculovirus was selected by plaque screening and biological assay, and high-titer virus was used to infect Sf21 cells in serum-free Excell 401 medium (Seralab, Crowley Down, UK) supplemented with 0.1% Pluronic F-68 (Life Technologies, Gaithersburg, MD). Conditioned medium was clarified after 3 d of culture, and neuregulin was purified on a heparin–Sepharose column (Pharmacia, Uppsala, Sweden) by the elution by a 0.4–1.2-M NaCl gradient. The fractions were tested for biological activity of neuregulin in the morphology assay on MDA MB 453 cells (Plowman et al., 1993) and for protein on a silver-stained gel. Neuregulin with highest biological activity eluted at 0.8–1.0 M NaCl.

Cell Culture on Matrigel

EpH4 cells are a derivative of IM-2 and were originally isolated from mammary tissue of a mid-pregnant mouse (Reichmann et al., 1989; Brinkmann et al., 1995; Lopez-Barahona et al., 1995). From EpH4 cells we subcloned the variant K6 (called EpH4/K6) which exhibits a pronounced morphogenic potential on matrigel.

Matrigel (basement membrane from Engelbreth-Holm-Swarm tumor) was obtained from Collaborative Biomedical Products (Serva, Germany), and 6-cm culture dishes were coated with 400 μl of this solution on ice. After incubation for 1 h at 37°C to allow the matrix to gel, 3.5×10^5 EpH4 cells were plated in DME containing 10% fetal bovine serum and the following hormones: 3 μg/ml bovine prolactin (Sigma Chemical Co., St. Louis, MO); 5 μg/ml insulin (Sigma Chemical Co.); 1 μg/ml hydrocortisone (Merck, Darmstadt, Germany), and neuregulin or HGF/SF at concentrations of 20 ng/ml (100 U/ml) or 3 nM (1 U/ml), respectively. After 1 d of culture, medium was replaced by serum-free DME containing factors and hormones and exchanged daily. Experiments were terminated after 6 d of culture. Inhibitors of the PI3 kinase (10 nM wortmannin and 25 μM LY294002) and MAPK kinase (50 μm PD98059) were added twice a day and experiments were terminated after 3 d of culture (Alessi et al., 1995; Derman et al., 1995; Keely et al., 1997; Khwaja et al., 1997).

Transfection of EpH4 Cells with cDNAs

Hybrid receptors containing the extracellular portion of trk (the nerve growth factor receptor) and the transmembrane and cytoplasmic region of epithelial receptor tyrosine kinases (trk/c-met, trk/KGFR, and trk/c-erbB2) have been described (Sachs et al. 1996). cDNA encoding a new hybrid receptor containing the cytoplasmic domain of c-erbB4, trk/c-erbB4, was

Published October 19, 1998

constructed. Mutations were introduced into the kinase domain of trk/c-erbB2 by an exchange of wild-type and mutant c-erbB2 sequences (Ben-Levy et al., 1994): trk/c-erbB2-P1 lacks all COOH-terminal substrate-binding domain of c-erbB2 but the sequences around the ultimate tyrosine residue, Y1253, which is directly fused to the c-erbB2 kinase domain; trk/c-erbB2Y1253F contains the complete substrate-binding region except the fifth tyrosine residue, which is mutated to phenylalanine (Y1253F). The cDNAs of these hybrid receptors and of Gab1 (Weidner et al., 1996) were cloned into the pBAT expression vector; the expression plasmids were cotransfected with pSV2neo into EpH4/K6 mammary epithelial cells by the calcium phosphate technique. Cell clones producing the trk-hybrid receptors were identified (Sachs et al., 1996) and characterized for their morphogenic response on matrigel after addition of nerve growth factor (NGF). Control transfections were performed with pSV2neo only.

Explant Cultures of Human Mammary Tumors

Human breast tumors cultivated as xenografts in the mammary glands of nude mice (Naundorf et al., 1992) were dissected and cut into 1-mm pieces in medium 199 (GIBCO BRL, Eggenstein, Germany) supplemented with 5 μg/ml insulin and recombinant HGF/SF or neuregulin at a concentration of 20 ng/ml (100 U/ml) and 3 nM (1 U/ml), respectively. The tissue pieces were placed into 25 cm^2 Falcon plastic flasks at a density of 30 explants per flask (Falcon Plastics, Cockeysville, MD). The explants were cultured for 8 d under an atmosphere of 5% CO_2, with daily medium changes (Binas et al., 1992). For histological analysis, explants were fixed in 4% formalin, embedded in paraffin, sectioned (5 μm), and then stained with hematoxylin/eosin (Rivera, 1971).

Northern Hybridization

Total RNA was isolated from dispase-treated EpH4 cell aggregates as described (Chomczynski and Sacchi, 1987). 5–20 μg RNA were electrophoresed in 1% agarose–formaldehyde gels and transferred to Hybond-C-extra membranes (Amersham, Little Chalfont, UK) and hybridized to ^{32}P-labeled cDNA probes for β-casein (Binas et al., 1992) and β-actin, respectively. The probes were labeled by random priming (10^9 cpm/μg; Feinberg and Vogelstein, 1984). Hybridization signals were autoradiographed and analyzed with a phosphoimager.

Light, Confocal, and Electron Microscopy

Cell aggregates of EpH4 cells after 6 d of culture were visualized using a Zeiss Axiovert 135 inverse microscope equipped with Nomarski optics (Carl Zeiss Inc., Thornwood, NY). For histological examination, organoids were fixed in situ with 2.5% glutaraldehyde, postfixed with OsO_4, and contrasted with tannic acid and uranyl acetate. Specimens were dehydrated in a graded ethanol series and embedded in Epon 812. For light microscopy, semithin sections (0.5 μm) were stained with toluidin blue and analyzed in a Zeiss Axiophot light microscope. Ultrathin sections (50–70 nm) were contrasted with lead citrate and analyzed in a Zeiss EM 10 electron microscope.

For immunohistological analysis, organoids were fixed with 4% formaldehyde in PBS and treated with 0.5% Triton X-100. Antibodies used were monoclonal rat anti–E-cadherin (DECMA-1, Vestweber and Kemler, 1985), rabbit anti–β-casein (Binas et al., 1992), CY3-conjugated goat anti–

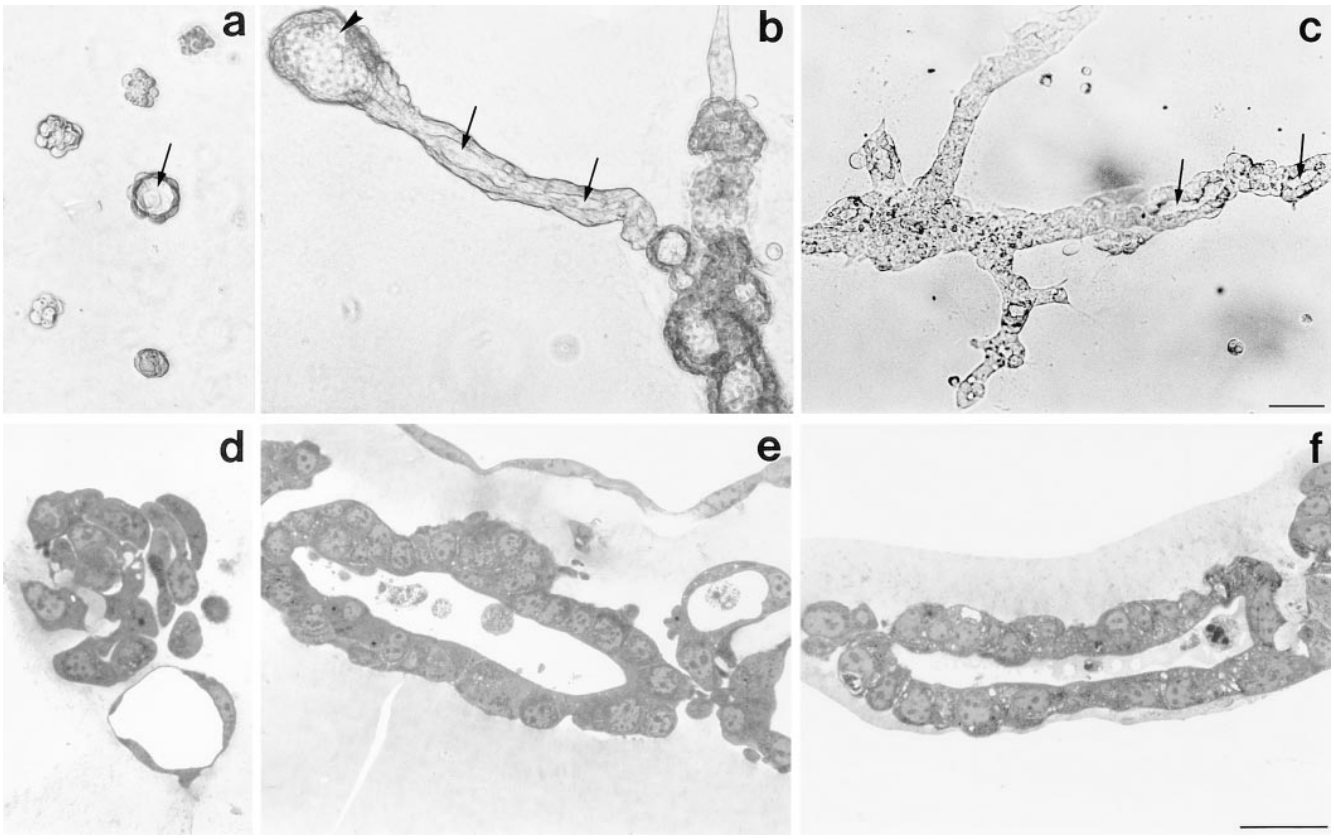

Figure 1. Effect of HGF/SF on the morphology of EpH4/K6 mammary epithelial cells on matrigel. Cells were cultured in medium containing hormones in the absence (*a* and *d*) or in the presence of HGF/SF (*b* and *e*). Small aggregates with (*arrow*) or without lumen were formed in the absence of factor. HGF/SF induced branching tubules with prominent lumina (*arrows* in *b*) and end buds (*arrowheads* in *b*). Gab1-transfected EpH4/K6 cells exhibited a similar morphogenic response in the absence of HGF/SF (*c* and *f*). *Top*, micrographs using Nomarski optics; *bottom*, semithin sections of Epon-embedded cultures. Bars: (*a*) 50 μm; (*c*) 25 μm.

Published October 19, 1998

rat and CY5-conjugated goat anti–rabbit antibodies. Nuclei were stained with quinacrine mustard or Sytox (Molecular Probes, Eugene, OR). The whole mounts were analyzed using a Leica TCS confocal microscope (Leica, St. Gallen, Switzerland).

Results

Effect of HGF/SF on Morphogenesis and Functional Differentiation of EpH4 Mammary Epithelial Cells

EpH4/K6 mouse mammary epithelial cells (Reichmann et al., 1989; refer to Materials and Methods) cultured on matrigel in the presence of hormones grow slowly, form small spheroids and secrete milk proteins. Lumina in these structures are surrounded by a single layer of epithelial cells (Fig. 1 *a*). This resembles the appearance of primary or CID9 mammary epithelial cells grown in matrigel (Bissell and Ram, 1989; Wicha et al., 1982; Wilde et al., 1984; Hahm and Ip, 1990*a*,*b*; Schmidhauser et al., 1992). When HGF/SF was added to the EpH4/K6 cell cultures, fast growing, long tubular structures that can reach a length of several millimeters were observed (Fig. 1 *b*). 44% of the HGF/SF-induced aggregates were tubular, of which 26% were branched (two to seven branches per structure, Fig. 2 *B*). In control cultures, 90% of the aggregates were small and round, and only 3% showed an elongated structure (Fig. 2 *A*). The K6 subline of EpH4 cells produces particularly pronounced tubular structures in the presence of HGF/SF; however, these structures were also observed with the original EpH4 cells, albeit at lower frequency. 20 ng/ml of HGF/SF produced an optimal morphogenic response of EpH4/K6 cells. Histological analysis confirmed that in the absence of HGF/SF, EpH4/K6 cells form loose aggregates or small spheroids, and that the lumina are lined by a layer of flattened cells (Fig. 1 *d*; see also below). The tubular structures induced by HGF/SF consist of several layers of cuboidal cells lining the elongated lumina (Fig. 1 *e*). We also tested other growth factors: epidermal growth factor (EGF) stimulated growth but did not induce branched tubules, whereas keratinocyte growth factor (KGF) had a moderate effect on growth but did not elicit morphogenic responses.

EpH4/K6 cells were transfected with the cDNA of the recently identified new substrate of c-met, Gab1 (Weidner et al., 1996; refer to Materials and Methods). When grown in matrigel, the transfected cells produced tubular and branched structures, now in the absence of HGF/SF (Fig. 1 *c*). This was not observed with cells transfected with control plasmids (pSV2neo). The histology of the Gab-1–induced structures was identical to those observed in the presence of HGF/SF (Fig. 1 *f*). Branched tubular structures could also be produced in EpH4/K6 cells transfected

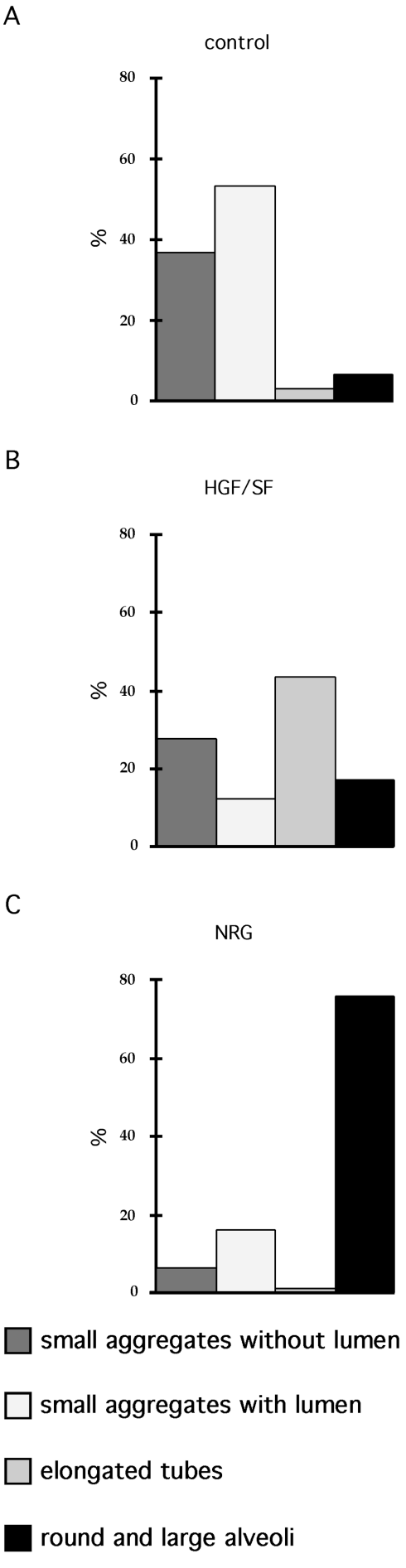

Figure 2. Statistical analysis of the effects of HGF/SF (*B*) and neuregulin (*C*) on the morphology of EpH4/K6 cells on matrigel. Control aggregates in the absence of the factors are analyzed in *A*. From three sets of experiments, a total of 1,050 structures were examined. The different groups of structures are indicated in the box, i.e., small aggregates without or with lumen (smaller than 50 μm), elongated tubes, and round large alveoli (larger than 50 μm).

Published October 19, 1998

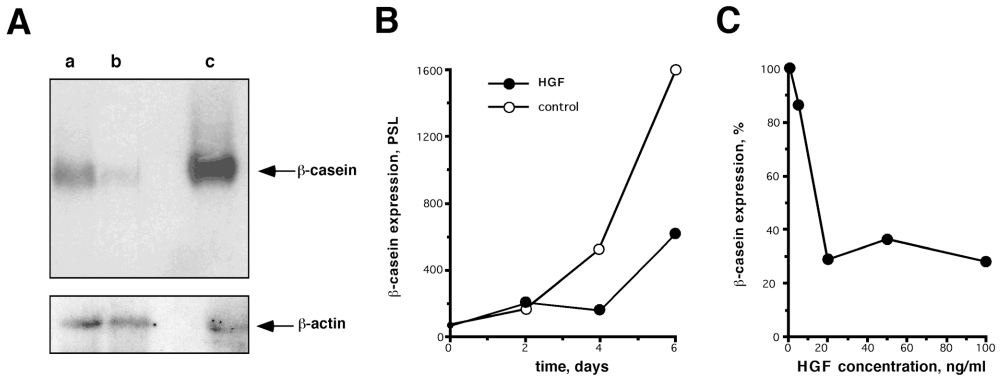

Figure 3. Effect of HGF/SF on the expression of β-casein in EpH4 mammary epithelial cells cultured on matrigel in the presence of lactogenic hormones. (*A*) Representative autoradiograph of a Northern blot showing the inhibition of β-casein expression by HGF/SF after 6 d of culture (lane *b*); control in the absence of HGF/SF (lane *a*). RNA from mammary glands of a pregnant mouse served as a control for β-casein expression (lane *c*). (*B*) Time course of expression of β-casein in EpH4 cells over 6 d of culture in the absence (○) or presence of HGF/SF (●), as analyzed by Northern blotting. Radioactive counts are expressed using a phosphoimager. (*C*) Concentration dependence on HGF/SF of the inhibition of β-casein expression. Control in the absence of HGF/SF was set to 100%.

with a trk/c-met receptor hybrid (Sachs et al., 1996) when the cells were stimulated by NGF (data not shown). EpH4/K6 cells transfected with trk/KGFR or trk did not produce branched tubules in the presence of NGF. Together, these data demonstrate that the formation of branching structures of EpH4 cells requires specific signals provided by the c-met receptor tyrosine kinase.

The effect of HGF/SF on the expression of β-casein during formation of tubules was analyzed by Northern blot hybridization (Fig. 3). HGF/SF strongly inhibited β-casein mRNA expression in a concentration-dependent fashion; inhibition was maximal at 20 ng/ml HGF/SF (Fig. 3 *C*). Apparently, formation of branching tubules of EpH4 mammary epithelial cells does not allow concomitant functional differentiation.

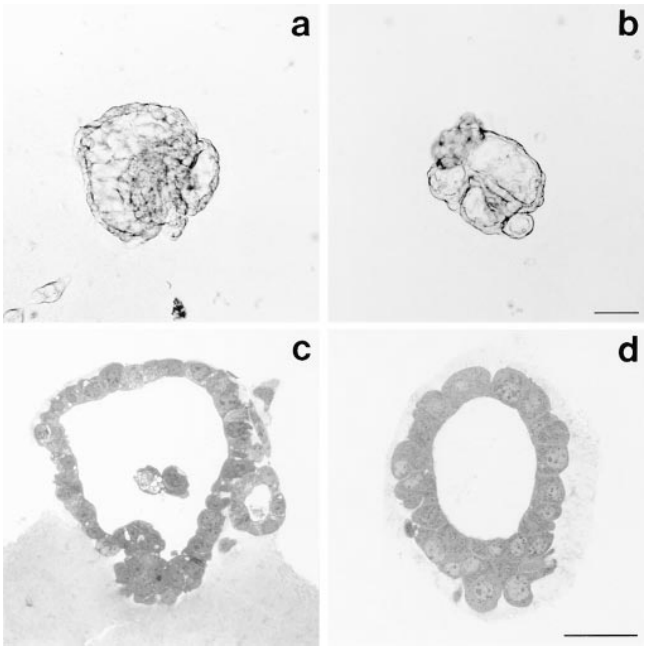

Figure 4. Effect of neuregulin on morphogenesis of mammary epithelial cells EpH4/K6 on matrigel. Cells were cultured for 6 d in medium containing hormones in the presence of neuregulin (*a* and *c*; for the control in the absence of neuregulin refer to Fig. 1 *a*). Large alveolar structures were formed which were predominantly built up by a single layer of columnar epithelial cells. Identical structures were observed in EpH4/K6 cells transfected with a trk/c-erbB2 hybrid receptor and stimulated with NGF (*b* and *d*). *Top*, micrograph using Nomarski optics; *bottom*, semithin sections of Epon-embedded cultures. Bars: (*b*) 50 μm; (*d*) 25 μm.

Effect of Neuregulin on the Morphogenesis of EpH4 Mammary Epithelial Cells

Neuregulin produces an entirely different response in EpH4/K6 cells: fast growing, large alveolar-like structures are formed (Fig. 4 *a*, compare with the small aggregates in the control in Fig. 1 *a*). The concentration of 3 nM of neuregulin was more effective than higher or lower concentrations. Histological analysis demonstrated that the alveoli consisted of single-layered, highly columnar epithelial cells (Fig. 4 *c*, see also below). Statistical analysis revealed that 75% of the structures had an alveolar-like morphology and contained a lumen (Fig. 2 *c*): the aggregates were generally larger than 100 μm, and 15% were larger than 200 μm. The original EpH4 cells produced the same structures, but at lower frequencies. In contrast, only 5% of the aggregates formed by cells grown in the absence of neuregulin exhibited large alveolar structures (Fig. 2 *A*). Other growth factors tested, like EGF or KGF, did not induce alveolar structures (data not shown).

EpH4/K6 cells were then transfected with the cDNAs of receptor hybrids consisting of extracellular trk (the NGF receptor) and intracellular c-erbBs (Sachs et al., 1996; see also Materials and Methods). Remarkably, stable clones of EpH4/K6 mammary epithelial cells which expressed the hybrid trk/c-erbB2 receptor produced alveolar-like structures in the presence of NGF (Fig. 4 *b*, see also the statistics in Fig. 5 *A*). These structures were histologically identical to the neuregulin-induced structures of EpH4/K6 cells (Fig. 4 *d*). Control transfectants with pSV2neo, trk, or a trk/KGFR hybrid (Sachs et al., 1996) did not show a morphogenic response in matrigel (data not shown). Similarly, stable cell clones expressing a trk/c-erbB4 hybrid receptor did not form alveolar structures in response to NGF, although NGF-dependent tyrosine phosphorylation of the receptor was observed (data not shown). We then

Published October 19, 1998

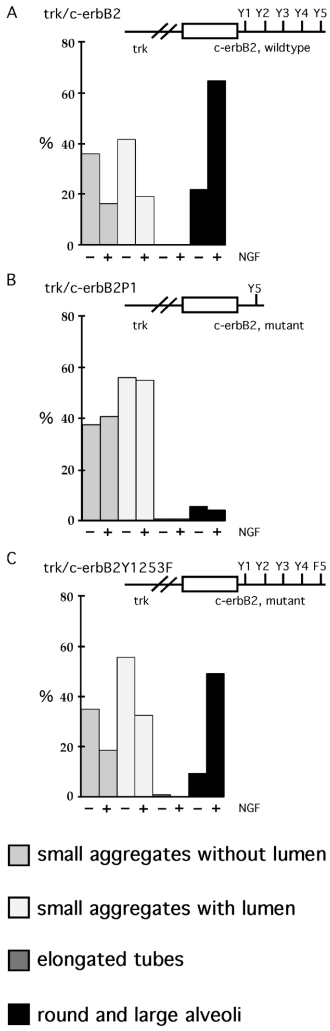

Figure 5. Statistical analysis of NGF-induced morphological responses of EpH4/K6 cells transfected with the hybrid receptors trk/c-erbB2 (*A*), trk/c-erbB2-P1 (*B*) or trk/c-erbB2Y1253F (*C*). For each set of experiments, a total of 450 structures from each of four different stably expressing clones were analyzed. The aggregates are defined as in Fig. 2 (*inset*).

examined EpH4/K6 cells expressing mutants of trk/c-erbB2 to identify essential docking sites in the erbB2 receptor that elicit this morphogenic response: a mutant of c-erbB2 that contains only the ultimate tyrosine residue P1 (Y1253; Ben-Levy et al., 1994) was inactive (Fig. 5 *B*), whereas a mutant containing the residual tyrosine residues (Y1028, Y1144, Y1201, and Y1226/27) produced alveoli (Fig. 5 *C*). This indicates that tyrosine residue Y1028, Y1144, Y1201, and Y1226/27 of c-erbB2, but not Y1253, are important for mediating the neuregulin-induced morphogenic response. Moreover, these findings demonstrate that an activated trk/erbB2 receptor is capable of eliciting a specific morphogenic response in mammary epithelial cells, formation of alveolar-like structures, in the absence of active c-erbB3 and c-erbB4 coreceptors.

Effect of Inhibitors of PI3 Kinase and MAPK Kinase on the Formation of Morphogenic Structures of EpH4 Cells

Addition of wortmannin and LY294002 to EpH4/K6 cells significantly reduced the production of tubular structures by HGF/SF, whereas PD9805 was without effect (Fig. 6). In contrast, PD98059 quenched the induction of large alveoli, whereas wortmannin was without effect. These results suggest that the formation of tubular structures by HGF/SF/c-met requires pathways involving PI3 kinase, whereas alveoli formation depends on the activity of MAP kinase pathways.

The effects of HGF/SF and neuregulin seen on matrigel reveal striking similarities to the results obtained in whole organ culture (Yang et al., 1995). Since HGF/SF and neuregulin are expressed sequentially during mammary gland development, we also added the factors in a consecutive manner to EpH4 cells. We thus cultured the cells on matrigel for 4 d first in the presence of HGF/SF, removed HGF/SF and cultured the cells in the presence of neuregulin for an additional 4 d. Complex structures were formed consisting of branched tubules with adherent alveoli along the tubes and at branching points (data not shown). In these sequential experiments, expression of β-casein was examined: HGF/SF inhibited β-casein expression in the first phase; removal of HGF/SF allowed re-expression of β-casein (Fig. 7, *A* and *B*). Addition of neuregulin increased β-casein expression slightly compared with control levels.

Differential Effects of HGF/SF and Neuregulin on the Polarity of EpH4 Cells and on Expression of Cell Adhesion Molecules

The ultrastructure of the HGF/SF and neuregulin-induced aggregates was examined, and the distribution of the cell adhesion molecule E-cadherin and the expression of the differentiation marker β-casein were visualized by immunofluorescence analysis. In the control aggregates, cells that surround lumina contain flat nuclei and many vesicles with electron-dense material, likely representing casein-containing vesicles (Fig. 8 *a*). Immunofluorescence analysis revealed that β-casein expression was high in the controls; E-cadherin was evenly distributed along all cell surfaces, suggesting only moderate polarization of the cells (Fig. 9, *a* and *arrows* in *d*);. The HGF/SF-induced tubular structures were composed of more cuboidal cells with round nuclei (Fig. 8 *b*). β-Casein production was reduced by HGF/SF, as assessed by the reduction in numbers of vesicles and by the reduction of immunofluorescence-staining intensity (Fig. 8 *b* and Fig. 9, *b* and *e*). E-cadherin was largely distributed along the whole cell surface (Fig. 9 *b*, *arrows* in *e*). However, the cells in neuregulin-induced alveoli showed higher degree of polarization, as revealed by the columnar cell shape and the elongation of nuclei along the apical–basal axis (Fig. 8 *c*). E-cadherin was now predominantly located at lateral membranes, in accordance with the more pronounced polarization of the cells (Fig. 9, *c* and *arrows* in *f*). β-Casein was strongly expressed, as assessed by the increased numbers of vesicles and the staining intensity observed by immunofluorescence analysis (Fig. 8 *c* and Fig. 9, *c* and *f*).

Morphogenesis Responses to HGF/SF and Neuregulin on Explants of Human Breast Carcinoma Cells

To analyze whether the different morphogenic responses are restricted to EpH4 cells, we examined morphogenic responses in explant culture of human breast carcinomas obtained as xenografts. Xenografts of estrogen-responsive and -nonresponsive tumors were dissected into 1 × 1-mm

Published October 19, 1998

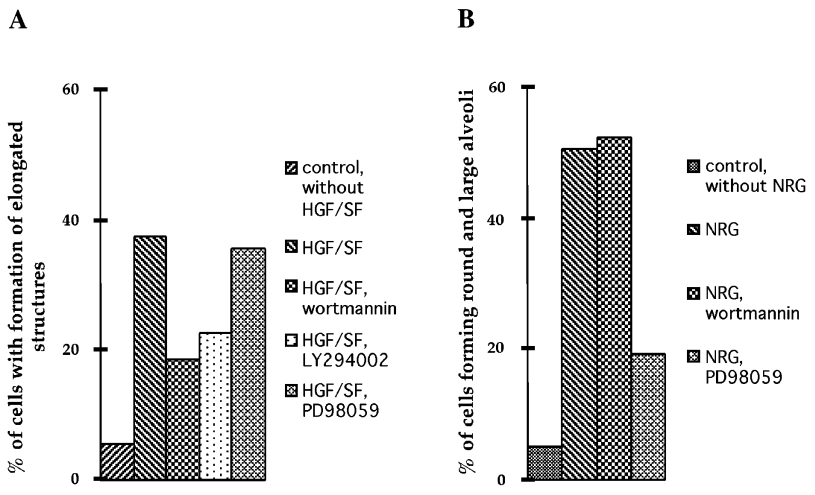

Figure 6. Effects of inhibitors of the PI3 kinase (wortmannin and LY294002) and MAPK kinase (PD98059) on the formation of elongated structures (*A*) and the formation of round, large alveoli (*B*). Experiments were terminated after 3 d of culture. From two sets of experiments, 350 cell aggregates each were analyzed.

pieces and cultured in the presence or absence of HGF/SF or neuregulin (refer to Materials and Methods for details). In the absence of factors, the explants from estrogen-dependent tumor 3366 (which was grown at minimal estrogen concentration in nude mice; Naundorf et al., 1992) were mainly composed of stromal tissue containing scattered epithelial islands that showed little mammary gland-specific differentiation (Fig. 10, *a* and *b* and Fig. 11, *a–c*). In the presence of HGF/SF, epithelial proliferation was strongly promoted and epithelial cells formed multilayered, cribiform, and papillary structures (Fig. 10, *c* and *d* and *c′* and *d′*). Duct-like structures were also observed. Thus, a similar multicellular organization was observed in HGF/SF-treated tumor tissue as in EpH4 cells grown on matrigel. No morphogenic response to HGF/SF was observed in the estrogen-independent human breast carcinoma 4151 (data not shown) (Naundorf et al., 1993). In contrast, addition of neuregulin to explants of the estrogen-responsive tumor 3366 produced an entirely different response: growth of epithelial cells was moderately promoted, epithelial sheets were predominantly single layered and cells lining the newly formed lumina were well polarized (Fig. 11, *d–f*), Thus, tumor explants and EpH4 cells on matrigel both form alveoli-like structures when exposed to neuregulin. No morphological response of tumor 4151 was detected in response to neuregulin (data not shown).

Discussion

We show here that EpH4 mammary gland epithelial cells on matrigel respond in distinct manners when stimulated with HGF/SF or neuregulin. HGF/SF induces branched multilayered tubes and inhibits the production of milk proteins. In contrast, neuregulin induces alveolar-like ag-

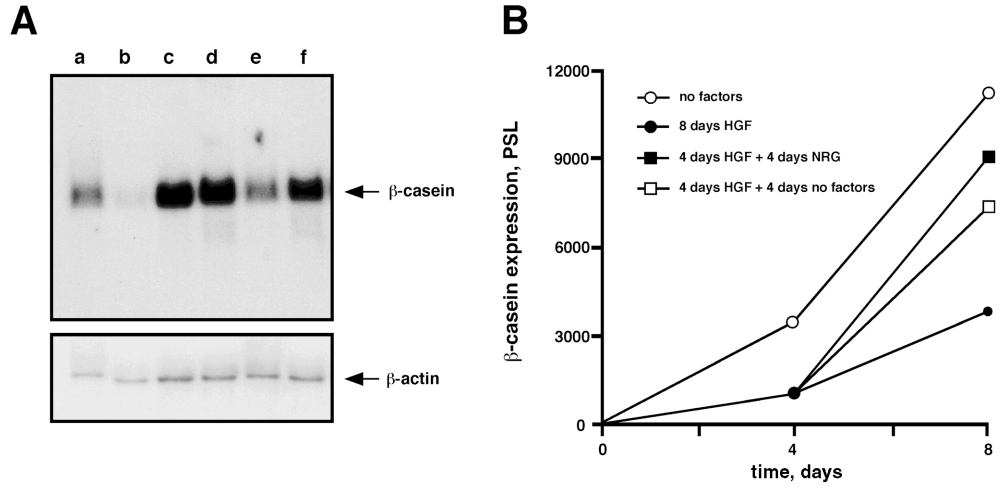

Figure 7. Effect of HGF/SF and neuregulin on the expression of β-casein in mammary epithelial cells EpH4 after their sequential application. (*A*) A representative Northern blot. (*B*) Quantification of Northern blot by phosphoimager analysis. Radioactive counts are expressed as PSL. Cells were cultured in medium containing hormones for 4 d in the presence of HGF/SF (lane *b*) and for an additional 4 d in the absence (lane *f*) or presence of HGF/SF (lane *e*) or neuregulin (lane *d*). Control cells were cultured in medium containing hormones without addition of factors (lanes *a* and *c*). *Inset* in *B* indicates the respective culture conditions.

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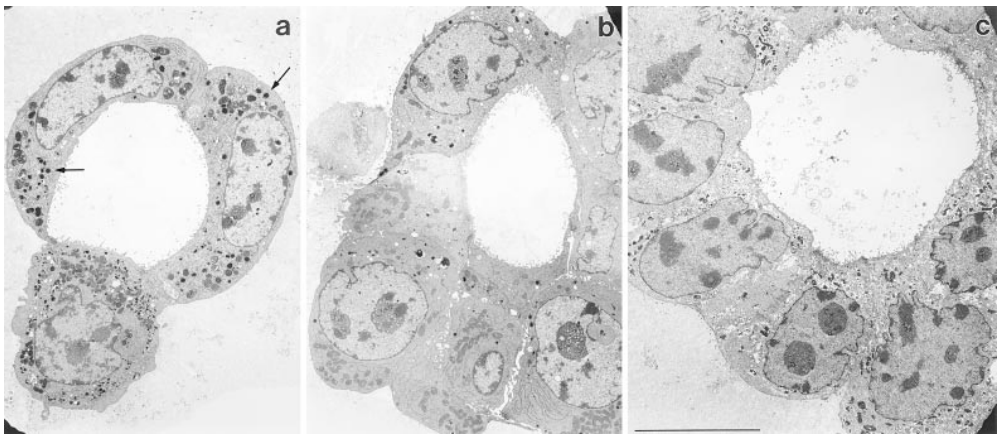

Figure 8. Ultrastructural analysis of EpH4/K6 cells grown under control conditions (*a*) and in the presence of HGF/SF (*b*) or neuregulin (*c*). Control aggregates often had small lumina lined by flattened cells containing numerous electron-dense granules (*arrows*). HGF/SF-induced tubules were lined by cuboidal cells with round nuclei and contained a reduced number of secretory granules. Neuregulin-induced alveoli had columnar epithelial cells with nuclei often elongated along the apical–basal axis and with abundant secretory vesicles. The lumina in most of neuregulin-treated structures were spacious; for better demonstrability, a small alveolus was selected here.

gregates which often consist of a monolayered epithelium, and allows β-casein expression. HGF/SF and neuregulin can thus induce complex morphogenic programs in mammary gland epithelial cells, which are also observed in the mammary gland during postnatal development. We used this cell culture system for the biochemical analysis of signals elicited by HGF/SF and neuregulin: the formation of branched tubules was found to be dependent on c-met and a pathway involving Gab1 and PI3 kinase, whereas alveolar formation required c-erbB2 and components of the ras/MAPK kinase pathway.

Morphogenic activities of various factors on mammary gland development have been examined; for instance, EGF (or TGF-α) and TGF-β influence ductal and alveolar development (Silberstein and Daniel, 1987; Vonderhaar, 1987; Coleman et al., 1988; Jhappan et al., 1990, 1993; Robinson et al., 1991; Snedeker et al., 1991; Daniel and Robinson, 1992; Pierce et al., 1993); these factors are predominantly produced in an autocrine fashion by epithelial cells and are expressed throughout mammary gland development. Since mesenchymal–epithelial interactions are essential for pre- and postnatal development of the mammary gland (Kratochwil, 1976, 1987; Sakakura et al., 1976, 1991; Durnberger et al., 1980; Cunha et al., 1992; Cunha and Hom, 1996), we have focused on the functional analysis of mesenchymal ligands (HGF/SF and neuregulin) and epithelial tyrosine kinase receptors (c-met and c-erbBs) in this process. In vivo, HGF/SF and neuregulin are expressed specifically during puberty and pregnancy in the mesenchyme of the mammary gland, and promote branching and lobulo-alveolar morphogenesis in whole organ culture, respectively (Yang et al., 1995). The HGF/SF receptor c-met and the neuregulin receptors (erbB2, erbB3, and erbB4) are produced in the outer epithelial cells of the ducts (Yang et al., 1995). Here we show that HGF/SF induces growth and morphogenesis of EpH4 mammary epithelial cells in matrigel; long multilayered tubes are formed from loose aggregates or small spheroids. Interestingly, HGF/SF inhibits the expression of β-casein, indicating that a morphogenic program without concomitant functional differentiation is activated. This is consistent with processes that are observed in vivo: milk production is blocked during tubular branching. HGF/SF-induced tubule formation and branching of mammary gland cells was also previously reported in collagen matrix (Berdichevsky et al., 1994; Brinkmann et al., 1995; Soriano et al., 1995); concomitant inhibition of functional differentiation had not been observed and might be a specific response to the specific substrate used here, i.e., matrigel. Neuregulin exerts an entirely different response of EpH4 mammary epithelial cells in matrigel: alveolar-like structures are induced which consist of single-layered epithelia. Thus, we assign here a morphogenic role to neuregulin in mammary epithelial cells; such a function is in accordance with previous whole organ culture experiments (Yang et al., 1995). Our data thus show that signaling of two different tyrosine kinase receptor types, c-met and c-erbB, elicits distinct morphogenic responses in a single mammary gland epithelial cell line. In whole organ cultures (Yang et al., 1995), HGF/SF and neuregulin stimulated preexisting structures, i.e., the factors enhanced either tubular or alveolar morphogenesis. Here we show that HGF/SF and neuregulin can induce these two morphogenic programs from cell aggregates that are apparently pluripotent.

Epithelial cells derived from the kidney, breast and other organs respond to SF/HGF and c-met by the formation of branched tubules when grown in a collagen matrix (Montesano et al., 1991*a*,*b*; Weidner et al., 1993; Berdichevsky et al., 1994; Soriano et al., 1995; Brinkmann et al., 1995). Furthermore, several tyrosine kinase receptors were reported to affect epithelial cells (e.g., trk, c-ros, the KGF-R) but only c-met induces tubulogenesis (Sachs et al., 1996). Although a variety of substrates were found to bind to the tyrosine phosphorylation sites in the COOH terminus of c-met (Ponzetto et al., 1994; Fixman et al., 1995; Weidner et al., 1995), a substrate which can mediate the signal responsible for branching morphogenesis, Gab1, has only recently been identified (Weidner et al., 1996). Gab1 binds specifically to c-met but not to various other receptor tyrosine kinases. We show here that Gab1 also promotes the formation of branched tubules from EpH4 mammary epithelial cells. Thus, Gab1 is an important substrate that transduces this morphogenic signal in various epithelial cell types. In a recent report it was shown that

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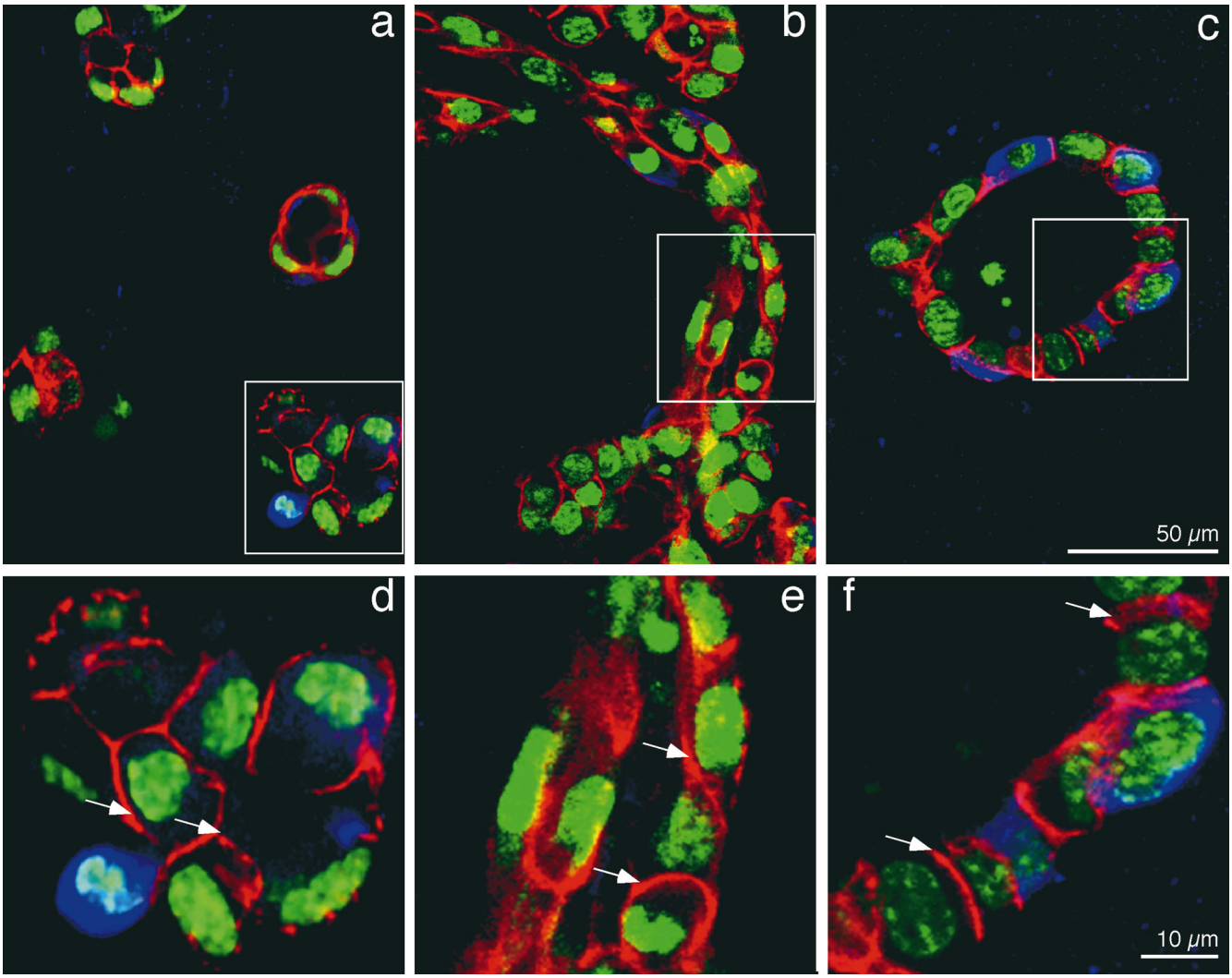

Figure 9. Scanning confocal micrograph of EpH4/K6 aggregates treated with HGF/SF (*b* and *e*) or neuregulin (*c* and *f*); controls are shown in *a* and *d*. *Green*, nuclei; *red*, E-cadherin; *blue*, β-casein. In control- and HGF/SF-treated cultures, E-cadherin is evenly distributed along all cell surfaces (*arrows* in *d* and *e*). Neuregulin-treated cells exhibit a stronger polarity resulting in a predominantly lateral localization of E-cadherin. β-Casein expression is strongly inhibited in HGF/SF-treated cells.

Stat 3 is also required for the formation of branching structures (Boccaccio et al., 1998). The production of milk constituents in the mammary gland is under tight hormonal control and influenced by extracellular matrix components. Stat 5 is an essential factor for the prolactin-controlled β-casein gene expression (Happ and Groner, 1993; Schmidhauser et al., 1992; Sympson et al., 1994; Groner et al., 1994; Wakao et al., 1995; Streuli et al., 1995). c-met signaling might thus interfere with the activity of Stat 5.

Neuregulin signaling requires the c-erbB3 or c-erbB4 receptors, which bind neuregulin with high affinity, and c-erbB2, that acts as an essential coreceptor (Kita et al., 1994; Carraway et al., 1994; Tzahar et al., 1994; Carraway and Burden, 1995; Marikovsky et al., 1995; Karunagaran et al., 1995; Meyer and Birchmeier, 1995; Lee et al., 1995; Gassmann et al., 1995; Riese et al., 1995; Pinkas-Kramarski et al., 1996; Riethmacher et al., 1997). The c-erbB2 receptor binds substrates like Shc (Segatto et al., 1993), Grb2 (D'souza and Taylor-Papadimitriou, 1994), PLC-γ (Songyang et al., 1993; Ben-Levy et al., 1994), and Grb7 (Stein et al., 1994). It has recently been shown that of the phosphorylated tyrosine residues found in the COOH-terminal substrate-binding domain of c-erbB2, tyrosine Y1144 (tyrosine 2) binds Grb2, and Y1227 (tyrosine 4) binds Shc (Dankort et al., 1997); both substrates signal through the ras pathway. The signaling capacities of Y 1201 (tyrosine 3) and Y1253 (tyrosine 5) are unknown. Tyrosines 1–4 were found to be necessary for the mitogenic response of c-erbB2 (Dankort et al., 1997); others observed that tyrosine residue 5 was required for this activity (Ben-Levy et al., 1994). By the use of a trk/c-erbB2 hybrid receptor, we demonstrate here that c-erbB2 induces alveolar-like differentiation in mammary epithelial cells without activation of coreceptors. Furthermore, a hybrid receptor of c-erbB2 containing tyrosines 1–4 is sufficient to induce alveolar structures; a hybrid containing only tyrosine 5 is not.

We have examined inhibitors that specifically interfere

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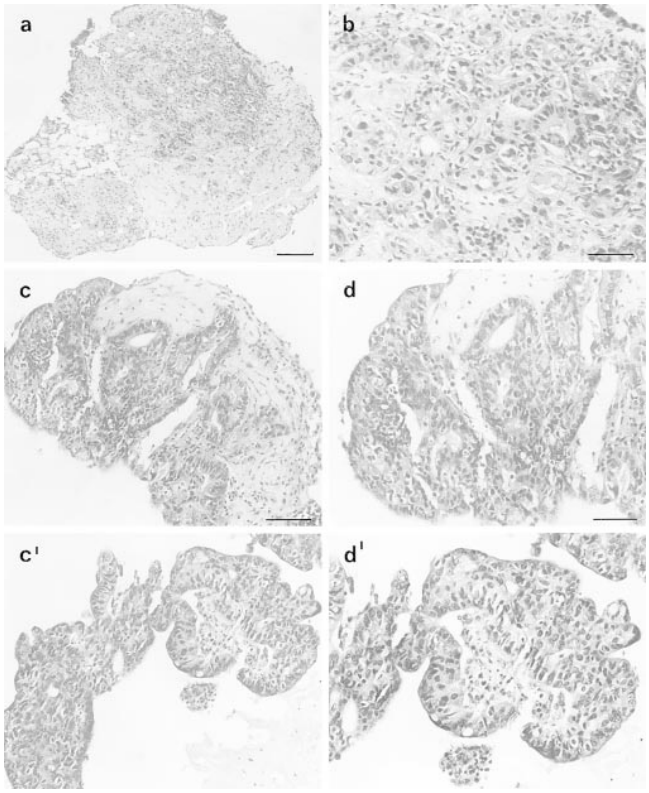

Figure 10. Effect of HGF/SF on morphogenesis of explants of the human mammary carcinoma 3366. Explants were cultured for 8 d in the absence (*a* and *b*) or presence of HGF/SF (*c*, *c′*, *d*, and *d′*). Hematoxylin/eosin-stained paraffin sections of the cultured explants are shown. Bars: (*a*, *c*, and *c′*) 100 μm; (*b*, *d*, and *d′*) 50 μm.

with signaling pathways activated by HGF/SF and neuregulin to analyze their effect on the morphogenic response evoked by these factors. The c-met receptor can potentially signal through ras and PI3 kinase (Hartmann et al., 1994; Ponzetto et al., 1994; Royal and Park, 1995; Royal et al., 1997). Signaling through ras and the resulting cellular growth require the substrate Grb2, which binds to tyrosine residue Y1354 of c-met (i.e., the second tyrosine residue of the bidentate docking site) (Ponzetto et al., 1994; Fixman et al., 1995; Nguyen et al., 1997). Motility and morphogenic responses evoked by c-met require the substrate Gab1, which binds strongly to Y1347 (the first residue of the bidentate docking site; Weidner et al., 1996; Nguyen et al., 1997). Gab1 can also bind to Y1354, but in the presence of Grb2 binding to Y1347 is preferred and stabilized (Nguyen et al., 1997). Furthermore, Gab1-binding blocks the SH3 domain of Grb2 (Holgado-Madruga et al., 1996), and Gab1 harbors three PI3 kinase-binding sites. Indeed, we find that inhibitors of PI3 kinase (wortmannin and LY294002) specifically reduce HGF/SF-induced formation of tubules. This suggests that branching morphogenesis depends on the activity of pathways requiring PI3 kinase. In contrast, alveolar morphogenesis of EpH4 cells by c-erbB2 could be blocked by inhibitors of MAPK kinase, i.e., PD98059. Thus, components of the ras/MAP kinase pathways play an essential role in alveolar morphogenesis. It will now be important to identify which substrates (or substrate combinations) elicit the alveolar morphogenesis that is induced by the c-erbB2 receptor.

The morphogenic activities of HGF/SF and neuregulin can be used to reconstitute further steps of postnatal mammary gland development in vitro. In the matrigel system established here, interactions of morphogenic factors with other mediators of growth and differentiation such as hormones, extracellular matrix components, or other mesenchymal factors can be studied in the future. Furthermore, downstream signaling processes that elicit the different morphogenic programs and eventually result in changes in gene expression are now amenable to investigation.

The authors would like to thank C. Birchmeier (Max-Delbrück-Center, Berlin, Germany) for critically reading the manuscript, E. Reichmann (ISREC, Lausanne, France) for providing the EpH4 cell line, Y. Yarden (Weizmann Institute of Science, Rehovot, Israel) for providing the mutant c-DNAs of c-erbB2, and H.R. Gelderblom (Robert Koch Institute, Berlin) for using his electron microscopic facilities. We also thank R. Franke,

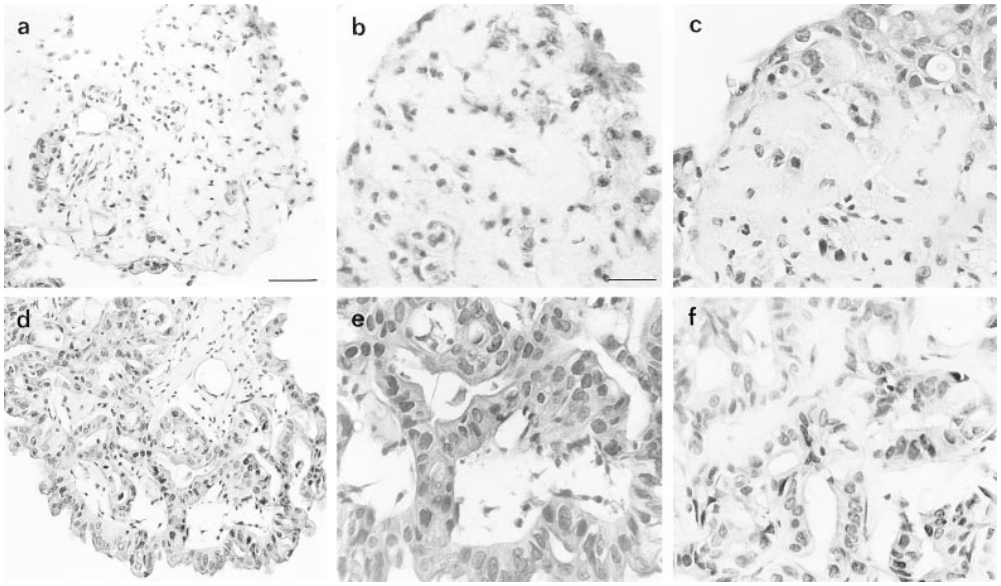

Figure 11. Effect of neuregulin on morphogenesis on explants of human mammary carcinoma 3366. Explants were cultured for 8 d in the absence (*a–c*) or presence of neuregulin (*d–f*). Hematoxylin/eosin-stained paraffin sections of explants are shown. Bars: (*a* and *d*) 50 μm; (*b*, *c*, *e*, and *f*) 25 μm.

R. Vogel, and B. Büttner for excellent technical assistance (all three from Max-Delbrück-Center). We dedicate this work to the memory of the late Prof. W. Zschiesche (Max-Delbrück-Center) who contributed invaluably to our work in mammary gland biology.

This work was supported by grants of the Deutsche Krebshilfe (Bonn, Germany).

Received for publication 21 March 1997 and in revised form 26 August 1998.

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