Epidermal growth factor and hepatocyte growth factor cooperate to enhance cell proliferation, scatter, and invasion in murine mammary epithelial cells

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Abstract

The development of the mammary gland requires an integrated response to specific growth factors and steroid hormones. Hepatocyte growth factor (HGF) and its tyrosine kinase receptor, MET, are expressed and temporally regulated during mammary development and differentiation. Epidermal growth factor receptor (EGFR) and its ligands have also been implicated in mammary gland growth and morphogenesis. Since both cytokines seem to exert a morphogenic program in this tissue, we have investigated the possible concerted action of EGF and HGF on the HC11 cell line, a widely used model of nontumorigenic mammary cells. Western blot analysis indicated that HC11 expressed MET and EGFR, and showed ERK1/2 and AKT activation following HGF or EGF treatment. Analysis by real-time PCR and western blot showed that after an EGF but not HGF or insulin-like growth factor-I treatment, HC11 mammary cells exhibited an increase in MET expression at both the mRNA and protein levels, which was dependent on the AKT pathway. Simultaneous treatment with HGF and EGF increased proliferation, scatter, and invasion as assessed by cell count, cell cycle, scatter, and transwell assays. AKT inhibition did not influence the cooperation on proliferation or invasion after HGF + EGF treatment, while ERK1/2 inhibition abolished MET/EGFR cooperation on proliferation. HGF + EGF treatment increased the duration of ERK1/2 and AKT activation compared to HGF or EGF alone. All these data indicate that a crosstalk between the EGF and HGF pathways in mammary epithelial cells may modulate the development of the mammary gland.

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Introduction

The mammary gland exhibits multiple remodeling events of the glandular tissue both during its development and in adulthood. Ductal elongation and branching occur mainly during puberty, whereas alveolar proliferation and differentiation take place during pregnancy. This phenomenon is very complex and requires the presence of multiple hormones, both of endocrine and of paracrine origin. Endocrine hormones, like estrogens and progesterone, have the ability to induce the release of locally produced cytokines (Brisken et al. 2000, Ciarloni et al. 2007). These factors, released by the both epithelial and mesenchymal compartments of the gland, activate mesenchymal–epithelial interactions that are essential for the correct development of this organ. Many locally produced signals and their receptors have been described. Among these, two well-described tyrosine kinase receptors, the epidermal growth factor receptor (EGFR; Stern 2003) and MET, the hepatocyte growth factor (HGF) receptor (Haslam et al. 2008), are of physiological significance during normal morphogenesis of the mammary gland.

On binding to one of its ligands (e.g. EGF, transforming growth factor-β, β-cellulin, and HB-EGF), EGFR dimerizes and activates multiple signaling pathways that are reported as major contributors to cell proliferation, survival, and motility. Waved-2 mice that carry a spontaneous mutation in the c-erbb gene encoding EGFR show impaired lactation (Fowler et al. 1995). Although several EGFR ligands can promote mammary development if given exogenously, only amphiregulin (Areg) is up-regulated at puberty. Moreover, Areg-null mutant mice, but not mice lacking one or more alternative EGFR ligands, display defective ductal outgrowth (Luetteke et al. 1999, Wiesen et al. 1999).

HGF was originally described as a potent growth stimulator for primary hepatocytes in culture and during liver regeneration (Nakamura et al. 1986), but HGF has a variety of supplementary biological activities including motogenesis and morphogenesis in a wide
variety of epithelial cells that express its receptor, MET (Birchmeier et al. 2003). In collagen gels, HGF induces proliferation and produces a tubulogenic response in cells derived from the mammary gland, the kidney, and other organs. HGF and MET are expressed and temporally regulated during mammary development and differentiation (Pepper et al. 1995). In this context, mammary fibroblasts produce HGF (Zhang et al. 2002) that is mitogenic, morphogenic, and motogenic for mammary epithelial cells (Berdichevsky et al. 1994, Soriano et al. 1995, Accornero et al. 2007).

Many studies have demonstrated multiple mechanisms of collaboration between MET and EGFR. Activation of multiple RTKs may potentiate some biological properties, each arising either from the independent activity of individual activated receptors or from an integrated signal arising from the combinatorial activation of multiple receptors. Since MET and EGFR are expressed and play an important role during mammary development, we investigated whether there was a cooperative effect between these receptors in cultured murine mammary epithelial cells. A primary attention has been put on this interaction in cell lines derived from tumors (Bergstrom et al. 2000, Ramos-Nino et al. 2003), while only few data are available on cells obtained from nontumorigenic tissues (Spix et al. 2007, Seki et al. 2008). Thus, we have investigated the possible common biological responses of HC11, a widely used model of mammary cell line (Civenni et al. 2003, Kabotyanski et al. 2006), to HGF and EGF stimulation.

Materials and methods

Reagents

All reagents, unless specified, were from Sigma–Aldrich; recombinant HGF, EGF, and insulin-like growth factor I (IGFI) were from Immunotools (Friesoythe, Germany) and were resuspended in PBS; UO126 and wortmannin were from LC Laboratories (Woburn, MA, USA); PHA-665752 was from Tocris Bioscience (Ellisville, MI, USA); PP2 was from EMD Biosciences (San Diego, CA, USA); NucleoSpin RNA II was from Macherey–Nagel (Duren, Germany); Bio-Rad iScript cDNA kit, iQ SYBR Green Supermix, and DC Protein Assay were from Bio-Rad Laboratories; Hybond-enhanced chemiluminescence (ECL) nitrocellulose membrane and Hyperfilm ECL were from GE Healthcare Bio-Sciences (Piscataway, NJ, USA); Super Signal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL, USA); transwell polycarbonate membranes were from Corning-Costar (Corning, NY, USA); Matrigel Basement Membrane Matrix was from BD Biosciences (Bedford, MA, USA).

Antibodies

Anti-MET mouse monoclonal antibody was from Zymed (South San Francisco, CA, USA); anti-EGFR rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-α-tubulin and anti-phospho-ERK1/2 mouse monoclonal antibodies were from Sigma–Aldrich; anti-ERK1/2 rabbit polyclonal, mouse monoclonal clone MK12 was from Upstate (Temecula, CA, USA) anti-phospho-AKT mouse monoclonal (ser-473), and anti-AKT rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA).

Cell culture

HC11 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS; Euroclone, Pero, Italy), insulin (5 µg/ml), and EGF (10 ng/ml). Cells were maintained in a 5% CO2–water-saturated atmosphere, and routinely passaged every 2–3 days by washing with PBS followed by trypsinization. Mouse liver extracts were kindly provided by Prof. C. Ponzetto (University of Torino, Italy).

For western blot and real-time (RT)-PCR analysis, HC11 cells were seeded in six-well plates and allowed to grow to 50% confluence. Then, the medium was removed and replaced with medium with no serum and 0.4% BSA for an additional 24 h. The indicated cytokines were then added, and cells were cultivated for the indicated time intervals (for immunoblotting) or other 16 h (for RT-PCR). UO126 (10 mM), wortmannin (100 nM), and PHA-665752 (250 nM) were resuspended in dimethyl sulfoxide (DMSO) in 10 mM solution and added 2 h before treatment with the cytokines. For control samples, an equivalent amount of DMSO was added.

For cell proliferation, HC11 cells were seeded in six-well plates at a density of 1×105 cells per well and allowed to grow. After 24 h, the medium was removed and replaced with medium containing 10% FBS and the indicated cytokines. For cell cycle analysis, HC11 cells were seeded in six-well plates in RPMI medium with 10% FBS and allowed to grow to 50% confluence, and then the indicated cytokines were added for 16 h. For the scatter assay, HC11 cells were seeded in 96-well plates at a low density of 300 cells per well and cultured until they formed tightly packed colonies (3 days). The medium was then removed and replaced with medium containing 10% FBS and the indicated cytokines.

Western blot analysis

Cells were washed with ice-cold PBS, lysed, and scraped in lysis buffer (20 mmol/l Tris (pH 7.5), 130 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 1 mmol/l h-glycerophosphate) with Protease Inhibitor Cocktail and 1 mmol/l sodium orthovanadate. Protein lysates (20 µg) were cleared of cellular debris by
centrifugation at 4°C for 10 min at 12,000 g, quantified using DC Protein Assay, resolved in 10% SDS-PAGE gels, and transferred to Hybond-C Extra nitrocellulose membranes. After the transfer of proteins, the membranes were blocked at room temperature for 2 h with Tris-buffered saline (TBS, 10 mM Tris and 150 mM NaCl, pH 7.4) containing 10% BSA and then incubated overnight at 4°C with the appropriate primary antibodies. The membranes were washed six times for 5 min each in TBS-Tween and then incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. The membranes were again washed six times in TBS-Tween and incubated for 5 min at room temperature with Super Signal West Pico ECL peroxidase substrate. The proteins were visualized by briefly exposing the membrane to an autoradiographic Hyperfilm ECL.

Preparation of the RNA and RT-PCR

Total RNA was extracted with NucleoSpin RNA II kit following the manufacturer’s protocol. One microgram total RNA was reverse transcribed with iScript cDNA kit following the manufacturer’s instructions. RT-PCR was used to measure the quantity of Met relative to the quantity of hypoxanthine phosphoribosyltransferase 1 (Hprt1) mRNAs. Hprt1 was used as a normalization gene to correct for RNA concentration and reverse transcription efficiency. Diluted cDNAs (1:5 and 1:50) were used for RT-PCR amplification using iQ SYBR Green Supermix. Primers for murine Met (GenBank accession number NM_008591) were forward 5'-CGC TAT GAC GTA CAC A-3', reverse 5'-TTG GGA AAC TGG TCT TCT GGA-3' (efficiency 90%); primers for Hprt (GenBank accession number NM_013556) were forward 5'-TGA CAC TGG TAA AAC AAT GCA-3', reverse 5'-GGT CCT TTT CAC CAG CAA GCT-3' (efficiency 94%). RT-PCR parameters were cycle 1, 95°C for 3 min and cycle 2, 95°C for 30 s, 60°C for 30 s for 40 cycles. The ΔΔCt method was used to analyze the data as described by Livak & Schmittgen (2001).

Cell proliferation assay

Cell proliferation was evaluated after 48 h by trypsinization, resuspension in PBS, and counting on Burker chambers. Nonviable cells were excluded by trypan blue staining. Each experiment was repeated three times independently, and in each experiment, each treatment was performed with two replicate culture wells.

Cell cycle analysis

Cells were trypsinized and washed with PBS, then treated with RNase (0.25 mg/ml) and stained with propidium iodide (50 μg/ml). The cell cycle distribution in G0/G1, S, and G2/M phases was calculated using the CellQuest program (BD Biosciences). Each experiment was repeated three times independently, and in each experiment, each treatment was performed with two replicate culture wells.

Scatter assay

After 16 h of cultivation, cells were fixed for 10 min with 11% glutaraldehyde dissolved in PBS, colored with crystal violet, stained with DAPI (0.5 μg/ml in PBS), and photographed with a Leica AF6000 LX (Leica Microsystems, Wetzlar, Germany) inverted microscope equipped with a Leica DFC350FX digital camera and a motorized stage controlled by the LAS AF software (Leica Microsystems). Images of the entire wells were captured using the tile scan feature for automatic scanning (4×5 fields). Tile scan image capture and merging were used to provide a single panoramic view of the well at 50× magnification. Quantitative analysis of the merged image (area occupied by all cell colonies in the well) was performed using the LAS AF software.

In vitro invasion assay

Invasiveness was examined by using the membrane invasion culture system (transwell polycarbonate membranes 6.5 mm diameter, 8 μm pore size). Briefly, 3×10⁴ HCl1 cells were seeded onto the upper chamber of the transwells previously coated with 25 μg Matrigel Basement Membrane Matrix. The lower chamber of the transwells was filled with medium containing 10% FBS, the indicated cytokines and inhibitors (UO126, wortmannin, and PHA-665752). After 48 h, the noninvasive cells on the upper surface of the membrane were removed with a cotton swab. Cells that solubilized the Matrigel, passed through the 8 μm pores of the transwell, and attached to the lower surface of membrane were fixed with 11% glutaraldehyde, stained with crystal violet, and photographed. For quantification, the transwell membranes were detached and solubilized in 10% acetic acid solution (90% deionized water and 10% glacial acetic acid), and the intensity of the colored solution was quantified by spectrophotometrical analysis (595 nm). The intensity of the untreated control transwell was set to 100%. Each experiment was repeated three times independently, and in each experiment, each treatment was performed with two replicate transwells.

Statistical analysis

Experimental data are presented as mean ± s.d. Statistical differences between treatments and interactions were calculated with one-way ANOVA using the Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffe’s F test.
Results

The HC11 murine cell line was derived from midpregnant BALB/c mouse mammary tissue and is considered a unique cell line that still retains important characteristics of normal mammary epithelial cells such as the ability to produce milk protein in response to lactogenic hormones without cultivation on exogenous extracellular matrix or cocultivation with adipocytes or fibroblasts (Marte et al. 1995).

To test the biological effects of HGF and EGF on HC11 mammary cells, we first verified, at the protein level, if this cell line expressed MET and EGFR by western blot analysis (Fig. 1A). Lysates obtained from mouse liver were used as a positive control for both receptors (Seki et al. 2008). Both the MET 190-kDa precursor and the mature 140-kDa MET receptor are visible. The 170-kDa EGFR receptor is clearly detected in both the liver and HC11.

In different cell lines, HGF- and EGF-induced activation of their respective receptors results in phosphorylation of many downstream effectors. The best studied pathways activated downstream of these receptors are the MEK-dependent phosphorylation of the MAP kinases ERK1 and ERK2 (ERK1/2), and the PI3K-dependent phosphorylation of PKB/AKT. We therefore decided to verify whether HGF and EGF could activate these two pathways in the HC11 cell line. Subconfluent HC11 cells were serum starved for 24 h and then stimulated for 10, 30, and 60 min with 10 ng/ml HGF or EGF (or stimulated with vehicle (PBS) control). Cells were lysed and protein lysates were subjected to immunoblotting (Fig. 1B). Both HGF and EGF activated ERK1 and ERK2 MAP kinases and the AKT protein kinase. In HGF- and EGF-treated cells, the phosphorylation intensity of ERK1/2 returned almost to a basal level after 60 min, whereas AKT activation persisted for a longer time only in HGF-treated cells.

For this purpose, cells were serum starved for 24 h and then treated for 16 h with HGF (10 ng/ml), EGF (10 ng/ml), or IGF-I (100 ng/ml). Cells were then lysed, and protein cell extracts were analyzed by immunoblotting for the expression of MET and EGFR. Tubulin was used to confirm that equal amounts of protein had been correctly loaded. EGF, but not HGF or IGF-I, increased the expression of MET, when compared to control cells (Fig. 2A). EGF also significantly lowered the expression of its own receptor, EGFR, a well-described mechanism that involves internalization and degradation (Sorkin & von Zastrow 2002). We then analyzed if the increased expression level of MET after EGFR activation could also be observed at the mRNA level. To this aim, cells were serum starved for 24 h and stimulated with HGF, EGF, or IGF-I for 16 h, and then Met relative mRNA levels were quantified by RT-PCR. In agreement with the data obtained by western blot analysis, we observed an

EGF, but not HGF or IGF-I, upregulates MET expression both at the mRNA and protein levels by using the AKT pathway

Multiple mechanisms of collaboration between EGFR and MET have been described among which ligand-dependent receptor upregulation or transphosphorylation. Thus, we first analyzed possible variations in the expression levels of MET and EGFR in HC11 cells after induction with HGF, EGF, or IGF-I. We used IGF-I because it is another locally released cytokine important during mammary development (Kleinberg et al. 2000).
increase in Met mRNA levels five- to tenfold in EGF-treated cells compared to the level of untreated (control) cells (Fig. 2B). HGF and IGF-I did not increase Met expression.

We finally analyzed the signaling pathway responsible for MET upregulation following EGFR induction. To test this hypothesis, we deactivated the ERK1/2 and the PI3K-AKT pathways by the use of their specific inhibitors UO126 (10 μM) and wortmannin (100 nM). For this purpose, cells were serum starved for 24 h, and the inhibitors were added. After 2 h, the cells were stimulated with EGF (10 ng/ml) for 16 h. Cell protein extracts (20 μg) were analyzed by immunoblotting with anti-MET antibodies, and total tubulin was used as a loading control. Wortmannin, but not UO126, inhibited MET upregulation (Fig. 2C). Also, Src inhibition by PP2 did not modify MET upregulation (data not shown).

HGF and EGF simultaneous treatment increases proliferation and scatter in HC11 murine mammary cells

Since both MET and EGFR are expressed and are functionally active in HC11 cells, we verified if the simultaneous addition of HGF plus EGF promoted an increase in proliferation compared with HGF or EGF alone. HC11 cells are highly responsive to EGF for proliferation but no data is available on HGF-induced proliferation in this cell line. For this purpose, cells were plated in six-well plates and cultured for 48 h in medium with serum alone (control) or medium with serum and HGF, EGF, or HGF + EGF (10 ng/ml each). To quantify growth, cells were trypsinized, stained with trypan blue, and counted. Nonviable, trypan blue-positive cells were equal under all conditions (3–6% average) and were not considered. HGF + EGF had the strongest potential to induce cell proliferation when compared with EGF or HGF (Fig. 3A).

We also assayed proliferation by propidium iodide staining and cell cycle analysis. HC11 cells were treated for 16 h with serum alone (control) or medium with serum and HGF (10 ng/ml), EGF (10 ng/ml), or HGF + EGF (10 ng/ml each). Cells were then trypsinized, stained with propidium iodide, and analyzed by FACS. HGF and EGF increased the number of cells in G2/S and M phases of the cycle, while simultaneously reducing cells in the G0/G1 phase. HGF + EGF treatment produced the higher percentage of cells in the G2/S and M phases (Fig. 3B).

To assess the potential collaborative effect of HGF and EGF, cell motility and dispersion (scatter) were also analyzed. For this assay, it was first verified if HC11 cells were treated with HGF or EGF alone (10 ng/ml) responded with scatter. For this purpose, cells were plated in 96-well plates at a low density (300 cells/well) and left to form colonies for 3 days. Cytokines were then added and scatter was analyzed after 16 h. At this concentration, HGF, but not EGF, greatly increased cell motility and dispersion. Interestingly, not all colonies

Figure 2 Effect of EGF, HGF, and IGF-I on MET and EGFR expression. (A) HC11 cells were serum starved overnight then either treated with PBS (control) or treated with EGF (10 ng/ml), HGF (10 ng/ml), or IGF-I (100 ng/ml) for 24 h. After lysis, 20 μg of total protein extracts were subjected to immunoblotting. The panel shows a representative image of MET and EGFR immunoblotting. Tubulin was used as loading control. (B) Met mRNA expression in HC11 cells treated with HGF, EGF, or IGF-I. Results are percentage relative to the untreated control and are expressed as means ± s.d. of three independent experiments. *P<0.05 versus control. (C) Inhibition of MET upregulation by the PI3K inhibitor wortmannin. HC11 cells were serum starved overnight, then treated with the indicated inhibitors with or without EGF (10 ng/ml). UO126 (ERK1/2 inhibitor; 10 μM) and wortmannin (PI3K inhibitor; 100 nM) were added 2 h before EGF. The panel shows a representative image of MET immunoblotting. Tubulin was used as loading control.
responded with scatter, confirming the heterogeneous population of this cell line (Deugnier et al. 1999). Thus, in order to verify a possible collaboration between HGF and EGF in this assay, we used suboptimal doses of HGF (0.5 ng/ml), a concentration at which this cytokine alone has no significant effect on scatter. Interestingly, the combination of EGF and HGF together significantly promoted cell scatter (Fig. 3C). To quantify differences between all treatment conditions, panoramic views of the entire wells were acquired (Fig. 3C see insets), and the average areas occupied by any single cell in the well were calculated. Coactivation of EGFR and MET enhanced the mean cell area when compared to control, EGF, or HGF treatments (Fig. 3D).

**HGF and EGF simultaneous treatment increases invasion in HC11 cells**

Invasive growth is a fundamental process in embryo development and organ formation (e.g. in the mammary gland). No data is available on the ability of HC11 mammary cells treated with either HGF or EGF to induce degradation of the extracellular matrix and move through the pores of the transwell membranes.
(invasion assay). Thus, we tested the invasive response of HC11 cells to HGF (10 ng/ml), EGF (10 ng/ml), and HGF + EGF (10 ng/ml each) in medium with serum (Fig. 4A). Untreated cells had a mild invasive potential, while HGF and, to a lesser extent, EGF increased the ability of HC11 cells to dissolve the matrix and pass through the pores of the transwells. Concurrent stimulation of HC11 cells with HGF and EGF increased the invasion (Fig. 4A). Quantification of cells that passed through the transwells confirmed that HGF + EGF had a synergistic effect on invasion (Fig. 4B). We also tested if HC11 cells could activate the invasion program in medium with no serum. No cell passed through the pores of the chambers under any condition (control, HGF, and EGF) except if stimulated concurrently by HGF + EGF (data not shown).

**Effects of MET, AKT, and ERK1/2 inhibition on HC11 cell proliferation and invasion**

To determine whether the EGFR–AKT-mediated MET upregulation was responsible for the observed cooperation in HC11 cells after the HGF + EGF treatment, we analyzed cell proliferation and invasion following MET and AKT inhibition using their respective inhibitors, PHA-665752 (Accornero et al. 2008) and wortmannin (a highly specific inhibitor of PI3K). As expected, MET inhibition by PHA-665752 at 250 nM abolished HGF-induced cell proliferation and invasion, and inhibited the cooperation between EGF and HGF in both assays (Fig. 5A and B). Wortmannin (100 nM) did not abolish the cooperation between EGFR and MET in proliferation and invasion assays ruling out the possibility that MET upregulation was responsible for the increase in HC11 responses following HGF + EGF treatment. We also analyzed the effect of ERK1/2 inhibition by UO126 (10 μM) on proliferation and invasion. Interestingly, UO126 abolished the EGFR–HGF cooperation on proliferation but not on invasion (Fig. 5A and B). As expected, both Wortmannin and UO126 reduced, but did not abolish, the overall capacity of HC11 cells to proliferate and invade, indicating these pathways as important but not fundamental for this process. PHA-665752 on the other side had a small inhibitory activity on HC11 proliferation but not on invasion, possibly due to very small quantities of HGF present in the added serum.

**Effects of EGF and HGF on MET–EGFR trans-phosphorylation, and AKT and ERK1/2 activation**

As already mentioned, another possible mechanism of collaboration between EGFR and MET is receptor heterodimerization and transphosphorylation. We thus tested whether HGF or EGF treatment could activate both MET and EGFR after short (10 and 60 min) or long (6 h) incubation times. Subconfluent HC11 cells were serum starved and then stimulated for 10, 60 min, and 6 h with 10 ng/ml HGF or EGF (or left unstimulated as control). Cells were lysed and protein lysates were subjected to immunoblotting. Under our experimental conditions, we could not detect transphosphorylation between MET and EGFR (Fig. 6A). Finally, ERK1/2 and AKT phosphorylation levels induced by simultaneous EGFR and MET activation were analyzed by western blot (Fig. 6B). To this aim, subconfluent HC11 cells were serum starved for 24 h and then stimulated for 1 and 4 h with HGF (10 ng/ml), EGF (10 ng/ml), or HGF + EGF (10 ng/ml each). A cooperation between EGFR and MET on ERK and AKT phosphorylation was observed only after a longer incubation times (4 h). HC11 cells retained an elevated level of ERK1/2 phosphorylation at 4 h only if stimulated concurrently with HGF + EGF, while AKT phosphorylation tended to lower progressively from 1 to 4 h under all conditions.
The present study shows that EGF and HGF collaborate to enhance proliferation, scatter, and invasion in the HC11 murine mammary epithelial cell line. The work also demonstrates that EGF upregulates the MET receptor by using the AKT pathway, although MET upregulation is not responsible for the HGF–EGF collaboration on proliferation and invasion. On the other hand, the ERK1/2 activation is responsible for MET/EGFR cooperation on proliferation but not on invasion. Finally, this study shows that in HC11 cells, HGF or EGF do not induce transphosphorylation between MET and EGFR, and that ERK and AKT activation are increased when the EGFR and MET pathways are activated simultaneously.

In vivo, MET is present in both the luminal and the myoepithelial compartments of the mammary gland. In particular, myoepithelial cells show higher MET expression levels and also exhibit a significant reduction during pregnancy and lactation as analyzed by immunofluorescence staining (Haslam et al. 2008). Here, we first demonstrated by western blot analysis that both MET and EGFR are expressed in HC11 cells. Subsequently, we showed that HGF or EGF activated the ERK/MAPK and the AKT pathways that have already been described downstream MET and EGFR in other cell lines (Zaczek et al. 2005, Benvenuti & Comoglio 2007). HGF- and EGF-induced stimulation of HC11 resulted in MEK-dependent phosphorylation of the MAP kinases ERK1 and ERK2 and PKB/AKT. Both HGF and EGF activated the ERK/MAPK and the PI3K–AKT pathways with a rapid increase in the phosphorylation levels (about 10 min) followed by dephosphorylation. In literature, ERK1/2 and AKT phosphorylation status following MET activation was shown to persist longer, compared to the levels induced by other receptors (Maroun et al. 2000). Our results show that HC11 cells respond to HGF treatment with longer AKT, but not ERK1/2, phosphorylation.

We then observed that EGF addition to HC11 cells induced MET upregulation both at the protein level and at the mRNA level, and that this event was dependent on the AKT pathway. MET upregulation downstream EGFR was a one-way process, as demonstrated by the fact that IGF1 and HGF were unable to
increase MET. A similar mechanism of cooperation has been described in thyroid carcinoma cells (Ramos-Nino et al. 2003) and in mesothelioma cells (Bergstrom et al. 2000). Another mechanism of EGFR/MET collaboration mediated by direct transphosphorylation of kinase domain tyrosines from heterodimerization between the two receptors was observed in corneal epithelial cells (Spix et al. 2007, Xu & Yu 2007). While in HGF-treated glioma cells, EGFR transactivation occurred in a transcription-dependent manner after EGFR ligand upregulation (Reznik et al. 2008). In HC11 cells, we did neither detect an activation of EGFR following HGF induction nor an activation of MET following stimulation with EGF both after short (0–60 min) or long (6 h) incubation times. Therefore, in our model, these means of transphosphorylation are ruled out.

Coordinated receptor coactivation may have considerable consequences on cell biology. In HC11 cells, we found that the simultaneous activation of EGFR and MET resulted in enhanced proliferation, scatter, and invasion. EGF and HGF possess potent mitogenic effects on different cell lines. EGF is a proliferative growth factor routinely added to the HC11 growth medium. Here, we demonstrated that HGF is also a strong proliferative agent. Increased cell proliferation after HGF addiction was also observed in primary mammary cells (Yant et al. 1998, Sunil et al. 2002). Interestingly, another nontumorigenic cell line, the canine kidney MDCK, though highly responsive to the morphogenic stimuli induced by HGF (Montesano et al. 1991, Soriano et al. 1995), is totally insensitive to MET-induced proliferation (P Accornero, personal observation), and therefore, not all cell lines that express MET react with similar biological responses to HGF stimulation.

Scatter is a complex mechanism that consists of a first step in which cells dissociate one from another, and a second phase in which the released cells begin to move. This complex program is important during several morphogenic processes that are also active during mammary gland development. HGF, at high concentrations, is a strong inducer of scatter in many epithelial cell lines (Benvenuti & Comoglio 2007) including mammary cells and thus, to analyze whether EGF and HGF might collaborate to evoke scatter, we lowered HGF concentrations to preclude mammary cells to scatter by means of this cytokine alone. During mammary gland morphogenesis, the local active concentrations of paracrine factors are not known. In fact, these factors are modulated, in vivo, by multiple extracellular barriers and by many activating enzymes. (Birchmeier et al. 2003, Harris et al. 2003, Sternlicht et al. 2005). Under our experimental conditions, we showed that EGF collaborates with HGF to induce scatter in HC11 cells.

In this work, we also demonstrated the collaboration between MET and EGFR in invasion assays. A similar cooperation between these two receptors has been described recently in ovarian carcinoma cells (Zhou et al. 2007). Metalloproteases are important regulators of matrix remodeling and play a fundamental role in mammary gland morphogenesis (Fata et al. 2004). Both EGF and HGF have been shown to induce the expression and activation of these molecules (Zhou et al. 2007). We are currently evaluating which metalloproteases are differentially modulated by HGF or EGF in nontumorigenic mammary epithelial cells.

We finally verified if MET upregulation via the EGFR–AKT pathway was responsible for the HGF–EGF
cooperation on proliferation and invasion of HC11 cells. MET inhibition by PHA-665752 blocked the HGF-mediated responses both when used as a single agent and when used as a cooperative agent with EGF. Interestingly, inhibition of the PI3K–AKT pathway with wortmannin did not reverse the cooperation between MET and EGFR ruling out MET upregulation as a prerequisite for this response. A possible explanation is that HC11 cells that express lower or higher MET levels have similar rates of HGF-mediated intracellular signaling. EGF-induced MET upregulation might represent a common physiological mechanism taken over by some tumorigenic cell lines during the process of transformation. Guo et al. (2008) showed recently that EGFR-addicted nonsmall cell lung cancer cells line when inhibited by the selective EGFR tyrosine kinase inhibitor Iressa exhibited a drastic reduction in MET levels. The role of this system in normal tissue and in mammary development is actually not known.

An interesting finding of this work is that UO126 treatment substantially abolished HGF–EGF cooperation on proliferation but not on invasion. Activated ERK1/2 have been directly linked to cell proliferation and motility (Migliore & Giordano 2008). Our observation that ERK1/2 phosphorylation levels following HGF+EGF treatment remain substantially unaltered even after long incubation times (4 h) indicate the possibility that the collaboration between EGF and HGF on proliferation is mainly driven by ERK1/2. On the other side, our results also indicate that other pathways other than ERK1/2 and AKT are more important for the synergism between MET and EGFR in invasion. A possibility is that MET and EGFR once activated drive their response through both common (like ERK1/2 and AKT) and unique effectors. This latter possibility is currently under investigation.

In conclusion, in this study, we demonstrated the collaboration between MET and EGFR in proliferation, scatter, and invasion of HC11 murine mammary epithelial cells, and we established that activation of the ERK1/2 pathway is fundamental for MET/EGFR cooperation on proliferation. All these effects justify a possible cooperative role of the EGFR–MET axis in the physiology of the mammary gland.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice their impartiality.

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