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**EPIDERMAL GROWTH FACTOR AND HEPATOCYTE GROWTH FACTOR
COOPERATE TO ENHANCE CELL PROLIFERATION, SCATTER AND
INVASION IN MURINE MAMMARY EPITHELIAL CELLS**

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Short title: **EGF-HGF collaboration in mammary epithelial cells**

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Cooperation

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 Epidermal Growth Factor (EGF) and HGF on the HC11 cell line, a widely used model of non-tumorigenic 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 a 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. Inhibition of the ERK1/2 or the AKT pathway showed that Met upregulation after EGF treatment was dependent on the AKT pathway. Simultaneous treatment with HGF and EGF increased proliferation, scatter and invasion as assessed by cell count, scatter and transwell assays. AKT inhibition did not modify the Met-EGFR cooperation on proliferation and invasion while ERK1/2 inhibition only impaired the HGF-EGF cooperation on proliferation. All these data outline a cooperative role between the EGF and the HGF pathways in non-tumorigenic mammary epithelial cells and indicate that a cross-talk between their respective receptors may modulate the development of the mammary gland.

Introduction

The mammary gland exhibits multiple remodelling events of the glandular tissue both during its development and in adulthood. Ductal elongation and branching occurs 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 paracrine origin. Endocrine hormones, like estrogens and progesterone have the ability to induce the release of locally produced cytokines (Briskin *et al.* 2000; Ciarloni *et al.* 2007). These factors, released both by the epithelial and the 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 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, TGF- α , betacellulin 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 (Luetkeke *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 produce 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 (Accornero *et al.* 2007; Berdichevsky *et al.* 1994; Soriano *et al.* 1995).

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 wanted to investigate 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 non-tumorigenic tissues (Seki *et al.* 2008; Spix *et al.* 2007). Thus we have investigated 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, antibodies and cell culture

All reagents, unless specified, were from Sigma-Aldrich (St. Louis, MO, USA); DAPI; recombinant HGF, EGF and Insulin-like Growth Factor-I (IGF-I) were from Immunotools (Friesoythe, Germany); UO126, Wortmannin and PP2 were from LC laboratories (Woburn,

MA, USA); PHA-665752 was from Tocris Bioscience (Ellisville, MI, USA); NucleoSpin® RNA II were from Macherey–Nagel (Duren, Germany); Bio-Rad iScript cDNA kit, iQ SYBR Green Supermix and DC Protein Assay were from Bio-Rad Laboratories (Hercules, CA); Hybond-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 (NY, USA); Matrigel Basement Membrane Matrix was from BD Biosciences (Bedford, MA, USA); 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 (St. Louis, MO, USA); anti-ERK 1/2 rabbit polyclonal, anti-phospho-AKT mouse monoclonal and anti-AKT rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA).

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). HC11 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS; Euroclone, Pero, Italy), insulin (5 μ g/ml) and EGF (10ng/ml). Cells were maintained in a 5% CO₂-water-saturated atmosphere and cells were 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).

Western blot analysis

HC11 cells were seeded in 6-well plates and allowed to grow to 50% confluency. Then the medium was removed and replaced with medium with no serum and 0.4% bovine serum albumin (BSA) for an additional 24 h. The indicated cytokines were then added and cells were cultivated for other 16 h. UO126 (10 μ M) and Wortmannin (100 nM) were added 2 h before treatment with the cytokines. Cells were washed with ice-cold PBS, lysed, and scraped in lysis buffer (20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1 mmol/L h-glycerolphosphate) with Protease Inhibitor Cocktail and 1 mmol/L sodium-orthovanadate. Protein lysates were cleared of cellular debris by centrifugation at 4°C for 10 min at 12,000 x 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, 10mM Tris and 150mMNaCl, pH 7.4) containing 10% BSA and then incubated overnight at 4°C with the appropriate primary antibodies. The membranes were washed 6 times for 5 min each in TBS-tween and then incubated for 1 h at room temperature with horseradish peroxidase conjugated secondary antibodies. The membranes were again washed 6 times in TBS-tween and incubated with Super Signal West Pico enhanced chemiluminescence (ECL) peroxidase substrate for 5 min at room temperature. The proteins were visualized by briefly exposing the membrane to an autoradiographic Hyperfilm ECL.

Preparation of the RNA and real-time PCR

HC11 cells were seeded in 6-well plates and allowed to grow to 50% confluency then the medium was removed and replaced with medium with no serum and 0.4% BSA for additional 24 h. The indicated cytokines were then added, cells were cultivated for other 16 h and total RNA was extracted with NucleoSpin RNA II kit following the manufacturer's protocol. 1 µg of total RNA was reverse transcribed with iScript cDNA kit following the manufacturer's instructions. Real-time PCR was used to measure the quantity of Met relative to the quantity of HPRT-1 (hypoxanthine phosphoribosyl-transferase 1) mRNAs. HPRT-1 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 real-time PCR amplification using iQ SYBR Green Supermix. Primers for murine Met (GenBank accession number NM_008591) were: forward 5'-CGC TAT GAC GCA AGA GTA CAC A-3', reverse 5'-TTG GGA AAC TGG TCT TCT GGA-3' (efficiency 89%); 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%). Real-time PCR parameters were: cycle 1, 95°C for 3 min; cycle 2, 95°C 60 s, 60°C 30 s for 40 cycles. The $\Delta(\Delta C_T)$ method was used to analyze the data as described by Livak and Schmittgen (Livak & Schmittgen 2001).

Cell proliferation assay

HC11 cells were seeded in 6-well plates at a density of 1×10^5 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. Cell proliferation was evaluated after 48 h by trypsinization,

resuspension in PBS and counting on Burker chambers. Non-viable cells were excluded by trypan blue staining. Each experiment was repeated 3 times independently and in each experiment each treatment was performed with 2 replicate culture wells.

Scatter assay

HC11 cells were seeded in 96-well plates at a low density of 500 cells per well and cultured until they formed tightly packed colonies (3 days). The medium was removed and replaced with medium containing 10% FBS and the indicated cytokines. After 16 h cells were fixed with glutaraldehyde 11% dissolved in PBS for 10 min, coloured with crystal violet, stained with DAPI (????? 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, Wetzlar, Germany). Images of the entire wells were captured using the Tile Scan feature for automatic scanning (4x5 fields). Tile Scan image capture and merging was used to provide a single panoramic view of the well at 50x magnification. Counting of cells of the entire well was made manually. 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, 3×10^4 HC11 cells were seeded onto the upper chamber of the transwells previously coated with 25 µg of Matrigel Basement Membrane Matrix. The lower chamber of the transwells was filled with

medium containing 10% FBS and the indicated cytokines. 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, 10% glacial acetic acid) and the intensity of the coloured solution was quantified by spectrophotometrical analysis (595 nm). The intensity of the untreated control transwell was set to 100%. Each experiment was repeated 3 times independently and in each experiment each treatment was performed with 2 replicate transwells.

Statistical analysis

Experimental data are presented as mean \pm SD. 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 Scheffè' F test.

Results

HC11 mammary cells express Met and EGF receptors and activate the ERK1/2 and AKT signaling pathways in response to HGF or EGF

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 both in 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' with 10 ng/ml HGF or EGF (or left unstimulated = Control). Cells were lysed and 20 µg of total 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' whereas AKT activation persisted for a longer time only in HGF treated cells.

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). 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 20 µg of total 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 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 then Met relative mRNA levels were quantified by real-time PCR. In agreement to the data obtained by western-blot analysis we observed an increase in Met mRNA levels 5 to 10 folds (mean=6.96 folds vs Control; $P < 0,05$) compared to the level of untreated (Control) cells (Fig. 2B). HGF and IGF-I did not increase Met expression (1.25 and 1.08 folds vs Control respectively).

To verify a possible cooperation mechanism between EGFR and Met mediated by transphosphorylation between these two receptors we also performed western blot analysis but with no results (data not shown, see also discussion).

We finally analyzed the signaling pathway responsible for Met upregulation following EGFR induction. To test this hypothesis we deactivated the ERK1/2 and the AKT pathways by 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 functionally active in HC11 cells we verified if the simultaneous addition of HGF plus EGF promoted an increase in proliferation compared to HGF or EGF alone. HC11 cells are highly responsive to EGF for proliferation but no data are available on HGF-induced proliferation in this cell line. For this purpose cells were plated in 6 well plates and cultured for 36 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. Non viable, 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 (220%, $P < 0,05$) when compared to EGF (179%) or HGF (138%; Fig. 3A).

To assess the potential collaborative effect of HGF and EGF, cell motility and dispersion (scatter) was also analyzed. For this assay it was first verified if HC11 cells treated with HGF or EGF alone (10 ng/ml) responded with scatter. For this purpose cells were plated in 96 well plates at 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 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. 3B see INSLETS). To quantify differences between all treatment conditions, panoramic views of the entire wells were acquired (Fig. 3B), cells were stained with DAPI and counted (on an average of 12.000 cells/well independently the treatments). The area occupied by all cell colonies in the well were calculated using the LAS AF software. The area was then divided by total cell number of each well to obtain the mean area of any single cell. Coactivation of EGFR and Met enhanced the mean cell area ($1155 \mu\text{m}^2$, $P < 0,05$) when compared to Control ($989 \mu\text{m}^2$ SD), EGF ($966 \mu\text{m}^2$ SD) or HGF ($991 \mu\text{m}^2$ SD) treatments (Fig. 3C).

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 are 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 invasion (Fig. 3A, HGF + EGF). Quantification of cells that passed through the transwells confirmed that HGF + EGF had a synergistic effect on invasion (EGF 5x, HGF 9,5x, HGF + EGF 19,4x relative to Control; Fig. 4B). We

also tested if HC11 cells could activate the invasion program in medium with no serum. No cells passed through the pores of the chambers under any condition (Fig. 4C, Control, HGF and EGF) except if stimulated concurrently by HGF + EGF.

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 EGF + HGF treatment we analyzed cell proliferation and invasion following Met and AKT inhibition using their respective inhibitors, PHA-665752 (Accornero *et al.* 2008) and Wortmannin. 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 5B, left panels). Wortmannin 100 nM did not abolish the cooperation between EGFR and Met in proliferation and invasion assays (Fig. 5A and 5B, central panels) ruling out the possibility that Met upregulation was responsible for the increase in HC11 responses following EGF + HGF treatment. We also analyzed the effect of ERK1/2 inhibition by UO126 (10 μ M) on proliferation and invasion. Interestingly, UO126 abolished the EGF-HGF cooperation on proliferation but not on invasion (Fig. 5A and 5B, right panels). Finally ERK1/2 and AKT phosphorylation levels induced by simultaneous EGFR and Met activation were analyzed by western-blot (Fig. 6). To this aim subconfluent HC11 cells were serum starved for 24 h and then stimulated for 1 h 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 apparent (non vuol dire nulla , o c'e' o non c'e', io metterei "observed") but only after longer incubation times (4 h). HC11 cells retained an elevated

level of ERK1/2 phosphorylation at 4 h only if stimulated concurrently with EGF + HGF while AKT phosphorylation tended to lower progressively from 1 h to 4 h under all conditions.

Discussion

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 EGF-HGF collaboration on proliferation and invasion. Finally this study shows that ERK and AKT activation is increased when the EGFR and Met pathways are activated simultaneously.

We first demonstrated by western-blot analysis that both Met and EGFR are expressed in this cell line. We used murine liver as internal positive control for Met expression because it is well documented that liver and kidney express Met constitutively in the adult but only liver expresses both EGFR and Met (data not shown). In vivo, Met is present both in the luminal and the myoepithelial compartments of the mammary gland. In particular, myoepithelial cells shows higher Met expression levels and also exhibit a significant reduction during pregnancy and lactation as analyzed by immunofluorescence staining (Haslam *et al.* 2008).

Subsequently, we explored whether HGF or EGF could activate two fundamental signaling pathways, the ERK/MAPK and the AKT pathways, that have already been described downstream Met and EGFR in other cell lines (Benvenuti & Comoglio 2007; Zaczek *et al.* 2005). Ligation of HGF or EGF results in dimerization of the corresponding receptor and

transautophosphorylation leading to full receptor activation and subsequent recruitment of numerous signaling mediators such as phosphoinositide 3-kinase (PI3K) and Src tyrosine kinase, as well as several adaptor proteins such as Grb2 (which activates the ERK pathway), Gab1, Shc and c-Cbl (Peschard & Park 2007; Zaczek *et al.* 2005). 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 minutes) 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 are currently addressing whether AKT, being differentially activated downstream HGF compared to EGF, might modulate cell spreading and motility in mammary cells.

We then observed that EGF addition to HC11 cells induced Met upregulation both at the protein and 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 IGF-I 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) but never in non tumorigenic mammary cells. 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 ligands

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 (1-12 h) incubation times (data not shown). Therefore, in our model, these means of transphosphorylation are ruled out.

Coordinated receptor coactivation may have considerable consequences on cell biology. Activated ERK1/2 have been directly linked to cell proliferation and motility while the PI3K pathway is also important for the disassembly of adherens junctions, cell spreading and motility (Migliore & Giordano 2008). 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 also HGF is a strong proliferative agent. Increased cell proliferation after HGF addition was also observed in primary mammary cells (Sunil *et al.* 2002; Yant *et al.* 1998). Interestingly another non tumorigenic 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 insensible to Met induced proliferation (Accornero P, personal observation) 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 thus, to

analyze whether EGF and HGF might collaborate to evoke scatter, we lowered HGF concentrations to preclude mammary cells to scatter by the mean of this cytokine alone. During mammary gland morphogenesis the local active concentrations of paracrine factors are not known. In fact, the biological availability of many cytokines is modulated, *in vivo*, by the presence of the basement membrane and multiple layers of cells that may modulate the diffusion of these hormones (Birchmeier & Gherardi 1998; Harris *et al.* 2003). Moreover the presence of TACE/ADAM17 for EGF ligands and urokinase plasminogen activator, coagulation factor XII, trombin, or XII-like factor for HGF are required for the local activation of these cytokines (Birchmeier *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 in which both migration and destruction of the extracellular matrix (matrigel) play a role. In the absence of serum invasion was exclusively present in cells stimulated concurrently with HGF and EGF. 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 remodelling 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 non-tumorigenic mammary epithelial cells.

We finally verified if Met upregulation via the EGFR-AKT pathway was responsible for the EGF-HGF cooperation in 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, AKT inhibition 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 with lower (before EGF treatment) or higher (after EGF treatment) Met levels have the same HGF-mediated intracellular signalling. Of interest, a recently published work by Guo *et al.* shows how EGFR-addicted non-small cell lung cancer cell lines, when inhibited by the selective EGFR tyrosine-kinase inhibitor Iressa exhibits a drastic reduction in Met levels (Guo *et al.* 2008). Therefore EGF induced Met upregulation might represent a common physiological mechanism taken over by some tumorigenic cell lines during the process of transformation. 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 EGF-HGF cooperation on proliferation but not on invasion. Activated ERK1/2 regulate ETS/API transcription factors which have been directly linked to cell proliferation (Wasylyk *et al.* 1998). Our observation that ERK1/2 phosphorylation levels following EGF+HGF 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 are more important for the synergism between Met and EGFR in invasion assays. It is possible that Met and/or EGFR ~~each~~ activate some unique effectors (Guo *et al.* 2008). It will be of interest to study some of these signaling transducers.

In conclusion in this study, we demonstrated that Met and EGFR are both present in HC11 mammary epithelial cells and respond to their respective cytokines with increased activation of the ERK1/2 and AKT pathways. We showed that, when simultaneously activated, Met and EGFR provide an increase in HC11 mammary cells proliferation, scatter and invasion. We also established that, although EGF treatment upregulates Met both at the mRNA and protein levels via an AKT dependent pathway, AKT inhibition did not influence the cooperation on proliferation or invasion after EGF + HGF treatment. On the other side we established that ERK1/2 inhibition abolished 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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Figure Legends

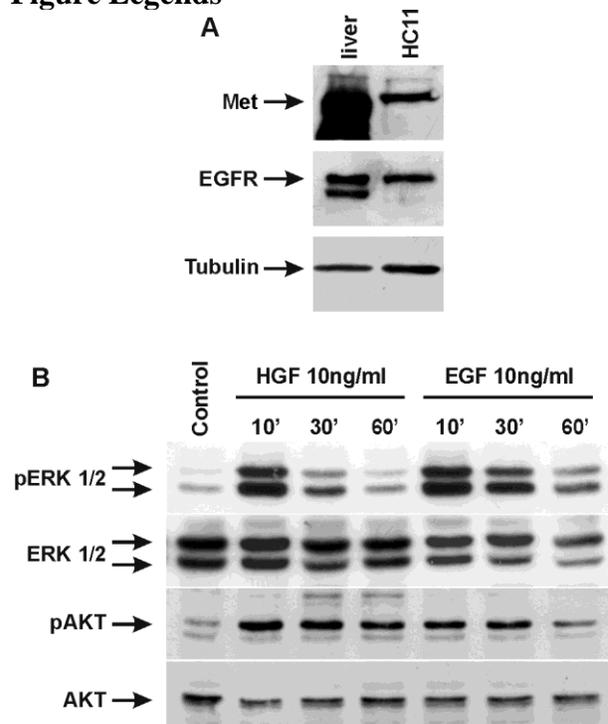


Figure 1 (A) Met and EGFR expression in HC11 cells as detected by immunoblotting. Mouse liver was used as a positive control for Met and EGFR. Tubulin was used as loading control. **(B)** Effects of EGF and HGF on ERK1/2 and AKT pathways activation in HC11 cell line. Cells were serum starved overnight then either left untreated (Control) or treated with HGF (10 ng/ml) or EGF (10 ng/ml) for the indicated times. The panel is a representative image of pERK1/2 and pAKT immunoblotting. Total ERK1/2 and AKT were used as loading controls. Western blots were repeated 3 times independently with similar results.

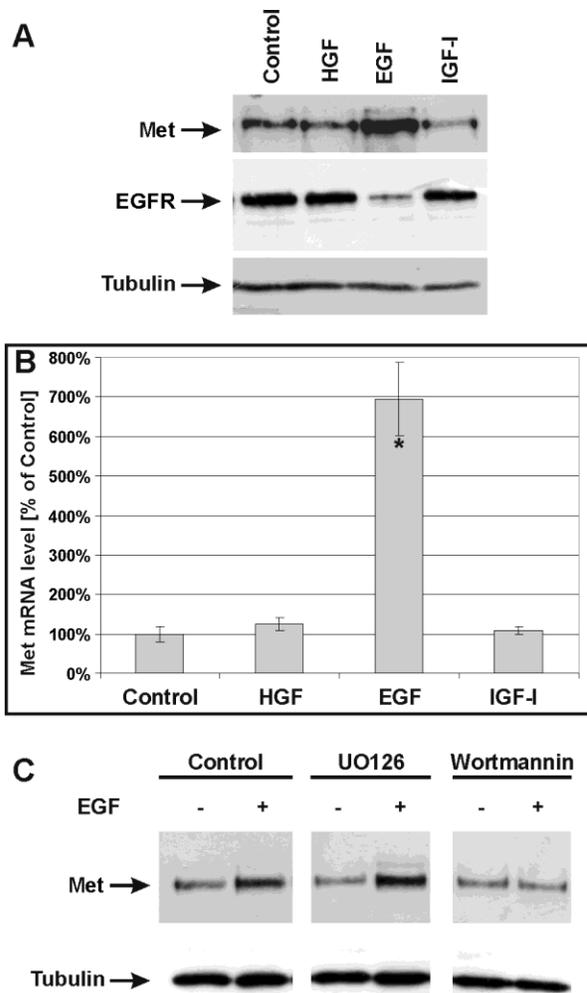


Figure 2 (A) Effect of EGF, HGF and IGF-I on Met and EGFR expression. HC11 cells were serum starved overnight then either left untreated (Control) or treated with EGF (10 ng/ml), HGF (10 ng/ml) or IGF-I (100 ng/ml) for 24 h. The panel shows a representative image of Met and EGFR immunoblotting. Tubulin 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 \pm SD of three independent experiments. * $P < 0.05$ vs 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 μ M) 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.

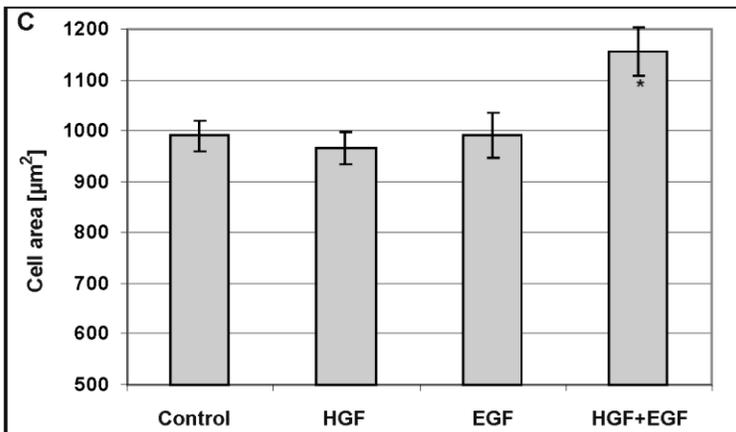
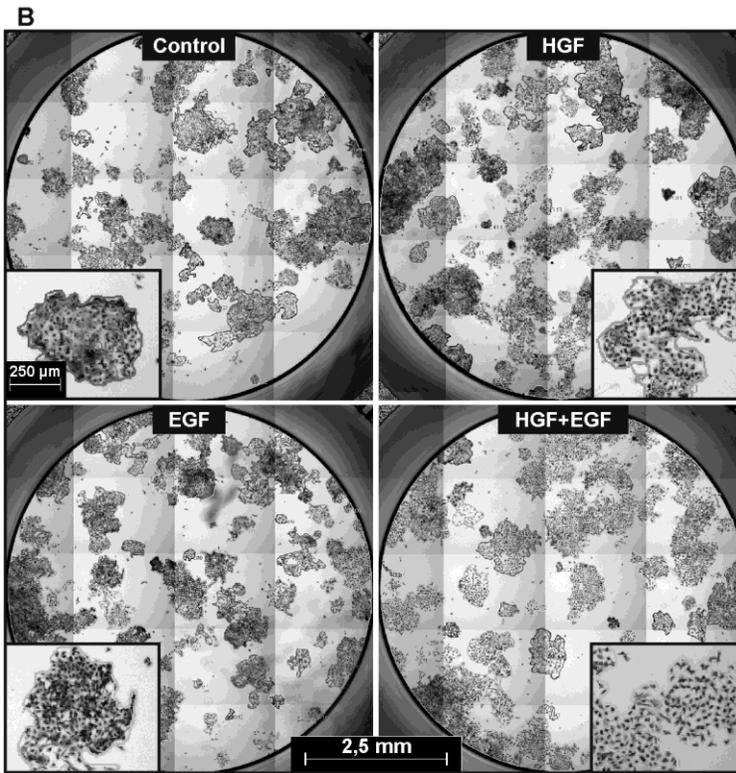
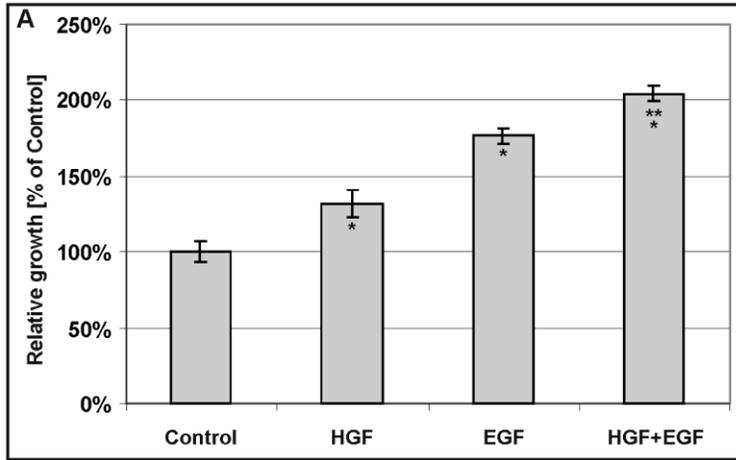


Figure 3 Effects of HGF, EGF or HGF + EGF on cell proliferation and cell scatter. (A) HC11 cells were cultured for 48 h in medium without supplements (Control) or with HGF (10 ng/ml), EGF (10 ng/ml) or HGF + EGF (10 ng/ml each). Cells were then trypsinized stained with trypan blue and counted. Non viable cells were excluded. Results are percentage relative to the untreated Control and are expressed as means \pm SD of 3 independent experiments. * $P < 0.05$ vs Control; ** $P < 0.05$ vs EGF. (B) HC11 cells were cultured for 16 h in medium without supplements (Control), with HGF (0,5 ng/ml), with EGF (10 ng/ml) or HGF (0,5 ng/ml) + EGF (10 ng/ml). Wells were fixed with glutaraldehyde, stained with crystal violet and the entire well was photographed at 50x magnification using the tile scan feature of a Leica DMI-6000 system (4x5 fields). The experiment was repeated 3 times and the panel is a representative image. The insets are a magnification of an area of the well. Metric bars for the entire well and for insets are included. (C). Quantification of the experiment in (B). Cells in the well were stained with DAPI and manually counted (~~about 12.000 cells/well~~), then all cell colonies were outlined using the Region of Interest command of the LAS software (see insets in B) and the calculated areas were summed. Areas of the wells were divided by total cell number to obtain the mean area occupied by any single cell expressed in μm^2 . * $P < 0.05$ vs Control.

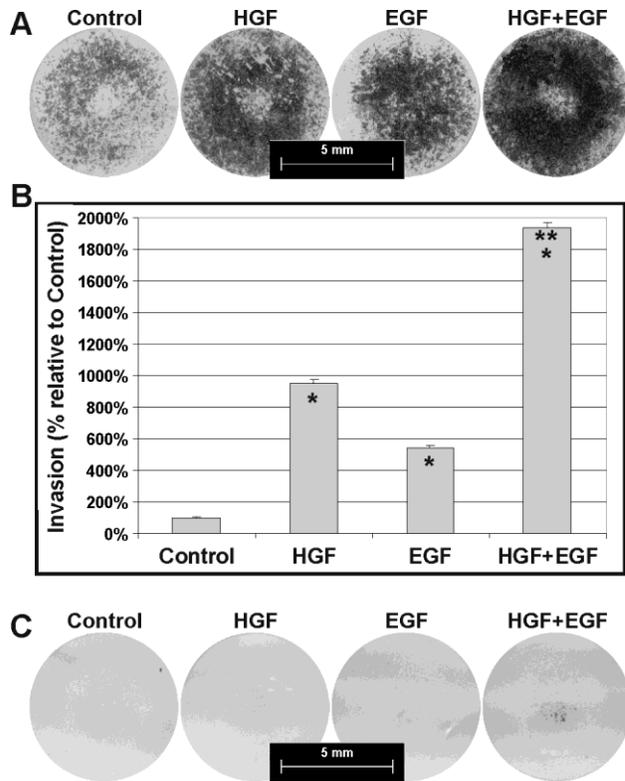


Figure 4 Effects of HGF, EGF or HGF + EGF on cell invasion. **(A)** HC11 cells were seeded in the upper side of the transwell and cultured for 48 h in RPMI medium with 10% FBS alone (Control) or in association with HGF (10 ng/ml), EGF (10 ng/ml) or HGF + EGF (10 ng/ml each). Cells in the upper side of the well were removed and the transwell was fixed, stained with crystal violet and photographed. The transwell assay was repeated 3 times with similar results. Metric bar is included. **(B)** Spectrophotometric quantification of the transwells in (A). Stained cells were dissolved in 10% acetic acid and the absorbance was read on a spectrophotometer set at 595nm. The values are expressed as percentage relative to the untreated Control. Error bars are the standard error of 3 independent experiments. * $P < 0.05$ vs Control; ** $P < 0.05$ vs HGF. **(C)** Transwell assay was

performed as in (A) but cell were seeded in medium with no serum. The panel is a representative image of 3 independent experiments. Metric bar is included.

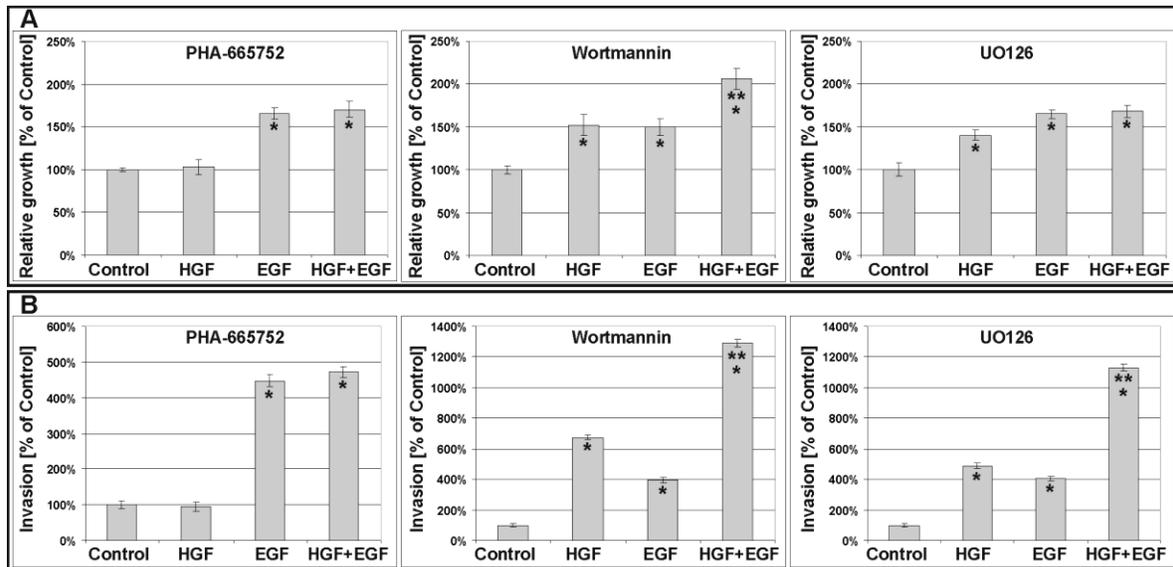


Figure 5 Effects of Met, AKT and ERK1/2 inhibition on cell proliferation and invasion. **(A)** HC11 cells were cultured for 48 h in medium containing the specific inhibitors for Met (PHA-665752 250nM), AKT (Wortmannin 100nM) or ERK1/2 (UO126 10 μ M) in RPMI + 10% fetal bovine serum alone (Control), with HGF (10 ng/ml), EGF (10 ng/ml) or HGF + EGF (10 ng/ml each). Cells were then trypsinized stained with trypan blue and counted. Non viable cells were excluded. Results are percentage relative to the untreated Control and are expressed as means \pm SD of 3 independent experiments. * $P < 0.05$ vs Control; ** $P < 0.05$ vs HGF. **(B)** Spectrophotometric quantification of transwell invasion assays on HC11 cells in presence of Met, AKT and ERK1/2 inhibitors. Cells were cultured for 48 h as described in Figure 4. The values are expressed as percentage relative to the

untreated Control. Error bars are the standard error of 3 independent experiments.

* $P < 0.05$ vs Control; ** $P < 0.05$ vs HGF.

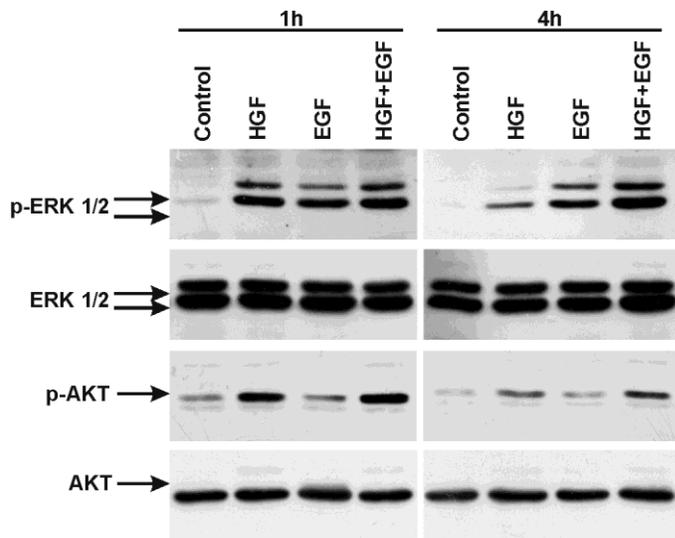


Figure 6 Effects of HGF, EGF or HGF + EGF on ERK 1/2 and AKT pathways activation in HC11 cell line. Cells were serum starved overnight then either left untreated (Control) or treated with HGF (10 ng/ml), EGF (10 ng/ml) or HGF + EGF (10 ng/ml each) for 1 h and 4 h. The panel is a representative image of pERK1/2 and pAKT immunoblotting. Total ERK 1/2 and AKT were used as loading controls. Western blots were repeated 3 times independently with similar results.