HGF Converts ErbB2/Neu Epithelial Morphogenesis to Cell Invasion

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Activation of the hepatocyte growth factor receptor Met induces a morphogenic response and stimulates the formation of branching tubules by Madin-Darby canine kidney (MDCK) epithelial cells in three-dimensional cultures. A constitutively activated ErbB2/Neu receptor, NeuNT, promotes a similar invasive morphogenic program in MDCK cells. Because both receptors are expressed in breast epithelia, are associated with poor prognosis, and hepatocyte growth factor (HGF) is expressed in stroma, we examined the consequence of cooperation between these signals. We show that HGF disrupts NeuNT-induced epithelial morphogenesis, stimulating the breakdown of cell-cell junctions, dispersal, and invasion of single cells. This correlates with a decrease in junctional proteins claudin-1 and E-cadherin, in addition to the internalization of the tight junction protein ZO–1. HGF-induced invasion of NT-expressing cells is abrogated by pretreatment with a pharmacological inhibitor of the mitogen-activated protein kinase kinase (MEK) pathway, which restores E-cadherin and ZO-1 at cell-cell junctions, establishing the involvement of MEK-dependent pathways in this process. These results demonstrate that physiological signals downstream from the HGF/Met receptor synergize with ErbB2/Neu to enhance the malignant phenotype, promoting the breakdown of cell-cell junctions and enhanced cell invasion. This is particularly important for cancers where ErbB2/Neu is overexpressed and HGF is a physiological growth factor found in the stroma.

INTRODUCTION

Normal epithelia are highly organized tissues composed of a single sheet of polarized cells connected by cellular junctions and attached to the extracellular matrix (ECM) via heteromeric protein complexes (Balda and Matter, 2003). This highly organized architecture suggests that dynamic and reciprocal interactions take place between epithelial cells and the surrounding ECM (Somasiri and Roskelley, 1999; Bissell et al., 2002). Tight regulation of these interactions dictates cell behavior and contributes to tissue homeostasis. Specific cellular processes during embryogenesis, such as neural crest cell migration, require a transient phenotypic modulation where epithelial cells lose their cell-cell junctions and acquire a motile mesenchymal phenotype (Savagner, 2001). This process, called epithelial-mesenchymal transition (EMT), can be initiated by different growth factors (hepatocyte growth factor [HGF], epidermal growth factor [EGF], transforming growth factor-β [TGFβ]) or extracellular matrix components, such as collagen. EMT also occurs in tumor dissemination and metastasis, where the phenotypic changes are more potent (Thiery, 2002).

The development of three-dimensional cell culture models that mimic the complexity of epithelial architecture, and the use of animal models, have allowed the identification of important modifiers of epithelial integrity, although for many, their mechanisms of action remain poorly understood (Schmeichel and Bissell, 2003; Kuperwasser et al., 2004; Wrobel et al., 2004). A role for the HGF/scatter factor (SF) receptor Met and HER2/ErbB2/Neu, a member of the EGFR family, in epithelial remodeling has been proposed from genetic ablation studies (Lee et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Ebens et al., 1996; Andrechek et al., 2002; Chan et al., 2002; Leu et al., 2003). Whole organ cultures and cell culture models suggest that Met and ErbB2/Neu are associated with the morphogenic and functional differentiation of normal breast epithelium (Yang et al., 1995; Niemann et al., 1998; Jones and Stern, 1999). Using a nontransformed kidney epithelial cell model (MDCK), we have previously shown that ligand-induced activation of Met and constitutive activation of ErbB2/Neu each promote the inherent morphogenic program of MDCK cells, that is the conversion of cysts of polarized epithelial cells into branching tubules in three-dimensional collagen gels (Maroun et al., 1999; Khoury et al., 2001).

HER2/ErbB2 and Met are independent prognostic markers for breast cancer (Yamashita et al., 1994; Ghoussoub et al., 1998). High levels of Met in human breast carcinomas and high levels of HGF in tumor stroma have been reported previously (Yamashita et al., 1994; Tuck et al., 1996; Kang et al., 2003). The amplification and overexpression of HER2/ErbB2 is associated with increased progression and metastasis in 25% of human breast carcinomas and is indicative of poor prognosis in breast, ovarian, and renal collecting duct

carcinomas (Selli *et al.*, 1997; Zhang *et al.*, 1997; Pegram *et al.*, 1998). Consistent with this, a constitutively activated ErbB2/Neu receptor, NeuNT, has been shown to induce mammary tumors in transgenic animals (Guy *et al.*, 1992, 1996; Dankort *et al.*, 1997). Transactivation of ErbB2/Neu by heregulin enhances cell invasion in some breast tumor cell lines (Meiners *et al.*, 1998; Brandt *et al.*, 1999), whereas in other cell lines, it promotes a remodeling of epithelial cell junctions consistent with a morphogenic response (Chausovsky *et al.*, 1998). Synergy between an ErbB2/Neu signal and TGFβ is associated with cell invasion in culture and animal-based models (Siegel *et al.*, 2003; Seton-Rogers *et al.*, 2004; Ueda *et al.*, 2004). These data suggest that the biological consequence of activation of ErbB2/Neu depends on how cells integrate an ErbB2/Neu signal in the context of other signals, and these may differ among different cancer cells. For example, activation of ErbB2/Neu may predispose epithelial cells to malignant transformation, where additional factors might be required to trigger the progression of invasive disease. Using stable MDCK cell lines, we show that the Met/HGF receptor converts an epithelial morphogenic program induced by activated ErbB2/Neu to cell invasion.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies used in this article are as follows: mouse anti-Neu antibodies antibody-3 and antibody-4 (Oncogene Science, Cambridge, MA), mouse anti-E-cadherin and β-catenin (BD Transduction Laboratories, Mississauga, Ontario, Canada), rabbit anti-ZO-1, occludin, claudin-1, and claudin-2 (Zymed Laboratories, South San Francisco, CA), and rabbit anti-phospho-Erk (New England Biolabs, Nepean, Ontario, Canada). The following antibodies were generously donated: mouse anti-gp135 from Dr. G. Ojakian (SUNY Health Science Center, Brooklyn, NY), mouse anti-p120catenin from Dr. A. Reynolds (Vanderbilt University School of Medicine, Nashville, TN), and rabbit anti-Erk from Dr. J. Blenis (Harvard University, Boston, MA). Secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 555 donkey anti-mouse, as well as Alexa Fluor 488 and 555 donkey anti-rabbit (Molecular Probes, Eugene, OR). HGF was a generous gift from Dr. G. Van deWoude (Van Andel Research Institute, Grand Rapids, MI). EGF and heregulin βl were purchased from Roche Diagnostics (Mannheim, Germany) and Neomarkers (Fremont, CA), respectively. Inhibitors for MEK (UO126), phosphoinositide 3-kinase (PI3K) (LY294002), Src (PP2), and RhoA (Exoenzyme C3) were purchased as follows: UO126 was from Promega (Fisher Scientific, Nepean, Ontario, Canada), LY294002 was from BIOMOL Research Laboratories (Plymouth Meeting, PA), PP2 was from Calbiochem (San Diego, CA), and Exoenzyme C3 from Upstate Biotechnology (Cedarlane, Hornby, Ontario, Canada).

Cell Culture and Scatter Assay

MDCK cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml Gentamicin (Invitrogen, Carlsbad, CA). Neu mutants (Dankort *et al.*, 1997) were transfected into MDCK cells by the calcium phosphate method, and stable cell lines were selected in G418 (400 μg/ml) (Geneticin; Invitrogen). Cells (5×10^3) were seeded in 24-well dishes (Nalge Nunc International, Naperville, IL) for cell scatter studies. After 48 h, HGF was added to the medium at 5 U/ml for 24 h.

Migration Assay

Cells (5×10^5/well) were plated on six-well Transwell filters (Costar, Cambridge, MA) in the presence or absence of HGF (5 U/ml). Twenty-four hours later, filters were submerged in formalin phosphate buffer (Fisher Scientific) for 15 min, washed twice with distilled water, and stained with crystal violet for 15 min at room temperature, followed by several washes with water. Nonmigrating cells were scraped off the upper layer of the filter by using a cotton swab. Filters were then air-dried and photographed. To quantitate cell migration, filters were cut, solubilized in 10% acetic acid, and absorbance readings were taken at 596 nm. The results plotted are average numbers of four experiments for each cell line.

Matrigel Invasion Assay

Transwell filters (24-well; Costar) were coated with Matrigel (50–100 μg/cm²; BD Biosciences, Mississauga, Ontario, Canada). Cells (8×10^4/well) were plated and processed as described above. Where indicated, cells were grown in the presence of HGF for 7–15 d before plating them on Matrigel. Alternatively, EGF (50 ng/ml) was added to the assay for 24 h, or cells were grown

in the presence of EGF for 7 d. For quantitation, filters were photographed and invading cells were counted using the Northern Eclipse image processing system (Empix Imaging, Toronto, Ontario, Canada).

Tubulogenesis Assay

MDCK cells were suspended in a collagen matrix as described previously (Maroun *et al.*, 1999). Briefly, 5×10^3 cells were resuspended in 500 μl of collagen solution (Vitrogen 100; Celtrix, Santa Clara, CA) prepared as specified by the manufacturer, and layered over 350 μl of the collagen solution in a 24-well plate. The cells were maintained in L-15 medium containing 5% FBS and allowed to form structures for 7 d. HGF (5 U/ml) and UO126 (5 and 20 μM) were then added to the medium either simultaneously or at a 2-d interval, as indicated. The medium was changed every 4 d. For the quantitation of the morphogenic response, 60 colonies in each of five independent cultures (wells) were scored for their ability to form branching tubules (structures whose length is 5 times their diameter), and the results were plotted as the average number of cysts able to undergo tubulogenesis per culture per 100.

Anoikis Assay

Anchorage-independent cell survival assay was performed as described previously (Frisch, 2000). Briefly, genomic DNA from normal (MDCK), wild-type Neu, and deregulated NeuNT-expressing cells maintained in suspension for 2 h was separated on a 1.5% agarose gel.

Immunoprecipitation and Western blotting

Subconfluent cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, and 1 mM Na_3VO_4). Equal amounts of total protein (20–50 μg) were separated by electrophoresis on 8% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and immunoblotted. Where indicated, cells were grown in the presence of HGF for 5 d. To quantitate Neu mutant expression levels, equal amounts of total protein (500 μg) were immunoprecipitated before gel separation.

Light Microscopy

Photographs of fixed collagen cultures and dispersed cells were taken using a digital image processing system (Northern Eclipse; Empix Imaging, Toronto, Ontario, Canada) at a magnification of 2.5× (bright field objective) and 10× (phase contrast).

Confocal Scanning Laser Microscopy

Collagen cultures were stained as described previously (Debnath *et al.*, 2003) Briefly, slices from collagen cultures were fixed in 2% paraformaldehyde for 20 min at room temperature, rinsed three times with phosphate-buffered saline (PBS), and then permeabilized with PBS containing 0.2–0.5% Triton X-100 for 10 min. The slices were then washed three times in PBS/glycine (100 mM glycine) for 5 min each time at room temperature, and then incubated in blocking solution (PBS, 2% bovine serum albumin, 0.2% Triton X-100, and 0.05% Tween 20) for 30 min. Incubation with primary antibody was performed for 1 h (room temperature) to overnight (4°C), after which slices were washed three times (5 min per wash) and incubated with the secondary antibody for 40–50 min. Antibodies were diluted in blocking solution. Collagen slices were then washed twice with water, mounted in Immu-mount, and allowed to dry overnight at room temperature.

Live/dead staining was performed on live cultures by using calcein and ethidium homodimers from Molecular Probes (Eugene, OR) (Debnath *et al.*, 2002). Images were photographed using Confocal LSM510 (Carl Zeiss, Thornwood, NY).

RESULTS

NeuNT Enhances Cell Migration

Stable expression of a constitutively activated ErbB2/Neu mutant receptor, NeuNT (NT-3, NT-6, and NT-9), but not a WT ErbB2/Neu receptor, Neu-1, induces a transient epithelial-mesenchymal (EM)-like transition in normal epithelial MDCK cells (Khoury *et al.*, 2001). This transition is characterized by the loss of cell-cell junctions and epithelial cell dispersal as well as enhanced cell migration and is comparable with HGF-induced cell scatter (Figure 1B) (Khoury *et al.*, 2001). To establish whether NeuNT-induced cell scatter is similar to that induced by HGF, we determined whether HGF synergizes with NeuNT to enhance cell migration by using a modified Boyden chamber. In the absence of HGF, MDCK cells overexpressing a WT Neu receptor (Neu-1)
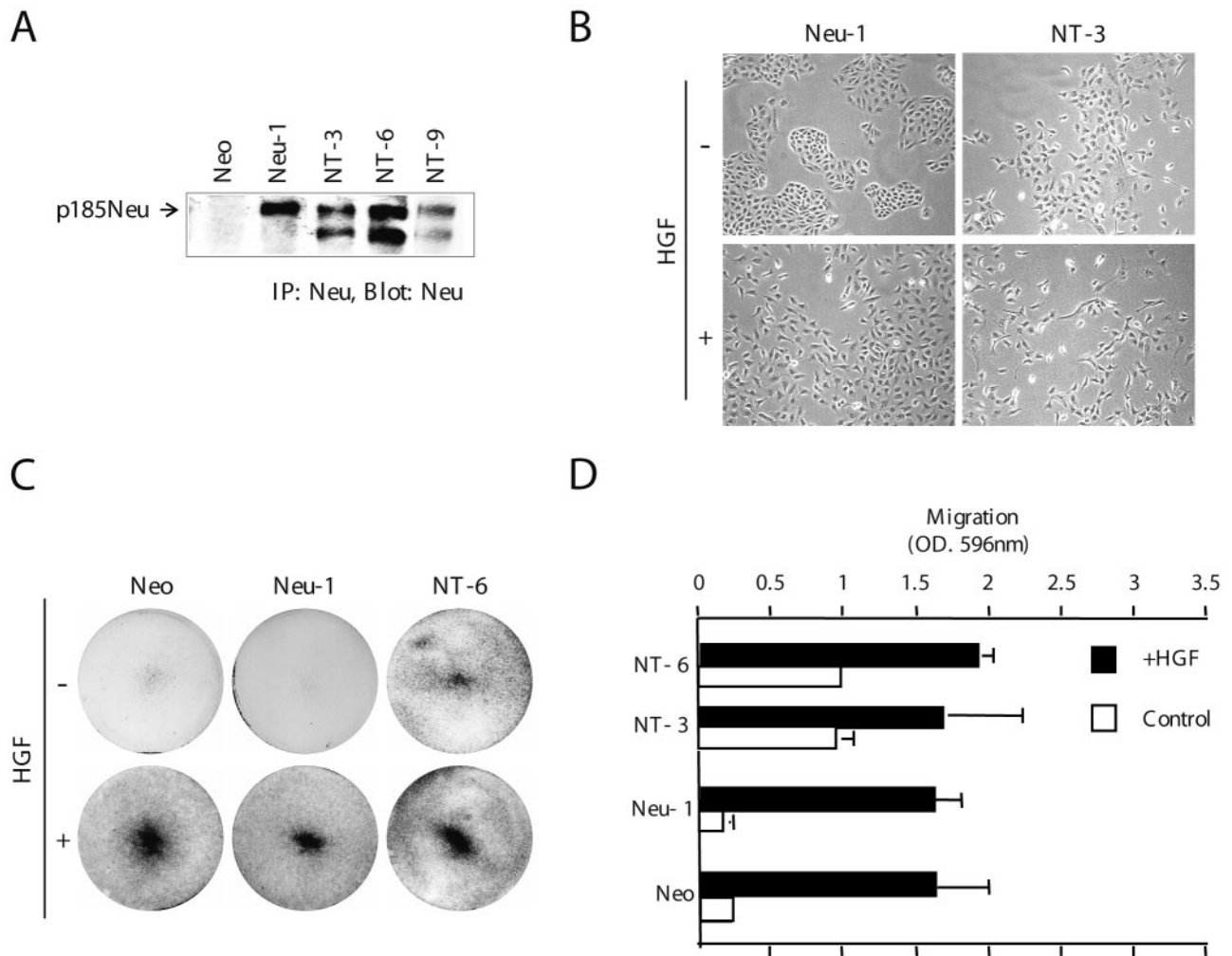
Figure 1. Additive effects of HGF and deregulated ErbB2/Neu enhance cell dispersal and motility. (A) Stable expression of ErbB2/Neu mutant receptors in MDCK cells. Neu protein was immunoprecipitated from RIPA cell lysates (500 μg of protein) with mouse antibody-4. Proteins were separated by SDS-PAGE (8%) and immunoblotted with antibody-3 (anti-Neu) antibody. (B) Stable MDCK cells expressing ErbB2/Neu mutants were grown in DMEM with 10% FBS in six-well plates, in the presence or absence of HGF. Representative clones were photographed using the Northern Eclipse image processing system (magnification, 10×). (C) Motility assays were performed using Transwell filters, as described in *Materials and Methods*. Representative photographs of crystal violet-stained filters were taken. (D) Filters were solubilized in 10% acetic acid, and the migration of Neu-expressing cells was determined by measuring the absorbance at a wavelength of 596 nm. Each value represents the average of four filters.

behave in a similar manner to vector-alone cells. In contrast, cells expressing an activated NeuNT (NT-6 and NT-3) show increased migration, where fivefold more cells have traversed the membrane 16 h postseeding (Figure 1, C and D). HGF treatment of NT-expressing cells enhances cell migration a further twofold, to a level that is comparable with, but not in excess of, HGF-treated control MDCK cells or cells expressing WT Neu (Figure 1, C and D). This demonstrates that although signals downstream from an activated ErbB2/Neu enhance cell migration, these signals are not as potent as HGF. Furthermore, signals downstream from NeuNT and HGF are not additive for cell migration.

HGF and NeuNT Synergize to Promote Disruption of Organized Epithelia

We have shown previously that deregulated ErbB2/Neu (NT) induces an invasive morphogenic program in MDCK cells in a three-dimensional collagen matrix and promotes

the formation of tubular structures (Khoury *et al.*, 2001). This response is similar in morphology to that induced by HGF in MDCK cells. To establish whether HGF and NeuNT cooperate to enhance the morphogenic response in normal epithelia, we allowed cells expressing NeuNT to form branching tubules and stimulated these with HGF. HGF promotes a morphogenic response in cells expressing WT Neu, or a Neu mutant receptor with impaired ability to couple with signaling molecules (NYPD) (Figure 2A). In contrast, stimulation with HGF promotes the disruption of NeuNT-induced branching tubules into single cells, which invade the collagen matrix (Figure 2, A and B). To establish which NeuNT-dependent signals collaborate with HGF to promote the breakdown of organized epithelia-stable MDCK cell lines expressing Neu tyrosine add-back mutants that couple to selected signaling molecules (Dankort *et al.*, 1997) were examined. Add-back mutants were generated using the *Neu Tyrosine Phosphorylation-Deficient* (NYPD) mutant by re-
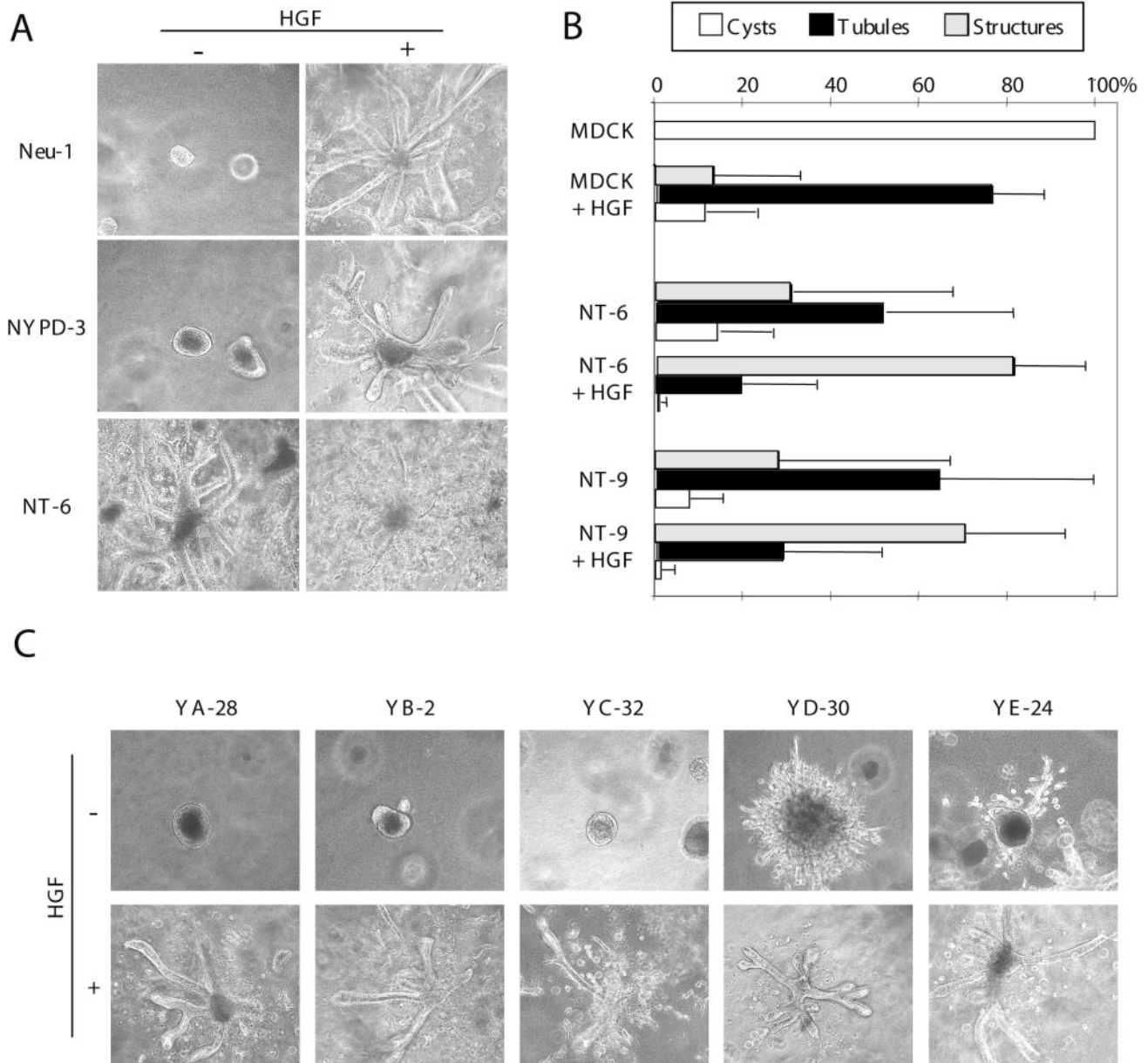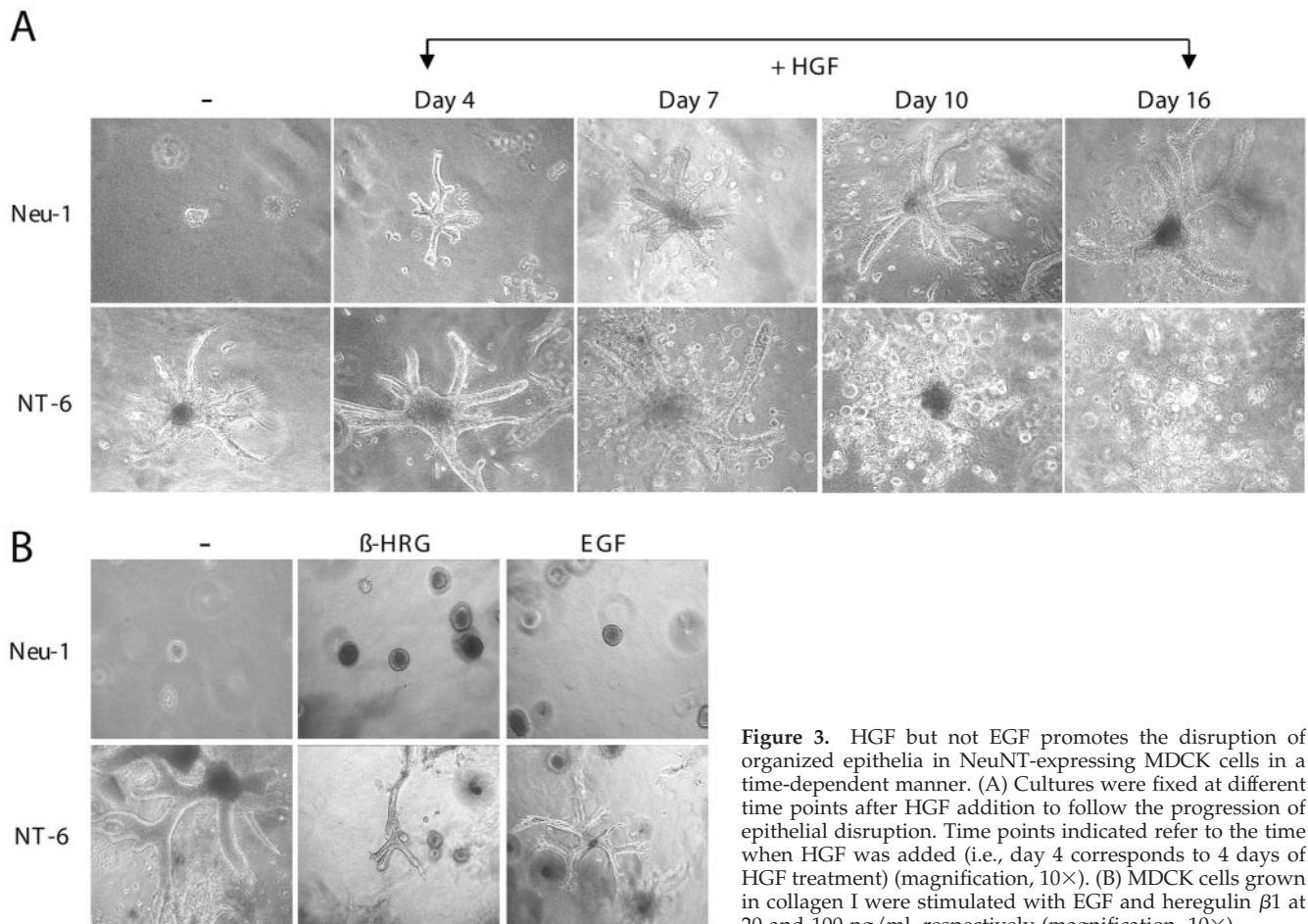
Figure 2. HGF promotes the disruption of organized epithelia in MDCK cells overexpressing deregulated ErbB2/Neu (NT). (A) Stable lines of MDCK cells expressing wild-type, mutant, and deregulated Neu receptors were grown in collagen I. Five days later, cultures were stimulated with HGF (5 U/ml) for 10 d, and then fixed and photographed. (B) Quantitation of the morphogenic response to HGF treatment in Neu-expressing MDCK cells. Data from representative clones are shown. Columns represent cysts (blank), tubules (solid), and various structures (striped). Results are plotted as the average number of structures per 100. Each value represents the mean average from at least four independent experiments. (C) Representative clones of stable MDCK cell lines expressing Neu add-back mutants (YA, YB, YC, YD, and YE) were grown in collagen I, stimulated with HGF (5U/ml) as in A, and then fixed and photographed. Tubules are structures whose length is 5 times their diameter. Structures represent mainly dispersed structures, but also include spikes, unbranched tubules, and protrusions.

verting individual phenylalanine substitutions to tyrosine residues (Dankort *et al.*, 1997). The resulting mutants YB, YC, YD, and YE are tyrosine phosphorylated and catalytically active (Dankort *et al.*, 1997). As shown previously (Khoury *et al.*, 2001), the YD and YE add-back mutants stimulate epithelial remodeling of MDCK cells when seeded in collagen, whereas MDCK cells expressing the YA, YB, and YC receptor mutants form cysts in a similar manner to control MDCK cells (Figure 2C). To establish whether specific NeuNT add-back mutants cooperate with HGF for breakdown of orga-

nized epithelia, we allowed cells expressing each of the add-back mutants to form cysts (YA, YB, and YC) or morphogenic structures (YD and YE) for 7 d and stimulated these with HGF. HGF promotes a morphogenic response in cells expressing each of the add-back mutants (Figure 2C), demonstrating that single carboxy-terminal tyrosine residues are not sufficient for the breakdown of organized epithelial structures observed with cells expressing NeuNT.

In cells expressing NeuNT, the breakdown of organized epithelia and cell dispersal observed in response to HGF,

Figure 3. HGF but not EGF promotes the disruption of organized epithelia in NeuNT-expressing MDCK cells in a time-dependent manner. (A) Cultures were fixed at different time points after HGF addition to follow the progression of epithelial disruption. Time points indicated refer to the time when HGF was added (i.e., day 4 corresponds to 4 days of HGF treatment) (magnification, 10×). (B) MDCK cells grown in collagen I were stimulated with EGF and heregulin β1 at 20 and 100 ng/ml, respectively (magnification, 10×).

occurs in a time-dependent manner, leading to the loss of organized epithelial structures within 10 d post-HGF treatment (Figure 3A), whereas HGF treatment of MDCK cells overexpressing WT Neu or any of the add-back mutants (YA, YB, YC, YD, and YE) promotes the formation of branching tubules over this time period, and no breakdown of epithelial structures is observed at later time periods (Figures 2C and 3A; our unpublished results). Moreover, heregulin and EGF, ligands for the EGFR family receptors, fail to induce the breakdown of organized epithelia observed with HGF in NeuNT-expressing cells, and the branched tubular structures are maintained (Figure 3B).

When seeded in a matrix of collagen I, MDCK cells form three-dimensional cyst structures. These cysts consist of a ball of polarized cells surrounding a hollow lumen formed through apoptosis of centrally located cells (Fournier *et al.*, 1996; Ojakian *et al.*, 1997; O'Brien *et al.*, 2002). Cell polarity and cell death can be monitored by immunofluorescence by using the apical marker gp135 and calcein/ethidium homodimer reagents that identify dead versus live cells (Debnath *et al.*, 2002). Using this assay, we show that gp135 localizes to the luminal side in NeuNT-induced tubular structures, similar to the fully polarized MDCK cysts and HGF-induced tubules (Figure 4). In all conditions, the lumen is filled with apoptotic cells as revealed by ethidium homodimer red staining, surrounded by live cells (green staining) (Figure 4). The single cells that disperse from NeuNT-dependent tubules in response to HGF stain for calcein (green), demonstrating that they are alive and invading the

collagen matrix (Figure 4). These data demonstrate that signals downstream from the Met receptor synergize with an activated NeuNT to promote the loss of epithelial integrity and cell invasion, whereas either receptor signal alone promotes a morphogenic program.

HGF and NeuNT Synergize to Enhance Cell Invasion

Malignant cells acquire the ability to invade the extracellular matrix and survive in the bloodstream in an anchorage-independent manner, allowing them to metastasize to distant organs. Using Matrigel-coated filters, we show that MDCK cells in the absence of HGF show no invasion over the time period assayed (16 h), whereas pretreatment of these cells with HGF (15 d) induces a modest invasive capacity (18 cells per average field; Figure 5, A and B). MDCK cells expressing NeuNT show increased levels of invasion (40 cells per field; Figure 5, A and B) compared with HGF-treated MDCK cells (18 cells per field; Figure 5, A and B). Notably, the most dramatic effect is observed after pretreatment of NeuNT-expressing MDCK cells with HGF, which increases their invasive potential by sixfold (250 cells per field; Figure 5, A and B) compared with untreated NeuNT cells. In contrast, EGF promotes invasion of parental MDCK cells when seeded as single cells. However, EGF pretreatment did not enhance the invasion of NeuNT-expressing cells (Figure 5C), demonstrating synergy between HGF and NeuNT for cell invasion but not between EGF and NeuNT.

HGF increases cell survival by protecting epithelial cells from anoikis or cell death induced by detachment from the
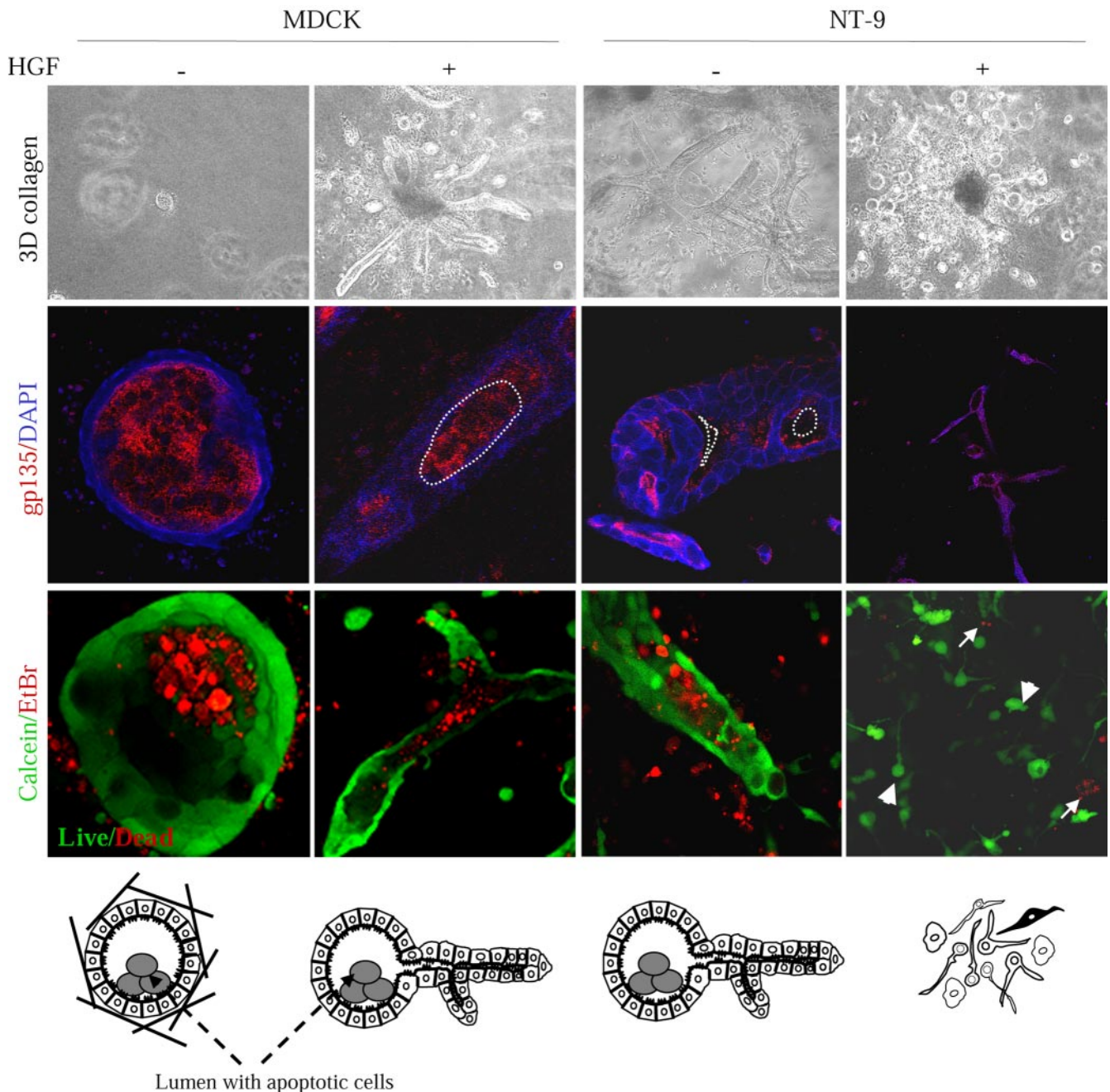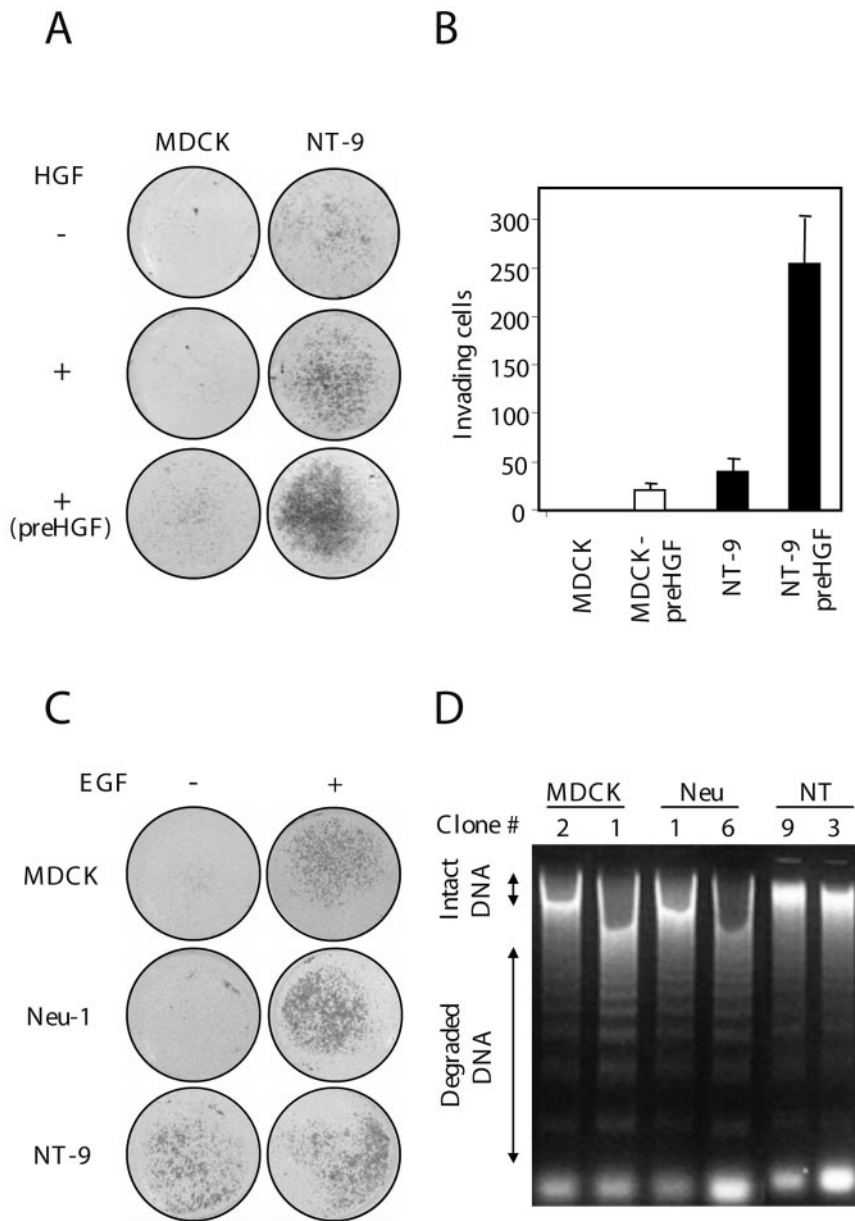
Figure 4. HGF and deregulated ErbB2/Neu synergize to promote collagen invasion. Live three-dimensional collagen cultures of MDCK cells and NeuNT-expressing cells were treated with HGF for 10 d and stained with calcein for live cells (green; arrowhead) and ethidium homodimers for dead cells (red; white arrow). LSM510 (magnification, 25×). Cells also were stained with the apical marker gp135 for luminal polarity, and the nuclei counterstained with 4,6-diamidino-2-phenylindole. Bottom, cartoon illustration of the different structures and single invading cells.

extracellular matrix. Anoikis can be monitored by increased genomic DNA degradation (Frisch, 2000). To assess whether deregulated NeuNT protects MDCK cells from anoikis, we isolated genomic DNA from NeuNT-expressing cells grown in suspension and followed DNA degradation on agarose gels. We show that under these conditions, wild-type Neu fails to prevent DNA degradation, whereas expression of NeuNT prevents DNA degradation in a similar manner to HGF pretreatment (Figure 5D; our unpublished results). Hence, activated ErbB2/Neu confers a cell survival advantage to epithelial cells in an anchorage-independent manner.

HGF and NeuNT Synergize to Break Cell-Cell Junctions

The development of invasive cancers correlates with the loss of epithelial organization, referred to as EMT (Boyer *et al.*, 1996; Thiery, 2002). This process involves, in addition to increased cell invasion and motility, the disruption of cell-cell junctions and cell dispersal. To assess the effect of HGF and NeuNT on the composition of cell-cell junctions, we have examined the levels of adherens (E-cadherin) and tight junction (claudin-1, occludin, and ZO-1) proteins in NeuNT-expressing cells grown in the presence or absence of HGF.

Figure 5. Synergy between HGF and deregulated ErbB2/Neu promotes Matrigel invasion. (A) Matrigel assay was performed in the presence or absence of HGF (5 U/ml), as described in *Materials and Methods*. In one case, NeuNT lines were maintained in HGF-containing medium for 15 d before seeding them on Matrigel (labeled as pre-HGF). Representative filters were scanned using a digital scanner. (B) Quantitation of the invasive response. Filters were photographed and cells counted using the Northern Eclipse image processing system. Values represent the average of three filters. (C) EGF promotes MDCK cell invasion. EGF (50 ng/ml) was added to parental MDCK cells or cells expressing WT or activated Neu in a Matrigel invasion assay for 24 h. Digital scans of representative filters are shown. (D) NT protects cells from anoikis. Stable MDCK cells expressing ErbB2/Neu receptor mutants were treated with different doses of HGF. Treated cells were then incubated at 37°C in suspension for 2 h and genomic DNA was extracted and separated on a 1.5% agarose gel.

E-cadherin and claudin-1 protein levels are consistently reduced in HGF-treated NeuNT cells, whereas levels of occludin or ZO-1 are not altered (Figure 6). This decrease occurs in a time-dependent manner, because prolonged treatment with HGF (5 d to 2 wk) enhances the reduction in E-cadherin and claudin-1 protein levels (Figure 6; our unpublished results). The decrease in E-cadherin and claudin-1 is specific to NeuNT-expressing cells, because HGF treatment of MDCK cells promotes no detectable change in the levels of E-cadherin or claudin-1. In addition, MDCK cells that have undergone an epithelial-mesenchymal transition after the expression of activated c-Src show a reduction in protein levels of occludin and ZO-1 but not E-cadherin or claudin-1 (Figure 6), indicating that these changes are not a general response to the breakdown of cell-cell junctions.

The down-regulation of E-cadherin at the transcriptional level has been observed after epithelial mesenchymal transitions (Thiery, 2002). No significant alterations of E-cadherin mRNA levels after long-term treatment with HGF were observed by quantitative real-time PCR analyses (our unpublished results), suggesting that posttranslational mechanisms might be involved in the observed reduction of E-cadherin protein levels. Consistent with this, immunofluorescence staining of NeuNT cells in three-dimensional collagen cultures reveals a reduction in E-cadherin protein levels, although E-cadherin is retained at cell-cell junctions. However, after stimulation with HGF, E-cadherin is redistributed from cell-cell junctions as observed in HGF-stimulated MDCK cells, to a cytosolic dot-like distribution in NeuNT-expressing cells (Figure 7). Similarly, indirect immunofluorescence shows that ZO-1 is redistributed from an apical junctional compartment localization in MDCK or NeuNT-expressing MDCK cells, to the cytosol in NeuNT-expressing cells in response to HGF (Figure 7). These data demonstrate that HGF and NT cooperate to destabilize junctional complexes in MDCK epithelial cells and that the expression of NeuNT alone is insufficient to promote the breakdown of cell-cell junctions in three-dimensional cultures.
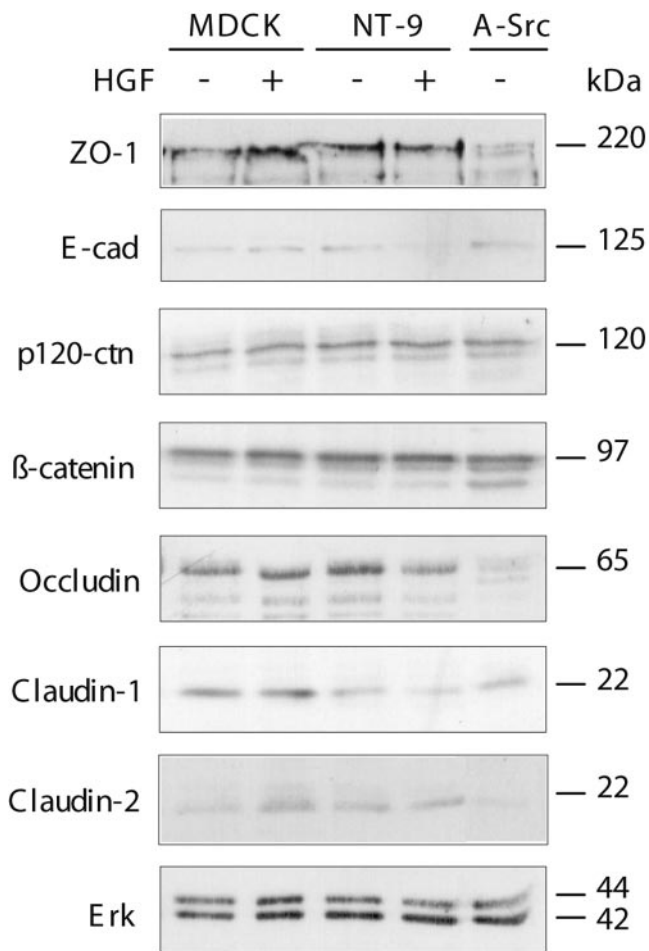
Figure 6. HGF and deregulated ErbB2/Neu synergize to down-modulate components of adherens and tight junctions. (A) Cells were maintained in the presence or absence of HGF for 5 d as indicated. RIPA cell lysates (25–50 μg) were separated on 8% polyacrylamide gels, transferred to nitrocellulose membranes, and probed for components of the adherens (E-cadherin, β-catenin, and p120 catenin) and tight junctions (ZO-1, occludin, claudin-1, and claudin-2) as indicated.

The MEK/MAPK Pathway Is Required for HGF-Induced Invasive Dispersal of NeuNT-expressing Cells

The breakdown of epithelial cell-cell junctions in response to HGF requires PI3K-, MEK-, Ras-, and Rho family-dependent signals (Royal and Park, 1995; Potempa and Ridley, 1998; Royal *et al.*, 2000). Moreover, it has been suggested that c-Src activity is required for HGF-induced cell dispersal (Elliott *et al.*, 2002). To examine the role of the MEK-Erk, PI3K, Rho, and Src pathways in HGF-induced disruption of tubular structures, NeuNT-expressing cells were treated in a dose-dependent manner with a MEK1 inhibitor (UO126), an inhibitor of PI3K (LY294002), A RhoA inhibitor (Exoenzyme C3), and a Src inhibitor (PP2). Pretreatment of NeuNT-expressing cells with low levels of UO126 (5 μM) before stimulation with HGF prevents the disruption of organized tubules, whereas pretreatment with LY294002, C3, or PP2 fails to prevent cell dispersal (Figure 8B; our unpublished results). After pretreatment with UO126, NeuNT branching tubules are retained in response to HGF, and E-cadherin and ZO-1 are maintained at cell-cell contacts as revealed by indirect immunofluorescence on three-dimensional struc-

tures (Figure 8C). However, when the NeuNT-expressing cells are pretreated with HGF before UO126 treatment, UO126 fails to block HGF-induced breakdown of tubular structures (Figure 8B). Consistent with a requirement for MEK-dependent pathways for a synergistic response, HGF stimulation of NeuNT-expressing MDCK cells induces further activation of extracellular signal-regulated kinase (Erk), which is downstream from MEK (Figure 8D). Erk phosphorylation is abolished upon pretreatment of the cells with UO126 before HGF stimulation. Together, these results demonstrate that MEK-dependent signals are required for the HGF-dependent breakdown of cell-cell junctions and cell invasion observed in NeuNT-expressing cells.

DISCUSSION

HGF induces transient morphological changes in MDCK epithelial cells that can be reversed after HGF withdrawal (Weidner *et al.*, 1993; Zhu *et al.*, 1994; Khoury *et al.*, 2001). Similarly, we have shown that a constitutively activated ErbB2/Neu receptor (NT) induces an EM-like transition in MDCK cells and promotes the inherent morphogenic program of these cells in a collagen matrix (Khoury *et al.*, 2001). However, the same NeuNT receptor induces metastatic mammary tumors in transgenic mice with long latency (Muller *et al.*, 1988), suggesting that other factors may play a role in the metastatic process.

We show in this article that signals downstream from the HGF/Met receptor convert a morphogenic signal, induced by an activated NeuNT receptor in MDCK cells, to an invasive response. This is specific to HGF and activated ErbB2/Neu. Neither EGF nor heregulin promote cell dispersal in MDCK cells, and HGF does not induce a similar phenotype in MDCK cells expressing the wild-type ErbB2/Neu receptor (Figures 2A and 3B). HGF fails to synergize with ErbB2/Neu for cell invasion in an immortalized mammary epithelial cell line (MCF10A), whereas TGFβ induces cell invasion in the same cell model (Seton-Rogers *et al.*, 2004). This difference probably reflects differences between cell lines. For example, MDA435 breast cancer cells transfected with HGF form tumors with metastasis to the lung in the absence of E-cadherin, but on the reintroduction of E-cadherin HGF promotes the formation of three-dimensional epithelial structures (Meiners *et al.*, 1998).

Loss of differentiation during cancer progression is accompanied by the breakdown of cell-cell junctions, a prerequisite for cell dissociation (Thiery, 2002). Several mechanisms lead to the breakdown of cell-cell junctions. For example, E-cadherin, a major component of adherens junctions, is down-regulated through transcriptional repression, genomic deletion and perhaps protein degradation, in many human cancers and in established cancer cell lines (Birchmeier and Behrens, 1994; Ji *et al.*, 1997). In addition, components of the tight junctions are destabilized during EMT and in some cancer cells (Li and Mrsny, 2000; Liebner *et al.*, 2000; Blanco *et al.*, 2004; Ohkubo and Ozawa, 2004; Tobioka *et al.*, 2004). Expression of claudins and occludin is repressed by the transcription factor Snail (Ikenouchi *et al.*, 2003).

In three-dimensional cultures, NeuNT-expressing MDCK cells retain E-cadherin at adherens junctions and ZO-1 at tight junctions (Figure 7). However, exposure to HGF disrupts the three-dimensional epithelial structures of NeuNT-expressing cells, promoting their conversion into single invading cells (Figure 7). In these cells, E-cadherin and ZO-1 seem internalized, as revealed by the vesicle-like staining pattern (Figure 7). Although the scatter factor, HGF or expression of NeuNT induces the redistribution of E-cadherin
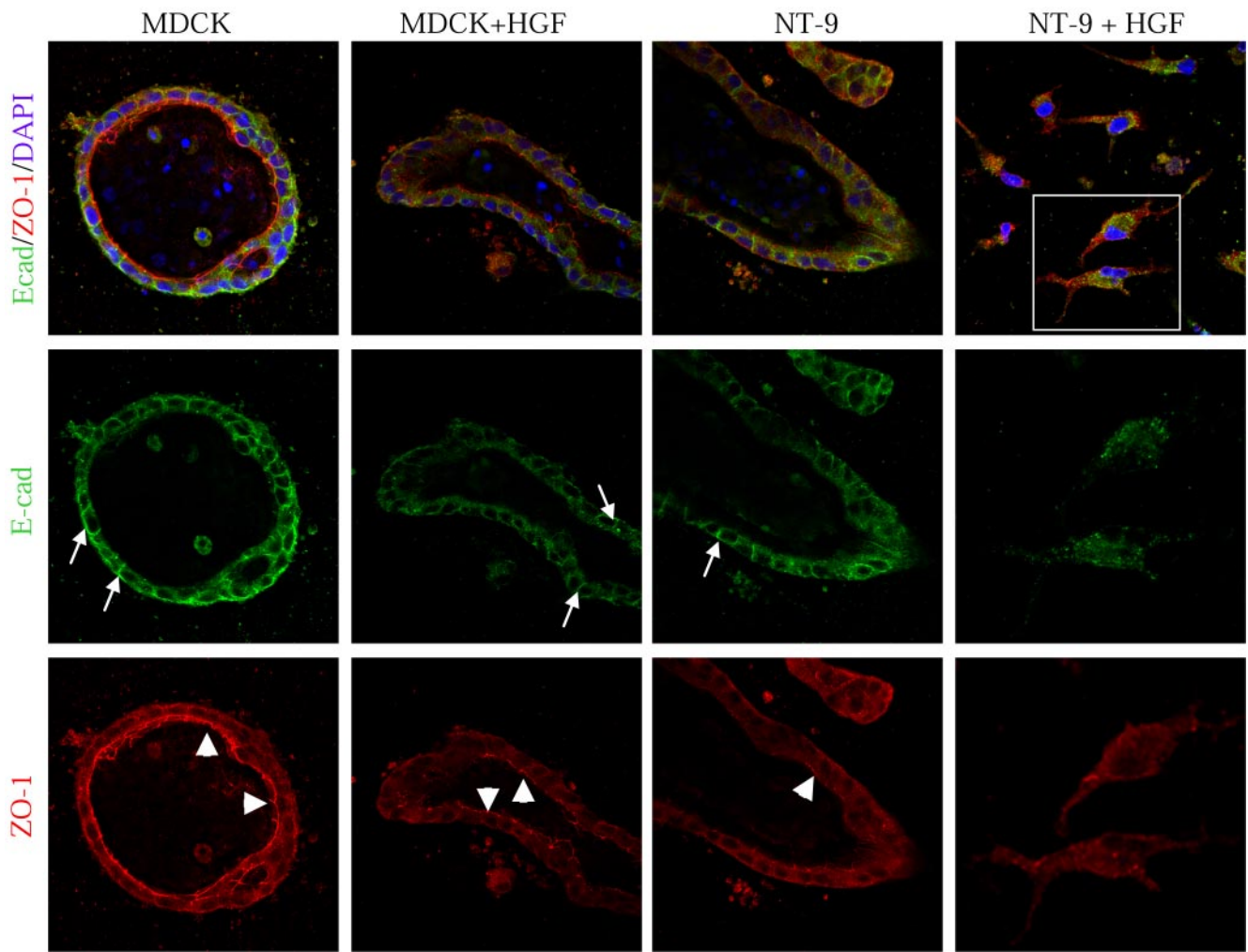
Figure 7. HGF induces E-cadherin internalization in MDCK cells expressing deregulated NeuNT. Collagen cultures of NT-expressing MDCK cells treated or not with HGF (5 U/ml) were labeled with anti E-cadherin (green) and anti ZO-1 (red). White arrows indicate basolateral distribution of E-cadherin. Arrowheads refer to the apical distribution of ZO-1. Confocal LSM510 (magnification 63×).

from the cell membrane into a cytoplasmic-soluble compartment in two-dimensional culture (Royal and Park, 1995; Khoury *et al.*, 2001), E-cadherin and ZO-1 are retained at cell-cell junctions in three-dimensional culture (Figure 7) (Pollack *et al.*, 1998). Protein levels of E-cadherin and claudin-1, a component of tight junctions, are consistently reduced when NeuNT-expressing cells, but not MDCK cells, are maintained in HGF-containing medium (Figure 6). The reduction in E-cadherin, a key component of adherens junctions, and claudin-1, which is important for the recruitment of ZO-1 to tight junctions, would promote the destabilization of both adherens and tight junctions. The reduction in E-cadherin is not due to transcriptional repression as revealed by quantitative real-time PCR (our unpublished results), and is consistent with previous reports where protein internalization and subsequent degradation have been reported as a mechanism for the down-modulation of E-cadherin (Fujita *et al.*, 2002). In this model, Src-mediated tyrosine phosphorylation leads to the association of E-cadherin with a ubiquitin ligase and subsequent endocytosis and degradation of the protein (Fujita *et al.*, 2002). Importantly, activated c-Src-transformed MDCK cells do not show altered levels of E-cadherin (Figure 6), suggesting that

other pathways downstream from HGF and deregulated ErbB2/Neu might be involved in the loss of E-cadherin.

The stable breakdown of cell-cell junctions, observed in NeuNT-expressing cells in response to HGF, is inhibited by a pharmacological inhibitor of MEK, UO126 (Figure 8). Moreover, pretreatment of NeuNT cells with UO126 prevents the internalization of E-cadherin and ZO-1 observed in untreated cells in response to HGF (Figure 8C). HGF synergizes with NeuNT to enhance Erk phosphorylation (Figure 8D), which is blocked by UO126. Consistent with this, sustained Erk activity is required for ErbB2-TGFβ cooperation for the induction of cell invasion in the MCF-10A mammary epithelial cell model (Seton-Rogers *et al.*, 2004), and the inhibition of the MEK pathway restores tight junctions in HGF-stimulated and Ras-transformed MDCK cells (Potempa and Ridley, 1998; Maroun *et al.*, 1999; Chen *et al.*, 2000; Khoury *et al.*, 2001). Furthermore, deregulated activation of the MAPK pathway alone is sufficient to induce permanent loss of epithelial structures in MDCK cells in collagen (Khwaja *et al.*, 1998), although Neu add-back mutants capable of activating Erk (YB, YC, YD, and YE) (Dankort *et al.*, 2001) are not sufficient, suggesting that a sustained threshold of Erk activation may be required. Erk activation is neces-
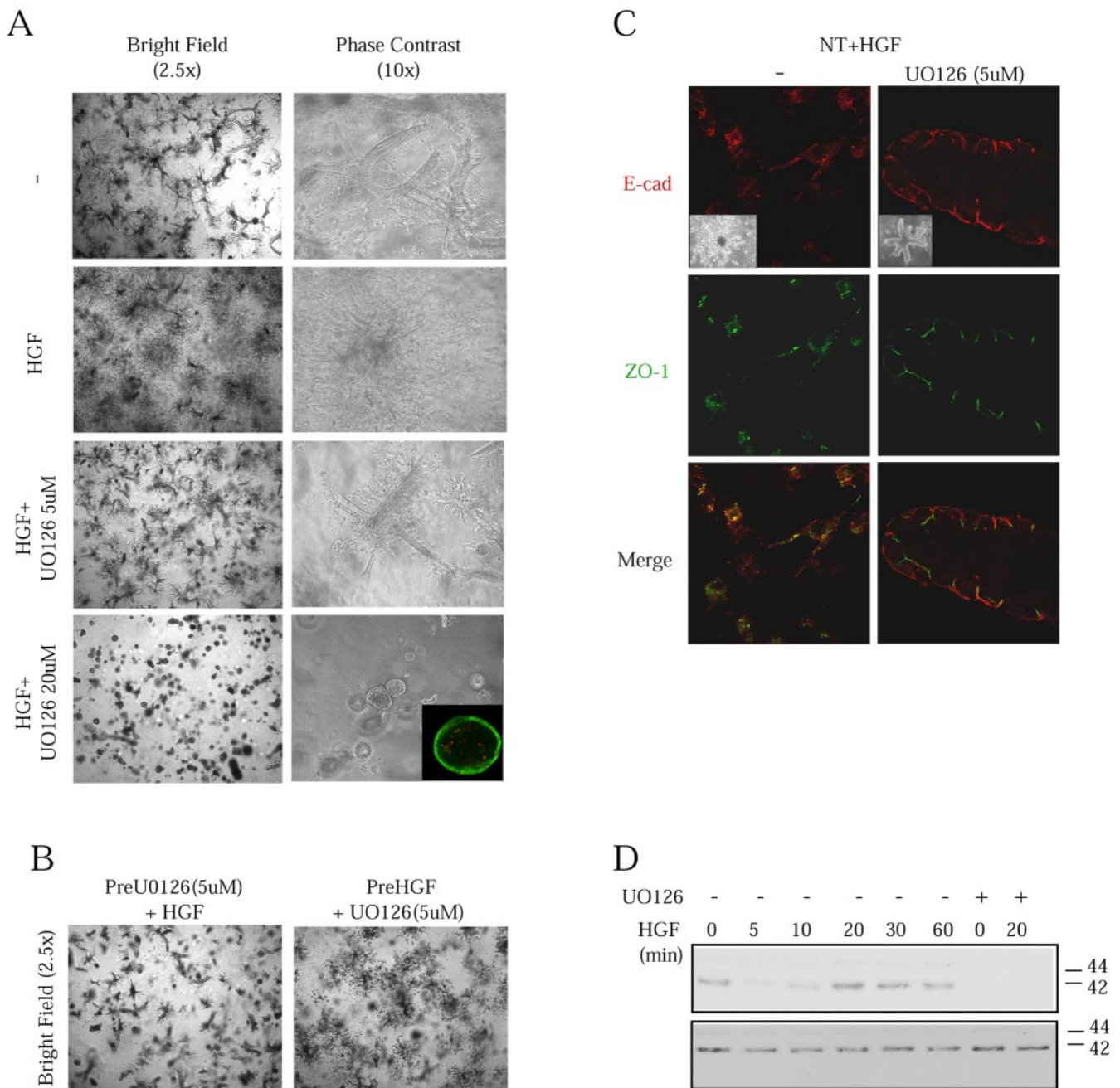
Figure 8. MEK inhibitor UO126 prevents HGF-induced epithelial disruption in MDCK cells expressing deregulated ErbB2/Neu. Stable lines of MDCK cells were grown in collagen I. Five days later, HGF (5 U/ml) and UO126 (5 and 20 μM) were added either simultaneously as in A, or at a 2-d interval where pretreatment was required, as in B. Seven days later, cultures were fixed in 4% paraformaldehyde. Representative pictures were taken using bright field (2.5×) and phase contrast (10×) objectives. Live/dead staining was performed on cultures after treatment with UO126. A representative picture is shown as an insert. (C) Representative collagen cultures from B stained for E-cadherin and ZO-1 show restored tubular structures with E-cadherin and ZO-1 staining at cell-cell junctions. Confocal LSM510 (magnification, 63×). (D) HGF and NeuNT synergize to enhance Erk phosphorylation. MDCK cells expressing deregulated ErbB2/Neu (NT9) were serum starved for 18 h, and then stimulated with HGF (10 U/ml) for the indicated times. Cells were pretreated with the MEK inhibitor UO126 (10 μM) 1 h before HGF stimulation where indicated. Proteins from total cell lysate (50 μg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted for phospho-Erk and total Erk.

sary to trigger the initial stage of HGF-induced tubulogenesis during which cell depolarization and remodeling occur without the loss of cell-cell contacts (Pollack *et al*., 1998; O'Brien *et al*., 2004). Consistent with our model, prolonged Erk activation prevented the formation of tubular structures in MDCK cells expressing activated Raf, an upstream acti-

vator of MEK/Erk; hence, synergistic Erk activation by HGF and deregulated ErbB2/Neu acts to promote the loss of cell-cell contacts, thus preventing cell redifferentiation. Sustained and prolonged Erk activation might be required to induce changes in gene expression resulting in the secretion of matrix degrading metalloproteases that promote cleavage

of junctional proteins, hence destabilizing cell-cell junctions (McCawley *et al.*, 1998; Liang and Chen, 2001; Tanimura *et al.*, 2002).

In conclusion, we provide evidence that synergy between HGF/Met and ErbB2/Neu promotes progression of the malignant phenotype of polarized MDCK epithelial cells through the destabilization of cell-cell junctions. A similar synergy between TGFβ and ErbB2/Neu was demonstrated where TGFβ was shown to promote pulmonary metastasis in transgenic mouse models of ErbB2/Neu-induced mammary tumorigenesis (Siegel *et al.*, 2003), as well as migration and invasion in mammary cell models in culture (Seton-Rogers *et al.*, 2004; Ueda *et al.*, 2004). Moreover, synergy between TGFβ and Ras or EGF has been reported to induce EMT in primary cultured cell models (Grande *et al.*, 2002; Janda *et al.*, 2002). HGF-induced cell dispersal correlates with metastatic behavior of cancer cells and highlights the importance of the cross-talk between normal and deregulated signals in the progression of invasive malignancies. This observation is particularly significant for breast cancer where overexpression of HER2/Neu and HGF/Met are independent markers for poor prognosis.

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