Synergy between HGF and ErbB2/neu promotes epithelial cell invasion

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Abstract

The development and progression of invasive carcinomas correlates with the loss of epithelial architecture and the acquisition of a motile phenotype. This process is known as epithelial-mesenchymal transition (EMT). EMT is also a common event in embryogenesis where a transient gain of cell motility is required for tissue remodeling. The ErbB-2/neu receptor tyrosine kinase is involved in normal tissue development. However, this receptor has been implicated in the genesis of human breast and renal carcinomas, where ErbB2 is amplified in 20-30 % of human breast cancers and correlates with poor prognosis. In addition, overexpression of ErbB2/Neu induces breast tumorigenesis in transgenic mice. More recently, the Met receptor tyrosine kinase and its ligand, Hepatocyte Growth Factor/Scatter Factor have been identified as independent markers that predict poor prognosis in breast cancer patients. Met is expressed in normal breast epithelia whereas HGF is expressed in tumor and normal stroma. Although both Met and ErbB2 are thought to be involved in normal tissue differentiation, the mechanisms through which these receptors contribute to loss of tissue architecture in tumors are poorly understood.

Using the non-transformed MDCK epithelial cell model, I have established that a deregulated activated ErbB2/Neu receptor (NeuNT) but not overexpression of the wild type (WT) receptor induces cell dispersal and motility, accompanied by the breakdown of cell-cell junctions and E-cadherin internalization, in addition to reorganization of the actin cytoskeleton. This phenotype can be reversed following treatment of the cells with a pharmacological inhibitor of MEK, indicating that MEK dependent pathways are involved in the NeuNT-induced remodeling of cell-cell junctions. In three-dimensional cultures of MDCK cells, NeuNT but not WT ErbB2 triggers a morphogenic response that correlates with recruitment and increased phosphorylation levels of the Shc adapter protein. This demonstrates that the deregulated ErbB2/NT receptor induces a distinct biological response when compared to the wild type receptor and induces the loss of epithelial architecture observed in carcinomas.

Invasive morphogenesis downstream from the Met/HGF receptor is modulated through a sustained phosphorylation of the Gab1 docking protein and of downstream

kinase (Erk). In contrast, a transient phosphorylation of Gab1 and Erk induced by EGF is not sufficient to promote a mophogenic response. In Chapter III, I demonstrate that NeuNT but not the WT ErbB2 receptor display elevated and sustained levels of Gab1 and Erk phosphorylation which correlates with their ability to promote invasive morphogenesis. In addition, co-immunoprecipitation analyses provide evidence for the recruitment of Gab1 to ErbB2/Neu in a Grb2-dependent and Grb2-independent manner.

To identify physiologically relevant factors that synergize with ErbB2, I established that HGF, the Met receptor ligand, promotes the disruption and invasion of NeuNT-induced epithelial structures in three dimensional matrix cultures. Moreover HGF synergizes with NeuNT, enhancing the invasive potential of NeuNT expressing cells ten fold through Matrigel. HGF treatment of NeuNT expressing cells promotes a decrease in E-cadherin protein, and can be blocked or reversed by treatment with the MEK inhibitor, UO126, establishing the involvement of MEK-dependent pathways in this process. These results demonstrate that physiological signals downstream from HGF/Met cooperate with deregulated ErbB2/Neu to enhance the malignant phenotype promoting a more stable epithelial-mesenchymal transition and enhanced cell invasion.

Sommaire

Les tissus épithéliaux sont caractérisés par une architecture tridimensionnelle bien organisée qui est perturbée au cours du développement et de la progression des carcinomes agressifs. Cette perte d'organisation correspond à une transition nommée transition épithélio-mesenchymateuse, où les cellules épithéliales acquièrent un phénotype qui ressemble aux fibroblastes, leur permettant de se déplacer à travers la matrice extracellulaire. HER2/ErbB2/neu est un récepteur à activité tyrosine kinase qui régule la différenciation des tissus épithéliaux dont les glandes mammaires. Cependant, ErbB2/neu est impliqué dans la genèse du cancer du sein et du rein. En effet, 20-30% des tumeurs mammaires surexpriment ErbB2/neu et présentent un mauvais pronostic. De même, Met, le récepteur du facteur de croissance hépatocytaire HGF/SF, et HGF lui-même sont-ils des marqueurs pronostiques indépendants pour le cancer du sein. Met est exprimé dans l'épithélium mammaire tandis que HGF est sécrété par les cellules mésenchymateuses. Le mécanisme par lequel Met et ErbB2/neu contribuent à la perte de l'architecture épithéliale reliée au cancer demeure inconnu.

En me servant du modèle de culture de cellules épithéliales normales MDCK, j'ai démontré que l'expression d'un récepteur ErbB2/neu hyperactif (nommé NeuNT) induit la motilité et la dispersion cellulaire, accompagnée par la dissolution des jonctions cellulaires et l'internalisation de E-cadherin, ceci contrairement au récepteur normal. Un inhibiteur pharmacologique de MEK renverse ce phénotype, montrant que les voies de signalisation activées par MEK sont impliquées en aval du récepteur hyperactif NeuNT. Quand les cellules MDCK sont cultivées dans du collagène (culture en trois dimensions), NeuNT, contrairement au récepteur normal, stimule une réponse morphogénique qui corrèle avec une élévation des niveaux de phosphorylation de la protéine d'ancrage Shc. Ces résultats montrent que le récepteur hyperactif NeuNT induit une réponse biologique distincte du récepteur normal, impliquant la perte de l'architecture épithéliale similaire à celle qu'on observe dans les carcinomes.

La réponse morphogénique induite par le récepteur Met est modulée par une phosphorylation maintenue de la protéine d'arrimage Gab1 et de la kinase Erk en aval de Gab1. Par contre, EGF entraîne une phosphorylation transitoire de Gab1 et Erk, qui ne

suffit pas pour promouvoir une réponse morphogénique. Dans le 3^{e} chapitre, je démontre que, contrairement au récepteur normal, l'expression de NeuNT entraîne une élévation des niveaux de phosphorylation de Gab1 et Erk, ce qui corrèle avec sa capacité d'induire la morphogenèse. De plus, Gab1 est recruté au récepteur ErbB2/neu de façon dépendante et indépendante de Grb2, tel que révélé par co-immunoprécipitation.

Afin d'identifier des facteurs qui pourraient agir en synergie avec ErbB2/neu, je montre que HGF induit la destruction des structures épithéliales induites par NeuNT en cellules solitaires qui envahissent la matrice de collagène. HGF promeut également l'invasion de ces cellules à travers une matrice de Matrigel. Dans ces cellules qui expriment NeuNT, HGF entraîne une réduction des niveaux protéiques de E-cadherin, ce qui peut être bloqué ou renversé par un inhibiteur de MEK, UO126, impliquant des signaux dépendant de MEK dans ce processus. Ces résultats démontrent que des signaux physiologiques en aval de Met et HGF coopèrent avec un récepteur ErbB2/neu hyperactif pour promouvoir une transition épithélio-mesenchymateuse plus stable, entraînant l'invasion cellulaire et par conséquent un phénotype malin plus prononcé.

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Au moment du dépôt de cette thèse, j'ai appris la nouvelle du décès de ma cousine Samar Yassa, à l'âge de 22 ans, à quelques mois de l'obtention de son diplôme en génie informatique. Samar avait un avenir prometteur, je lui dédie donc cette thèse en espérant que la recherche scientifique aboutira bientôt à améliorer la santé des jeunes et des moins jeunes.

Preface

The guidelines concerning thesis preparation issued by the Faculty of Graduate and Postdoctoral Studies at McGill University reads as follows:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following:

1. a table of contents;
2. a brief abstract in both English and French;
3. an introduction which clearly states the rational and objectives of the research;
4. a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
5. a final conclusion and summary;
6. a thorough bibliography;
7. Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

I have chosen to write my thesis according to these guidelines, with one published article and two manuscripts in preparation. The thesis is organized in six chapters: (I) a general introduction and literature review, (II-IV) manuscripts, each with their own abstract, introduction, materials and methods, results, discussion and references, (V) a general discussion of all results with references, and (VI) claims to original research.

Publications arising from work of the thesis

1- **Khoury, H**, Dankort, D.L., Sadekova, S., Naujokas, M.A., Muller, W.J. and M. Park. **2001**. *Distinct tyrosine autophosphorylation sites mediate induction of epithelial-mesenchymal like transition by an activated ErbB-2/neu receptor*. Oncogene **20**: 788-799.

2- **Khoury H.**, Lock, L.S., Naujokas, M.A., Dankort, D.L., Maroun, C.R., Muller W.J. and M. Park. *Invasive morphogenesis downstream from a constitutively activated ErbB-2/Neu correlates with increased phosphorylation of the Gab1 docking protein and Grb2-dependent recruitment of Gab1* (manuscript in preparation).

3- **Khoury, H.**, Naujokas, M.A., Zuo, D., Petkiewicz, S., Sangwan, V., Muller, W.J. and M. Park. *HGF/Met and deregulated ErbB2/Neu cooperate to promote breakdown of tubular epithelia and cell invasion.* (manuscript in preparation).

The specific contributions of various authors to these manuscripts are as follows:

- D.L. Dankort generated the ErbB2/Neu mutants in W.J. Muller's lab.
- M.A. Naujokas performed morphogenesis assays in all three manuscripts.

- In manuscript one (Chapter II), S. Sadekova performed immunostaining assays and took confocal photographs (Figures 3 and 6).

- In manuscript two (Chapter III), L.S. Lock generated the Gab1 constructs; C.R.Maroun initiated kinetics of Gab1 phosphorylation downstream from HGF and EGF.

- In manuscript three (Chapter IV), D. Zuo performed immunofluorescence assays and took confocal pictures for figures 2 and 6. S. Petkiewicz demonstrated by real-time PCR that E-cadherin was not downmodulated at the transcriptional level (data not shown in Chapter IV). V. Sangwan performed the anoïkis assay (Figure 3).

Other publications:

- Kamikura, D.M., **Khoury, H.**, Maroun, C., Naujokas, M.A. and M. Park. **2000**. *Enhanced transformation by a plasma membrane-associated Met oncoprotein: activation of a phosphoinositide 3'-kinase-dependent autocrine loop involving hyaluronic acid and CD44.* Mol Cell Biol, **20,** 3482-96.

- Saucier, C., **Khoury, H.**, Venus Lai, K-M., Peschard, P., Dankort, D.L., Naujokas, M.A., Holash, J., Yancopoulos, G.D., Muller, W.J., Pawson, T. and M. Park. *The Shc adapter protein plays a critical role in VEGF induction by the Met and ErbB2 receptor tyrosine kinases: A critical function of Shc for early onset of tumor angiogenesis*. Proc Natl Acad Sci, *in press.*

Contributions to original research

- Demonstrated that deregulated ErbB2/neu promotes the inherent morphogenic program of non-transformed epithelial cells in a three-dimensional collagen matrix, indicating that signals downstream from a deregulated RTK are qualitatively different from those activated upon transient ligand-induced activation
- Identified specific signaling pathways involved in deregulated ErbB2/neu-induced redistribution of cell-cell junctions and cell dispersal, implicating a role for MEK/Erk and PI3K
- Demonstrated that deregulated ErbB2/neu promotes tyrosine phosphorylation of Gab1
- Established that Gab1 co-immunoprecipitates with ErbB2 both in a Grb2-dependent and Grb2-independent manner, suggesting possible direct recruitment of Gab1 to ErbB2
- Demonstrated that HGF and Met synergize with deregulated ErbB2/neu to disrupt epithelial cell integrity and promote cell invasion
- Identified the MEK/Erk pathway as being involved in the synergy between HGF and deregulated ErbB2/neu
- Showed that HGF and Met synergize with oncogenic ErbB2/neu to reduce E-cadherin levels
- Showed that deregulated ErbB2/neu promotes subnuclear localization of the ZEB1 transcription factor

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Chapter II Distinct tyrosine autophosphorylation sites mediate induction of epithelial-mesenchymal like transition by an activated ErbB-2/neu receptor

List of Abbreviations

ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
FA	Focal adhesion
Gab1	Grb2-associated binder
HGF/SF	Hepatocyte growth factor/Scatter factor
HRG	Heregulin
MBD	Met binding domain
MDCK	Madin-Darby canine kidney
NDF	Neu differentiation factor
RTK	Receptor tyrosine kinase

1. INTRODUCTION

Epithelial tissue architecture is modulated by different factors from the microenvironment, including interactions with extracellular matrix components through integrins, as well as growth factors released from the stroma. Deregulation of these interactions perturbs tissue homeostasis and contributes to the loss of epithelial differentiation observed in cancer. Numerous studies have focused on the consequence of a single signal alteration on epithelial cell integrity. Recently, the contribution of the cell microenvironment to the progression of the malignant phenotype has been addressed. For example, within the tumor microenvironment the cell response to unaltered signals can differ, contributing further to the malignant phenotype, as has been reported for TGFβ (Janda et al., 2002a). The development of three-dimensional cell culture models that mimic the complexity of epithelial architecture and the use of animal models have identified important modifiers of epithelial integrity although for many their mechanisms of action remain poorly understood.

2. EPITHELIAL ORGANIZATION

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). Apical-basal polarity characterizes epithelial cells, with the apical side facing the lumen (Figure 1). Epithelial cells receive signals from the environment and translate them into biological outcomes such as cell proliferation, survival, differentiation or migration (Balda and

Matter, 2003; Drubin and Nelson, 1996). Cell-cell junctions include tight and adherens junctions as well as desmosomes. They maintain epithelial integrity through stable yet dynamic interactions between their protein components (Gumbiner, 1996); tissue remodeling and morphogenetic events during development depend on the breakdown and reconstitution of adherens junctions (Gumbiner, 1996; Schock and Perrimon, 2002). Components of cell-cell junctions are regulated by phosphorylation/dephosphorylation and constant recycling (Le et al., 1999). Decreased expression of genes encoding for junctional proteins results in the disruption of epithelial organization and contributes to cancer progression (Hajra et al., 2002; Ikenouchi et al., 2003).

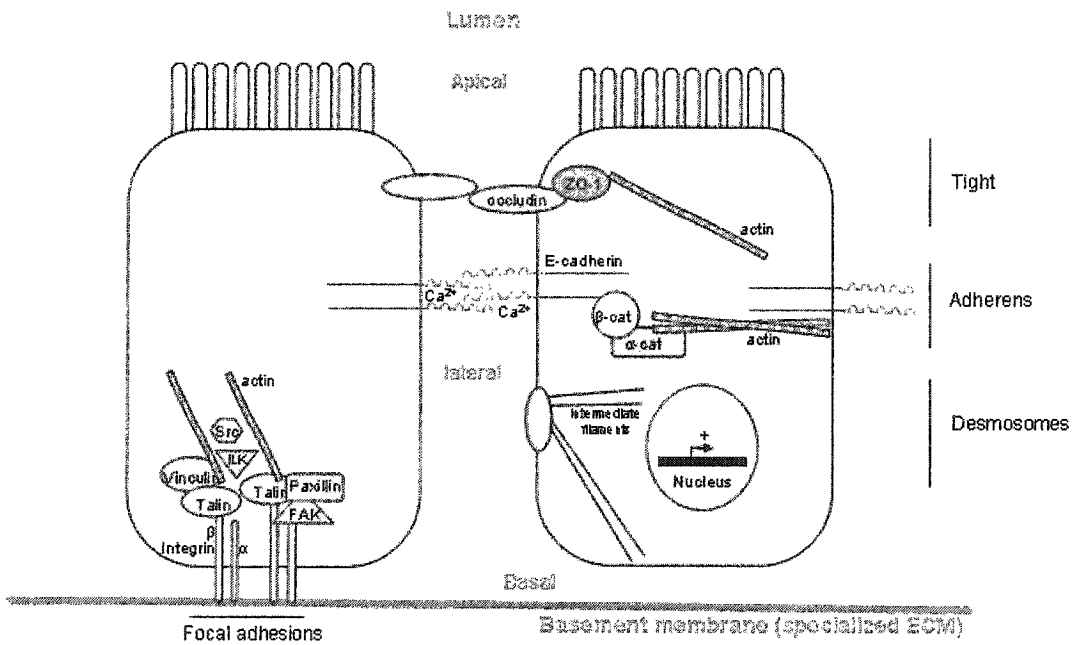

Figure 1: Epithelial cell architecture. Epithelia are composed of a single sheet of polarized cells connected to each other by cell-cell junctions (tight, adherens and desmosomes) and attached to the extracellular matrix via focal adhesion complexes.

2.1 Cell-cell junctions

2.1.1 Tight junctions

Located in the apical region of cell-cell contacts (Figure 1), tight junctions constitute a multiprotein complex that acts as an intramembrane diffusion barrier between apical and basal sides, preventing the free movement of molecules (Balda and Matter, 2003). Major protein components of tight junctions include claudins, occludin, ZO-1 and ZO-2. Tight junctions contribute to the maintenance of epithelial cell polarity. This role is further supported by the observation that Snail, a repressor of the epithelial phenotype, represses the expression of occludin and claudin, two components of tight junctions (Ikenouchi et al., 2003). In addition, ZO-1, a PDZ domain-containing protein, might be involved in the modulation of cell proliferation by preventing the nuclear accumulation of ZONAB, a Y-box transcription factor that interacts with the G1/S-phase regulator CDK4 (Balda and Matter, 2003). ZONAB represses the expression of the ErbB2 receptor tyrosine kinase (Balda and Matter, 2000). These results indicate that in addition to their role in cell adhesion, adhesion complexes contribute to the regulation of gene expression by modulating the transmission of signals from the plasma membrane to the nucleus (Balda and Matter, 2003).

2.1.2 Adherens junctions

Adherens junctions are the main adhesive junctions in epithelial cells. They consist of stable junctional complexes that form upon Ca^{++}-dependent homotypic interactions between cadherins of neighboring cells. E-cadherin recruits β and α-catenin

which link E-cadherin to the actin cytoskeleton (Figure 1) (Balda and Matter, 2003). This results in the formation of a continuous adhesion belt, the zonula adherens.

Alterations in the cadherin-catenin complexes result in the disruption of epithelial integrity which correlates with cancer progression (Hajra et al., 2002). Hence a role for E-cadherin as a tumor suppressor gene has been proposed. Consistently, introducing E-cadherin into E-cadherin negative breast cancer cells switches the cell response to HGF (hepatocyte growth factor) from a metastasis-inducing to a remodeling response, allowing them to form organized structures (Birchmeier et al., 1997). E-cadherin is downmodulated in many human cancers via different mechanisms that will be discussed in section 2.3. E-cadherin sequesters β-catenin to cell junctions, thus preventing the accumulation of free β-catenin and its subsequent translocation to the nucleus through its association with the LEF/TCF family of transcription factors and induction of gene expression (Balda and Matter, 2003).

The c-Src tyrosine kinase and the EGFR are enriched at adherens junctions, these are thought to be involved in signal transduction where they act as cellular signaling centers (Gumbiner, 1996; Kirkpatrick and Peifer, 1995).

2.1.3 Desmosomes

Desmosomes are associated with intermediate filaments, providing a structural framework for the cytoplasm (Figure 1). Desmosomal cadherins include desmocollin and desmoglein which interact with plakoglobin (γ-catenin) and plakophilin, these form a bridge to intermediate filaments through desmoplakin (Kirkpatrick and Peifer, 1995).

Desmosome disruption occurs in several autoimmune blistering diseases (Vasioukhin and Fuchs, 2001).

2.2 The Extracellular Matrix

2.2.1 Function and composition

The extracellular matrix (ECM) plays a key role in tissue architecture and homeostasis. Composed of fibers of proteins, the ECM helps maintain tissue integrity and polarity, regulates cell migration, in addition to providing a reservoir of cytokines and growth factors (Stamenkovic, 2003). The ECM comprises two specialized domains, the basement membrane adjacent to epithelial cells and the interstitial matrix (Bosman and Stamenkovic, 2003). Major components of the ECM are collagens, considered as the structural elements; laminins and tenascin are adhesive glycoproteins involved in cell adhesion, migration and differentiation through integrin-mediated interactions. Proteoglycans are involved in cell adhesion and epithelial cell differentiation; heparin sulphate proteoglycans bind different cytokines and growth factors (Bosman and Stamenkovic, 2003). Various biological responses such as cell migration and tissue remodeling require alterations of the composition and structure of the ECM. ECM remodeling is regulated by metalloproteases and other proteases (Stamenkovic, 2003). TGFβ and HGF regulate protease expression (McCawley *et al.*, 1998; Verrecchia and Mauviel, 2002).

2.2.2 Cell-ECM interactions

The highly organized architecture of normal epithelia suggests that dynamic and reciprocal interactions take place between epithelial cells and the surrounding ECM

(Bissell et al., 2002; Somasiri and Roskelley, 1999). Tight regulation of these interactions dictates cell behavior and contributes to tissue homeostasis. Consequently, deregulation of such interactions disturbs tissue homeostasis leading to a disorganized phenotype. Architectural disruption of the cell microenvironment is a common epigenetic alteration in animal models of breast cancer (Roskelley and Bissell, 2002). The use of organotypic three-dimensional cell culture assays allowed the identification of important factors that modulate tissue organization and specificity (Schmeichel and Bissell, 2003). It has been shown that the ECM component laminin regulates the functional differentiation of mouse mammary epithelial cells by the induction of beta-casein expression in the presence of lactogenic hormones (Streuli et al., 1995). In addition, laminin rich basement membrane (lrBM) promotes structural differentiation of nontumorigenic epithelial cells into polarized acini (Petersen et al., 1992), however tumorigenic cells fail to organize under the same conditions. Further investigation using co-cultures with myoepithelial cells as a source of laminin established that tumor-derived myoepithelial cells do not produce laminin (Gudjonsson et al., 2002). These studies identified laminin as a modulator of cell polarization.

Cell-ECM interactions are mediated in part by integrins which activate survival signals and prevent apoptosis (Stupack and Cheresh, 2002). Receptor tyrosine kinases (RTK) are one class of proteins that modulate the cell response to growth factors secreted in the ECM (Birchmeier and Birchmeier, 1993). The role of the Met and ErbB2/neu RTKs in epithelial organization is discussed in sections 4-6. Additionally, members of the TGFβ family of cytokines regulate tissue remodeling through interactions with their transmembrane serine/threonine receptors.

2.2.2.1 Integrins

Integrins are transmembrane receptors for ECM components (Sastry and Horwitz, 1993). They transmit signals from the extracellular matrix to the cell interior and mediate cell migration and anchorage to the ECM. The human integrin family includes 24 different functional receptors, arising from the combination of 18 α and eight β subunits (Danen and Sonnenberg, 2003). $\alpha_2\beta_1$ is the predominant receptor for collagen I.

Upon ligand binding, integrins cluster into focal adhesions (FA) and hemidesmosomes, connecting the ECM to the actin cytoskeleton (Figure 1). Focal adhesions are multiprotein complexes including kinases (Integrin-linked kinase ILK, focal adhesion kinase FAK, Src) and cytoskeletal linkers (vinculin, talin, α-actinin, paxillin) (Burridge and Fath, 1989; Eide *et al.*, 1995). FAK is a phosphotyrosine protein that recruits, in addition to Src, signaling proteins such as PI3K, p130Cas, Crk, Sos, Csk and Grb2 (Cary and Guan, 1999). FAK and Src activities are required for the turnover of FA, a process required during cell migration. Paxillin is also critical for FA turnover; paxillin-null fibloblasts and mouse embryonic stem cells display abnormal focal adhesions and reduced migration consistent with reduced tyrosine phosphorylation of FAK (Hagel et al., 2002; Wade et al., 2002).

Integrin signaling is important for cell survival, spreading and migration (Almeida et al., 2000; Brakebusch and Fassler, 2003; Stupack and Cheresh, 2002). Moreover, integrin function is critical for epithelial cell polarity and differentiation (Gudjonsson et al., 2002; Ojakian et al., 2001). Perturbation of β_1 integrin impairs the development of the murine mammary gland (Faraldo et al., 1998). Mutation-induced deregulation of β_1 integrin found in squamous cell carcinoma of the tongue fails to promote cell

differentiation (Evans *et al.*, 2003). In addition, α_1 and α_2 integrins confer invasive behavior to mouse mammary carcinoma cells by upregulating stromelysin-1 and promoting cell motility (Lochter et al., 1999), suggesting a role for integrins in tumor cell invasion. Accordingly, overexpression of the integrin-linked kinase ILK induces a mesenchymal phenotype in epithelial cells accompanied by E-cadherin repression (Guaita et al., 2002; Somasiri et al., 2001; Wu et al., 1998), enhanced cell invasion (Troussard et al., 2000) and suppression of anoikis (Attwell et al., 2000).

2.3 Tissue remodeling

2.3.1 Epithelial mesenchymal transition

Epithelial tissue remodeling during embryonic and adult life requires coordinated cell movement and changes in cell morphology. Examples include neural crest cell migration and tubular morphogenesis of kidney and breast epithelia. These processes involve a transient phenotypic modulation, known as Epithelial-Mesenchymal Transition (EMT) where polarized epithelial cells acquire a motile mesenchymal phenotype involving the breakdown of cell-cell junctions and cytoskeletal changes associated with cell migration (Boyer *et al.*, 1996; Meiners *et al.*, 1998; Savagner, 2001; Thiery, 2002).

In addition to its role in tissue remodeling and morphogenesis, EMT induction correlates with the progression of invasion carcinomas (Meiners et al., 1998; Thiery, 2002), where it is characterized by the loss of epithelial differentiation (Figure 2). In this case, the dedifferentiation process involves the repression of epithelial markers, namely E-cadherin, and the upregulation of mesenchymal markers such as the transcription factor Snail (Thiery, 2002). Therefore, a more potent EMT accompanies cancer progression, as

opposed to tissue remodeling which involves the redistribution rather than repression of E-cadherin.

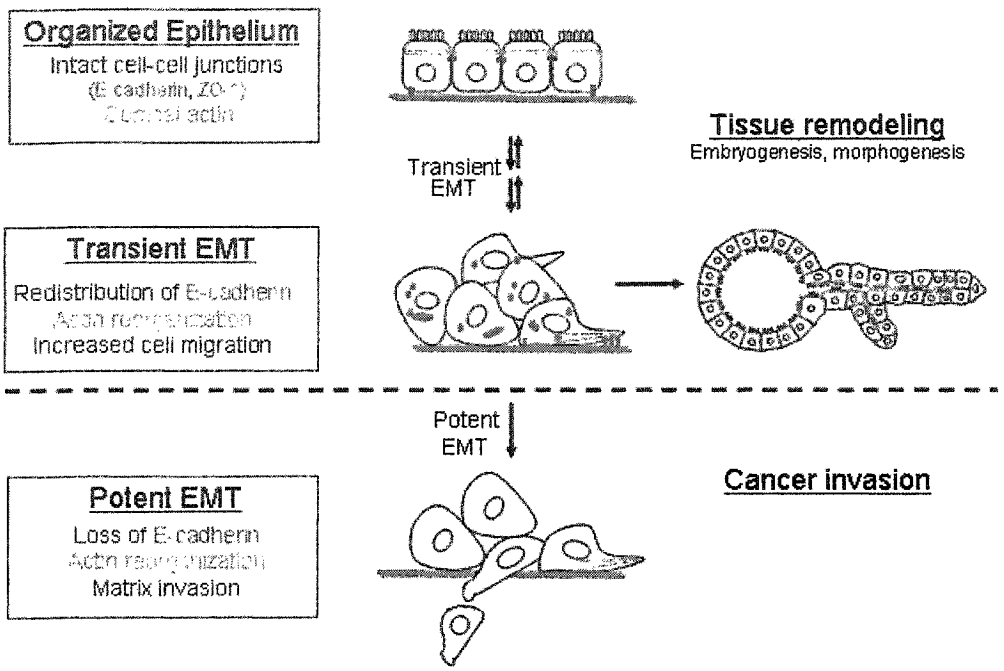

Figure 2: Epithelial mesenchymal transition (EMT). EMT is a common process in embryonic and adult life. During this process, polarized cells acquire a motile phenotype accompanied by a transient loss of polarity and redistribution of cell-cell junctions.
Bottom panel: A potent induction of EMT is associated with tumor dissemination and metastasis. This process involves transcriptional repression of epithelial markers such as E-cadherin.

E-cadherin is downregulated in many human cancers and in established cancer cell lines (Ji et al., 1997). Transcriptional repressors of E-cadherin include the transcription factor Snail that is upregulated in tumors and carcinoma cell lines (Batlle *et al.*, 2000; Cano *et al.*, 2000). Other repressors include Slug (Hajra et al., 2002), Snail-induced δEF1/ZEB1 (Sekido et al., 1994) and SIP1/ZEB2 (Comijn et al., 2001). It has been shown that overexpression of ILK promotes the loss of E-cadherin mediated by Snail and ZEB upregulation (Guaita et al., 2002).

Post-translational regulation of E-cadherin occurs as well, involving protein internalization and protease-mediated cleavage (Fujita et al., 2002). Recently, the apoptosis-regulating gene Bcl-2 was reported to decrease ZO-1 and cadherin-mediated cell adhesion (Li et al., 2003a). In addition to E-cadherin, occludin and claudins are downregulated by oncogenic Raf-1 and Snail (Ikenouchi et al., 2003) (Li and Mrsny, 2000).

Growth factors, such as HGF and TGFβ, and extracellular matrix components (collagen) promote EMT in tissue culture models (Khoury et al., 2001; Weidner et al., 1993a; Zhu et al., 1994). HGF, the Hepatocyte Growth Factor, also known as Scatter factor (SF), is a potent inducer of EMT. HGF induces transient morphological changes in normal Madin-Darby canine kidney (MDCK) epithelial cells that can be completely reversed following HGF withdrawal. These changes include the breakdown of cell-cell junctions and reorganization of the actin cytoskeleton, leading to MDCK cell dispersal and morphogenesis in three-dimensional cultures (refer to Figure 3, section 2.3.3). HGF-induced EMT in MDCK cells will be used here as a model to describe biological processes involved in tissue remodeling.

2.3.2 TGFβ in tissue remodeling

The TGFβ superfamily of secreted polypeptide factors comprises multiple functionally related factors including TGFβ1-3, bone morphogenetic proteins and activins (Attisano and Wrana, 2002). TGFβ plays an essential role in the development of many tissues where it is believed to regulate cell fate by affecting differentiation, proliferation

and cell death (Massague, 1998). TGFβ is also implicated in human cancer where it acts to induce metastasis (Massague et al., 2000; Siegel et al., 2003).

The TGFβ receptor consists of two distinct transmembrane proteins, type I and type II receptors that dimerize upon ligand binding and phosphorylate the Smad transcription factors. The Smad family consists of eight members; Smad 2 and 3 are phosphorylated downstream of TGFβ. Phosphorylated Smads form heterodimers with Smad4 and translocate to the nucleus where they interact with numerous nuclear factors to regulate gene transcription (Attisano and Wrana, 2000). Hence, Smads act as transcriptional co-modulators. A Smad-interacting protein SIP1, also known as ZEB2, represses E-cadherin expression (Comijn et al., 2001). SIP1 antagonizes with TGFβ-mediated transcriptional regulation by recruiting the CtBP co-repressor to Smads (Postigo, 2003; Postigo et al., 2003). However, the SIP1-related protein ZEB1, a transcriptional repressor of E-cadherin, synergizes with TGFβ to mediate transcription, osteoblast differentiation and growth arrest (Postigo, 2003). This is mediated by ZEB1 recruitment of coactivators p300 and P/CAF to Smads (Postigo et al., 2003).

Transcriptional profiling of human epithelial cells allowed the identification of the inhibitors of differentiation Id1, Id2 and Id3 as target genes repressed downstream from TGFβ (Kang et al., 2003b). Moreover, TGFβ induces the expression of ECM proteins in mesenchymal cells and stimulates the production of protease inhibitors that prevent enzymatic breakdown of the ECM (Verrecchia and Mauviel, 2002). However, in renal tubular epithelial cells, TGFβ induces ILK-mediated MMP-2 expression and promotes EMT (Li et al., 2003b). Modulation of the ECM composition may contribute to the ability of TGFβ to regulate tissue remodeling. TGFβ regulates lobuloalveolar

differentiation of the breast epithelium during pregnancy and induces apoptosis during involution (Pollard, 2001). Overall, TGFβ-mediated biological responses depend on the cell context, where it either promotes proliferation or induces growth arrest (Massague et al., 2000; Pollard, 2001). Examples for differential TGFβ responses are presented in section 7.

2.3.3 HGF-induced EMT: the MDCK cell model

HGF, the Hepatocyte Growth Factor, is a plasminogen-related growth factor. It was originally identified as a fibroblast-derived scatter factor that promotes endothelial and epithelial cell dispersal and motility (Gherardi et al., 1989; Naldini et al., 1991b; Stoker et al., 1987). HGF genetic ablation results in embryonic lethality due to placental defects and impaired migration of muscle precursor cells, supporting a role for HGF in epithelial tissue remodeling and cell migration (Bladt et al., 1995; Ebens et al., 1996; Maina et al., 1997; Schmidt et al., 1995; Uehara et al., 1995).

HGF is involved in liver regeneration (Zarnegar and Michalopoulos, 1989) and initiates the intrinsic morphogenic program of kidney, breast and lung epithelia in matrix culture (Brinkmann et al., 1995; Weidner et al., 1993b). In addition to its morphogenic potential, HGF acts as a potent mitogen and motogen for epithelial cells in culture (Montesano et al., 1991; Weidner et al., 1993c; Zhu et al., 1994).

Much information on the molecular mechanisms by which HGF induces epithelial cell dispersal and morphogenesis were obtained using the MDCK epithelial cell model (Figure 3). MDCK epithelial cells are derived from the renal collecting ducts, they are non-transformed cells that grow as polarized epithelial sheets with mature cell-cell

junctions (Rodriguez-Boulan and Nelson, 1989). When embedded in a collagen matrix, cells initially form a small mass. Interaction with the extracellular matrix promotes the formation of polarized spherical cysts, with a hollow lumen formed by cell apoptosis. These structures remodel to form branching tubules in response to HGF. This represents the inherent morphogenic program of renal collecting ductal epithelia (Brinkmann et al., 1995; Weidner et al., 1993c; Zhu et al., 1994). The MDCK cell model has been used in many studies to identify the mechanisms controlling epithelial cell movement and morphogenesis (Potempa and Ridley, 1998).

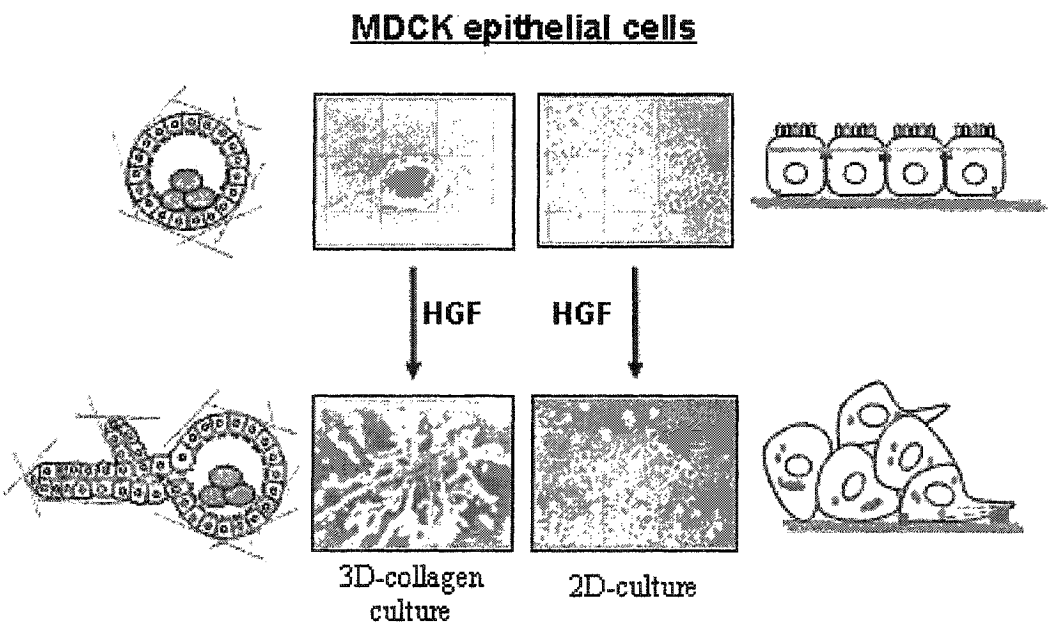

Figure 3: Growth factor-induced transient EMT as a model for tissue remodeling.

2.3.4 Biological processes involved in tissue remodeling

2.3.4.1 Cell dispersal and migration

Cell migration is an essential process for embryogenesis, angiogenesis and wound healing. It is also involved in tumor invasion and metastasis which involve cell dispersal and detachment from the extracellular matrix. In MDCK cells, HGF-induced cell dispersal is a multi-stage process that initially involves changes to the actin cytoskeleton including membrane ruffling and loss of cortical actin, in addition to the formation of focal adhesions, resulting in cell spreading (Potempa and Ridley, 1998; Royal et al., 1997; Royal et al., 2000; Royal and Park, 1995). These events depend on MEK and PI3K activities as well as RhoA GTPases Rac1 and Cdc42, as revealed by the use of specific pharmacological inhibitors and dominant negative reagents (Potempa and Ridley, 1998; Ridley et al., 1995; Royal et al., 1997; Royal et al., 2000; Royal and Park, 1995). HGF-mediated events include redistribution of E-cadherin and desmoplakin from cell-cell contacts into a soluble cytosolic compartment, followed by the breakdown of tight junctions (Royal and Park, 1995). Breakdown of cell-cell adhesions requires the activation of multiple pathways including Ras, PI3K, MEK and Rac1 (Potempa and Ridley, 1998; Ridley et al., 1995; Royal et al., 1997; Royal et al., 2000; Royal and Park, 1995). The Ras-related GTPase ARF6 regulates this process by inducing E-cadherin internalization via endocytic vesicles (Palacios et al., 2001). Loss of cell-cell junctions leads to cell dispersal accompanied by actin polymerization into stress fibers. Cell migration requires the coordinated activation of the Rho small GTPases that modulate the formation of membrane extensions and cell contractility (Pozzi and Zent, 2003). Rac1 activation induces lamellipodia formation involving reorganization of the actin

cytoskeleton at the cell edge, accompanied by Cdc42-induced filopodia formation. These membrane extensions serve as active sites for focal adhesion assembly. Cas/Crk coupling is involved during these processes (Klemke et al., 1998). Generation of cell contractility through the assembly of actin-myosin contractile units is dependent on the phosphorylation of myosin light chain kinase downstream from Erk (Klemke et al., 1997). Cell contraction and release of the rear edge are required to propel the cell forward. Active turnover of focal adhesions occurs during this process (Pozzi and Zent, 2003).

2.3.4.2 Cell invasion

Cell invasion is involved in tissue remodeling and in metastasis. Invasion requires the alteration of the extracellular matrix, mediated by metalloproteases and other proteases (Stamenkovic, 2003). HGF treatment of MDCK cells upregulates the expression of matrix metalloproteases and urokinase (Pepper et al., 1992), and stimulates the invasion of epithelial and endothelial cells through collagen gels *in vitro* (Weidner et al., 1990; Weimar et al., 1997). Prolonged Erk phosphorylation is required for enhanced transcription of MMP9 and cell invasion downstream of HGF (Liang and Chen, 2001; McCawley et al., 1998; Tanimura et al., 2002).

2.3.4.3 Morphogenesis

In addition to its motogenic potential, HGF is a morphogenic factor for kidney, breast and lung epithelia (Brinkmann et al., 1995; Weidner et al., 1993c). HGF promotes the inherent morphogenic program of MDCK cells that is branching tubulogenesis

(Montesano et al., 1991). HGF-induced branching is a multi-step process that involves changes in cell shape and junction reorganization, cell proliferation and matrix degradation (Pollack et al., 1998). In three-dimensional collagen matrix cultures, MDCK cells form hollow cysts with conserved apical and basolateral polarity, the apical side facing the lumen where apoptotic cells are expelled. In the presence of HGF initially, a partial EMT is initiated (Figure 4) resulting in the formation of membrane protrusions from individual cells that proliferate to form chains. During this step, cells transiently lose their polarity with E-cadherin being randomly distributed around the cell surface rather than being predominantly at lateral membranes. Cell proliferation leads to the formation of cords that are two or three cells thick. This process does not depend on HGF signals. Cords develop discontinuous lumens which enlarge and join the lumen of the cyst (Pollack et al., 1998). Basolateral polarity, with cell apoptosis is restored in the forming tubules and is dependent on interactions with the matrix but independent of HGF (Pollack et al., 1998).

HGF-induced tubulogenesis in MDCK cells is dependent on PI3K and Erk activation (Khwaja et al., 1998; Maroun et al., 1999). Moreover, recruitment and phosphorylation of the Gab1 docking protein is essential for this process (refer to section 4.3). The requirement for sustained Erk phosphorylation to induce tubule formation (Maroun et al., 1999) correlates with the enhanced transcription of metalloproteases downstream from prolonged Erk phosphorylation (Liang and Chen, 2001; McCawley et al., 1998; Tanimura et al., 2002), since matrix degradation is involved in the morphogenic response.

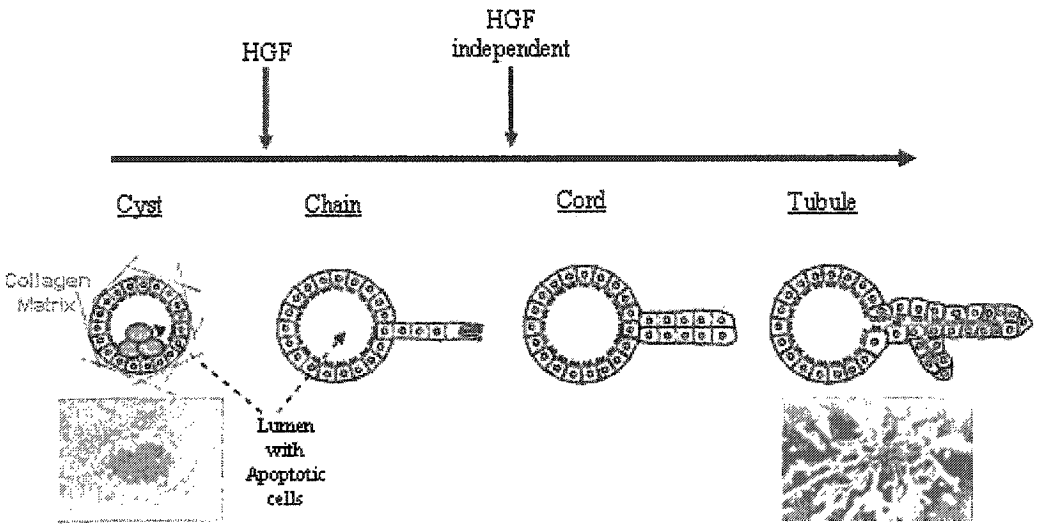

Figure 4: Branching morphogenesis in MDCK epithelial cells. Branching morphogenesis is a multi-step process that involves changes in cell shape, remodeling of cell-cell junctions, cell proliferation, matrix invasion and lumen formation. HGF initiates this process through its ability to promote a transient EMT.

3. RECEPTOR TYROSINE KINASES

3.1 Structure

Receptor tyrosine kinases (RTK) are cell surface receptors possessing intrinsic tyrosine kinase activity. They mediate the cell response to growth factors from the extracellular matrix (Birchmeier and Birchmeier, 1993).

Fifty-eight receptor tyrosine kinases have been identified; they can be classified into 20 subfamilies based on ligand specificity and structural similarity (Blume-Jensen and Hunter, 2001). All RTKs share some common structural features. These include an extracellular ligand-binding domain, a single transmembrane domain and an intracellular

domain containing the kinase domain as well as multiple potential sites for tyrosine phosphorylation. The kinase domain is well conserved among tyrosine and serine/threonine kinases and consists of two subdomains (Johnson et al., 1996). The N-terminus mediates binding to ATP through a consensus Gly-X-Gly-X-X-Gly sequence and carries out the ATP-mediated phosphotransfer reaction (Hanks et al., 1988), whereas the C-terminal region mediates substrate peptide binding and catalysis (Johnson et al., 1996).

3.2 Kinase activation

Most RTKs are monomers in the absence of ligand, except for the insulin receptor and the insulin-like growth factor-1 receptor. Activation of RTKs is a multi-step process initiated by ligand binding which promotes receptor dimerization or oligomerization. Oligomerized receptors are transphosphorylated on tyrosine residues within the kinase domain. This is essential for full catalytic activity of the receptor and acts to stabilize the receptor in an active conformation. Following receptor activation, tyrosine residues outside the catalytic domain are phosphorylated in trans (Jiang and Hunter, 1999; Ullrich and Schlessinger, 1990). These phosphorylated tyrosine residues provide specific binding sites for signaling proteins that contain Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Pawson and Nash, 2000; Weiss and Schlessinger, 1998). Recruited proteins include proteins with enzymatic activities (PI3K), modular domains (Grb2) and motifs such as tyrosine residues or proline rich regions that recruit additional signaling proteins. The activated receptor can phosphorylate some of these proteins or their associated partners, thereby increasing their catalytic activity and/or creating

docking sites for additional proteins to bind. In this manner, an activated RTK can assemble a complex of proteins involved in signal transduction through a series of specific protein-protein interactions. This coordinated recruitment of signals to the receptor transmits signals that modulate cellular responses such as cell proliferation, differentiation, cell migration and survival.

Activation of RTKs is a tightly regulated event. However, activation has also been shown to occur in the absence of ligand binding, through receptor overexpression, or in the presence of inhibitors of protein tyrosine phosphatases (Schlessinger, 2000). Receptor overexpression is frequently reported in human cancers and may result in deregulated receptor activation (Lamorte and Park, 2001). This leads to deregulated propagation of downstream signaling pathways. Signal deregulation perturbs tissue homeostasis and contributes to the loss of epithelial differentiation observed in cancer.

3.3 Oncogenic activation of RTKs

Oncogenic activation of RTKs is frequently found in cancer, resulting in constitutive kinase activation of the receptor with altered/constitutive downstream signaling. Mechanisms of oncogenic activation include receptor amplification and overexpression, the acquisition of an autocrine loop where the ligand and the receptor are expressed in the same cells, point mutations, or chromosomal translocation.

Receptor amplification and overexpression is thought to increase the concentration of receptors at the membrane, leading to amplification of the signal following ligand activation or, in other cases, to ligand-independent receptor clustering and activation. For example, EGFR amplification in glioblastomas is associated with

deletions of the extracellular domain, leading to receptor activation (Wong et al., 1992). Overexpression of the EGFR is associated with poor prognosis in breast cancer (Harris et al., 1992). A common example of receptor amplification and overexpression is the HER2/ErbB2/Neu receptor, overexpressed in 20-30% of breast tumors and in ovarian and renal collecting duct carcinomas (Pegram et al., 1998; Selli et al., 1997; Slamon et al., 1987; Zhang et al., 1997)

Another mechanism of oncogenic activation is the creation of an autocrine loop where a receptor and its ligand are co-expressed in the same cell, resulting in persistent receptor activation. An autocrine loop of HGF and its receptor Met was identified in mouse mammary carcinoma cells (Rahimi et al., 1996), leading to the constitutive tyrosine phosphorylation of several signaling molecules including PI3K, Src and FAK. Moreover, co-expression of HGF and its receptor Met was reported in infiltrating ductal carcinoma, suggesting the presence of autocrine activation (Tuck et al., 1996).

Point mutations that promote kinase activation or constitutive dimerization and subsequent activation have been reported for multiple receptors. A mutant ErbB2/Neu receptor was isolated from a rat neuroblastoma, containing a single point mutation in the transmembrane domain (Bargmann et al., 1986). This mutation promotes receptor dimerization through an inter-chain hydrogen bond with a neighboring mutant receptor (Smith et al., 1996). In addition, activating mutations within the Met receptor kinase domain have been reported in sporadic and inherited renal cell carcinoma, hepatocellular, gastric and head and neck squamous carcinomas (Jeffers et al., 1997; Schmidt et al., 1997; Schmidt et al., 1999). In general, these mutations lead to an elevated catalytic activity of the receptor.

Oncogenic activation by chromosomal translocation involves the fusion of a dimerization motif with the kinase domain of a receptor, leading to constitutive receptor dimerization and activation. Tpr-Met, the oncogenic variant of the HGF receptor Met, was isolated from an *N*-methyl-*N*'-nitronitrosoguanidine-treated human osteogenic sarcoma cell line (Cooper et al., 1984). This oncogene is generated following a chromosomal rearrangement that fuses *tpr* sequences encoding a protein-protein dimerization motif (leucine zipper) to the juxtamembrane and kinase domains of Met (Rodrigues and Park, 1994b). This results in constitutive activation of the kinase in the absence of ligand. Although activation of the Met receptor following chromosomal translocation has not been observed in human tumors, this is now regarded as a prototype for activation of many receptor tyrosine kinases (Ret, TrkA, PDGF) in human tumors following chromosomal translocations (Lamorte and Park, 2001).

4. THE MET RECEPTOR TYROSINE KINASE

The Met receptor tyrosine kinase is the receptor for the Hepatocyte Growth Factor/Scatter Factor (HGF/SF) (Figure 5). Met is a member of a RTK family that includes Ron and Sea, a Ron homologue from chicken. Met is highly conserved in vertebrates but is absent in Drosophila melanogaster and C-elegans (Rubin et al., 2000). The Met receptor is synthesized as a single chain precursor that is proteolytically cleaved to generate a mature receptor comprising an extracellular α subunit of 45 kDa and a 145 kDa β subunit spanning the membrane (Giordano et al., 1989). Met localizes to basolateral membranes in polarized epithelial cells (Crepaldi et al., 1994).

4.1 Biological functions

Met is predominantly expressed in epithelial cells including breast and kidney epithelia, whereas HGF is expressed in the surrounding mesenchyme (Gonzatti-Haces et al., 1988; Prat et al., 1991b; Rosen et al., 1994b; Yang and Park, 1995a; Yang and Park, 1995b). Animals null for either HGF or Met are embryonic lethal at day 13.5 and demonstrate identical phenotype (Ebens et al., 1996; Schmidt et al., 1995; Uehara et al., 1995). These studies have established that Met and HGF are required for the development of liver and placenta, the development and innervation of skeletal muscle, and are involved in neuronal chemotaxis (Ebens et al., 1996; Schmidt et al., 1995; Uehara et al., 1995). In addition, Met and HGF are involved in organ regeneration through their mitogenic potential (Bussolino et al., 1992; Igawa et al., 1991; Matsumoto et al., 1991); increased HGF expression was observed following liver injury (Matsumoto and Nakamura, 1993). HGF and Met also promote angiogenesis (Bussolino *et al.*, 1992; Rosen *et al.*, 1997) and the survival of epithelial cells as they are detached from extracellular matrix (anoikis) (Frisch and Francis, 1994). HGF and Met regulate the development of the nervous system by stimulating the migration, survival and outgrowth of different neuronal subpopulations (Maina et al., 1998; Powell et al., 2001; Yang et al., 1998).

As discussed earlier, stimulation of the Met receptor with HGF promotes epithelial cell dispersal and morphogenesis in a cell culture model. This requires multiple signals dependent on recruitment of the Gab1 docking protein, that promote the remodeling of cell-cell junctions, reorganization of the actin cytoskeleton as well as the induction of proteases involved in the degradation of the extracellular matrix (Liang and

Chen, 2001; Maroun et al., 1999; McCawley et al., 1998; Pollack et al., 1998; Tanimura et al., 2002). These are all signals that correlate with enhanced invasion of tumor cells and HGF is a potent inducer of the migration and invasion of carcinoma cells (Bellusci et al., 1994; Weidner et al., 1990; Weimar et al., 1997). Moreover, HGF is found at the invading edges of breast carcinomas (Edakuni et al., 2001) where it is thought to act to enhance tumor invasion. The involvement of Met in breast cancer progression will be discussed in section 6.

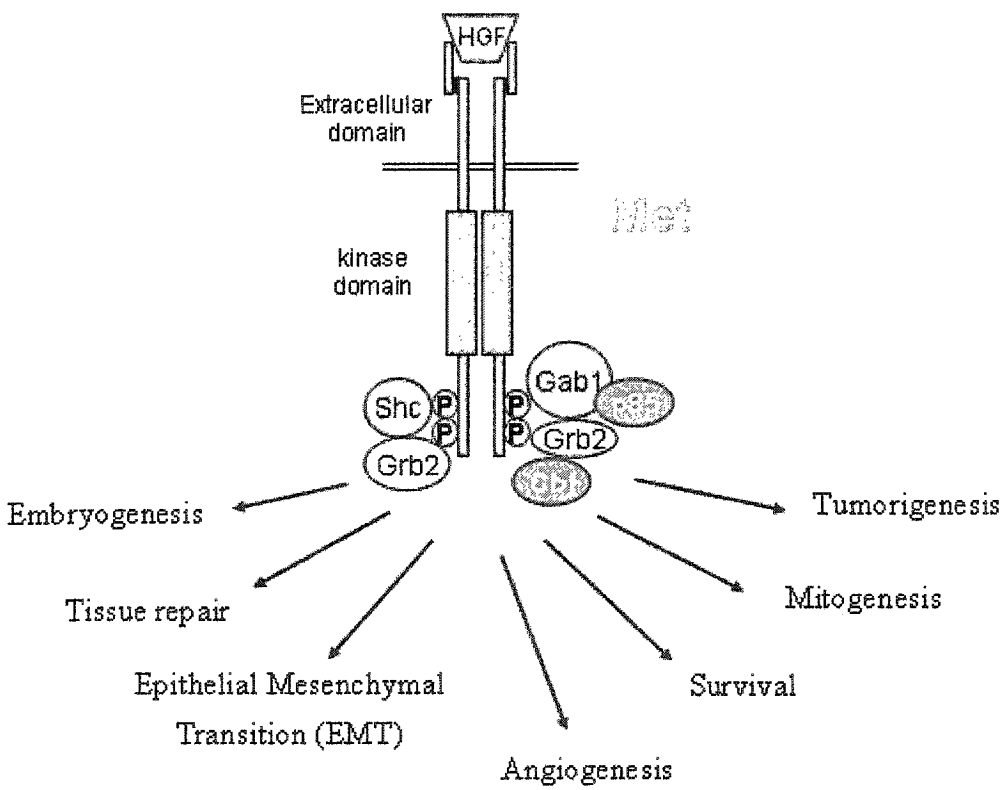

Figure 5: The Met receptor tyrosine kinase. Upon HGF-induced activation, Met recruits signaling proteins and mediates multiple biological processes including cell dispersal and morphogenesis. Met is also involved in tumorigenesis.

4.2 Downstream signaling

The Met receptor contains 16 tyrosine residues within its cytosolic domain. Two tyrosine residues within the kinase domain are required for full catalytic activation of the receptor whereas two tyrosine residues within the carboxy terminus of the receptor, Y1349 and Y1356, are essential for all known biological responses downstream from Met, by providing docking sites for signaling proteins (Figure 5) (Naldini *et al.*, 1991a; Rodrigues and Park, 1994a). Multiple signaling proteins are recruited to the Met receptor. These include the adapter proteins, Grb2 and Shc, enzymes, phosphatidylinositol 3' kinase (PI3K), phospholipase C γ (PLCγ), the tyrosine phosphatase SHP2, the Src tyrosine kinase, as well as the multisubstrate docking protein Gab1 and the ubiquitin ligase Cbl.

Tyrosine Y1356 (NAT**Y**VNV), when phosphorylated, provides a direct binding site for the Grb2 and Shc adapter proteins (Fixman et al., 1996; Fournier et al., 1996; Ponzetto et al., 1994). Grb2 acts as an adapter to indirectly recruit the Gab1 docking protein and the c-Cbl ubiquitin ligase. Gab1 has no enzymatic activity but once phosphorylated by the Met receptor acts as a docking protein to recruit the p85 subunit of PI3K, PLCγ, SHP2 as well as the adapter protein Crk (Bardelli et al., 1997; Gual et al., 2000; Holgado-Madruga et al., 1997; Lamorte et al., 2000; Maroun et al., 1999). In addition, Gab1 is recruited to Y1349 in a direct mechanism (Lock et al., 2000; Nguyen et al., 1997; Weidner et al., 1996). This is through a novel interaction of the Gab1 Met binding domain with the carboxy terminal helix of the Met kinase domain (Lock et al., 2003). Substitution of Y1356 with phenylalanine interferes with all Met mediated biological responses, including cell transformation, motility, invasion and morphogenesis, whereas substitution of Y1349 has limited effect on cell transformation

only (Fixman et al., 1995; Maina et al., 1996; Ponzetto et al., 1994; Weidner et al., 1995; Zhu et al., 1994).

Direct recruitment of the adapter protein Grb2 to Met is required for Met-induced morphogenesis and for efficient cell transformation by the Tpr-Met oncogene (Fixman et al., 1997; Fournier et al., 1996). Grb2 can also be recruited to Met through the Shc adapter protein. Using mutants of the Tpr-Met oncogene, the direct recruitment of Shc or Grb2 was shown to be sufficient to induce cell transformation (Saucier et al., 2002). However, recruitment of Shc but not Grb2 is essential for Met-induced angiogenesis mediated by the increased production of vascular endothelial growth factor (VEGF) (Saucier et al., 2004). Shc recruitment is also required for Met-induced MDCK cell dispersal (Fournier et al., 1996).

Grb2 mediates the recruitment of c-Cbl and Gab1 to Met. The involvement of Gab1 in Met physiology is discussed in the following section. Cbl functions as an E3-ubiquitin ligase, hence it is involved in the negative regulation of RTKs (Joazeiro et al., 1999; Levkowitz et al., 1999; Meisner et al., 1997; Yokouchi et al., 2001). In addition to Grb2-mediated recruitment, Cbl can be recruited directly to the Met receptor through the Cbl TKB domain (Peschard et al., 2001). Cbl has been shown to promote ubiquitination and internalization of Met, uncoupling Cbl from the receptor enhances its transforming potential (Peschard et al., 2001; Petrelli et al., 2002).

4.3 The Gab1 docking protein

Gab1 (Grb2-associated binder) is a member of the IRS-1 multisubstrate docking protein family which includes IRS-1, IRS-2, p62dok and DOS, in addition to Gab2 and

Gab3 (Carpino *et al.*, 1997; Yenush and White, 1997). These proteins share similar structural motifs, such as tyrosine residues, proline-rich motifs and/or PTB domains. Gab1 contains 16 tyrosine residues that may be potential binding sites for SH2 and PTB domain containing proteins, as well as several proline-rich regions that could interact with SH3 domain-containing proteins such as Grb2 (Figure 6) (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997). Gab1 is generally recruited to RTKs through association of its proline-rich region with Grb2 (Bardelli et al., 1997; Lock et al., 2000; Nguyen et al., 1997). An amino-terminal pleckstrin homology (PH) domain is involved in membrane phospholipid binding and recruitment of Gab1 to the membrane (Maroun et al., 1999).

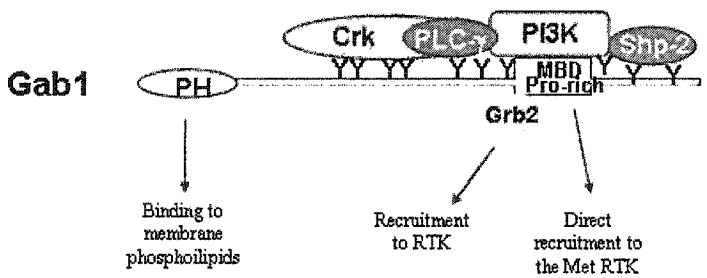

Figure 6: The Gab1 multisubstrate docking protein. Gab1 is a common substrate for RTK and interleukins. It recruits multiple signaling proteins to RTK and mediates multiple biological processes in embryonic and adult life.

Gab1 was identified as the major tyrosine phosphorylated protein in MDCK epithelial cells following HGF stimulation (Fixman et al., 1997; Nguyen et al., 1997). In MDCK cells, phosphorylated Gab1 recruits the p85 subunit of PI3K, PLCγ, SHP2 and the Crk adaptor to the Met receptor (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Lamorte et al., 2000; Lamorte et al., 2002; Maroun et al., 1999; Maroun et al.,

2000). It is believed that Gab1 recruits these signaling proteins and links them to the activation of downstream signaling pathways including Ras, JNK and Erk. Gab1-Crk association correlates with JNK activation in cells transformed with oncogenic Tpr-Met (Lamorte et al., 2000). The involvement of Erk downstream from Gab1 has been reported in Gab1 -/- fibroblasts that show reduced Erk activation (Itoh et al., 2000; Shi et al., 2000; Yart et al., 2001).

Animals null for Gab1 are embryonic lethal due to heart, placenta and skin defects. In addition Gab1-/- embryos show impaired migration of myogenic precursor cells into the limb anlage (Itoh et al., 2000; Sachs et al., 2000). These phenotypes evoke the Met and HGF knockout phenotypes (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995), providing genetic evidence that Gab1 is essential for Met signaling in vivo. Consistently, *in vitro* studies using the MDCK cell model show that Gab1 is a key mediator of Met-induced biological processes including cell transformation, cell dispersal and morphogenesis (Fournier et al., 1996; Maina et al., 1996; Maroun et al., 1999). Overexpression of Gab1 rescues the morphogenic response downstream from a Met receptor mutant that fails to bind Grb2 (Maroun et al., 1999). Moreover, expression of Gab1 in mouse mammary epithelial cells cultured in Matrigel mimics the HGF effects in promoting branching morphogenesis (Niemann et al., 1998). This provides further evidence that Gab1 is required for the morphogenic response downstream from the Met receptor. Association of PLCγ and Crk with Gab1 seems to be essential for this process (Gual et al., 2000; Lamorte et al., 2002), whereas interaction of Gab1 with the p85 subunit of PI3K is not required for morphogenesis (Maroun et al., 1999). Gab1-p85

interaction is thought to enhance Met-dependent synthesis of hyaluronic acid (HA) (Kamikura et al., 2000).

In addition to Met, Gab1 is phosphorylated downstream from the EGFR, the insulin receptor, the TrkA receptor, as well as interleukins (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Takahashi-Tezuka et al., 1998). Gab1 plays a significant biological role downstream from these receptors, it is involved in neuronal cell survival, neurite outgrowth, DNA synthesis and epithelial cell morphogenesis (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Korhonen et al., 1999; Weidner et al., 1996). However, induction of epithelial morphogenesis seems to be unique to Gab1 and Met, since other RTKs fail to induce branching in MDCK cells (Maroun et al., 1999; Sachs et al., 1996).

Gab1 interaction with Met is distinct from the other RTK in two aspects: the mode of recruitment and phosphorylation kinetics. It has been shown that in addition to the Grb2-mediated interaction, direct binding of Gab1 to Met accounts for 20% of Gab1 recruitment to the receptor (Lock et al., 2000; Nguyen et al., 1997). This direct binding is mediated via the Gab1 proline-rich Met Binding Domain (MBD) (Lock et al., 2000) and is required for Met-induced morphogenic response, since Gab1 fails to rescue this response in the absence of a functional MBD (Lock et al., 2002). Moreover, Met induces a prolonged and sustained phosphorylation of Gab1 and downstream kinases Erk1/2, whereas EGF induces transient phosphorylation of these proteins and fails to promote tubule formation (Maroun et al., 1999), suggesting that a prolonged phosphorylation is required for the morphogenic response to be triggered.

Gab2, a member of the Gab1 protein family is recruited to RTKs and phosphorylated on tyrosine residues in response to different stimuli including interleukins, EGF, heregulin and HGF (Lock et al., 2002; Lock et al., 2000; Lynch and Daly, 2002). Although it shares structural similarities with Gab1 (Figure 6), Gab2 mediated cellular responses are different from Gab1. Gab2 is not involved in Met-mediated morphogenesis (Lock et al., 2002); it is involved in the differentiation of hematopoietic cells and genetic deletion leads to defects in allergic responses (Gu et al., 2001). Negative feedback regulation of Gab2 downstream from the ErbB receptors has been reported (Lynch and Daly, 2002).

5. THE HER2/ERBB2/NEU Receptor Tyrosine Kinase

p185 HER2/ErbB2 (the human homologue of rat Neu) is a member of the EGF receptor (EGFR) family which includes EGFR (ErbB1), the kinase-inactive ErbB3, and ErbB4 (Figure 7) (Burden and Yarden, 1997). A ligand for ErbB2/neu has not yet been identified. However, the receptor is activated through the formation of heterodimers with other EGFR family members following stimulation of these members with their corresponding ligands (Burden and Yarden, 1997; Klapper *et al.*, 1999). ErbB2/neu is the preferred heterodimerization partner for all the EGFR family members (Graus-Porta et al., 1997; Muthuswamy et al., 1999; Pinkas-Kramarski et al., 1996b; Qian et al., 1999). It has been shown to potentiate signaling downstream from these receptors by preventing receptor degradation and increasing recycling to the membrane (Wang et al., 1999;

Waterman et al., 1998; Worthylake et al., 1999). Hence, ErbB2/neu acts as an amplifier for other EGFR family receptors.

ErbB receptor ligands belong to two families of structurally related proteins, the EGF-like growth factors and the neuregulins (Chang et al., 1997; Lee et al., 1995; Peles and Yarden, 1993). The first family includes EGF, TGFα, amphiregulin, heparin-binding EGF (HB-EGF), betacellulin (BTC) and epiregulin (EPR). These ligands bind the EGF receptor. In addition, HB-EGF, BTC and EPR bind ErbB4. The neuregulin family includes various isoforms derived from four independent genes by alternative splicing (Peles and Yarden, 1993). Neuregulin 1 is also called Neu differentiation factor (NDF) or heregulin (HRG). Neuregulins specifically bind ErbB3 and ErbB4, inducing heterodimer formation between these receptors and ErbB2 (Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996a). The ErbB ligands bind to their receptors with varying affinities

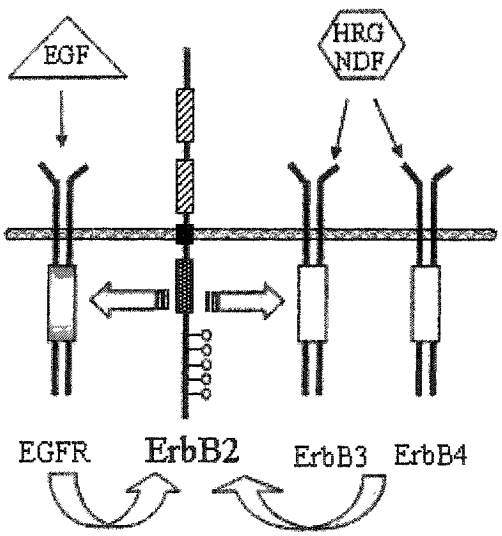

Figure 7: The EGFR family of RTKs. ErbB2 is an orphan receptor that is activated following ligand-induced heterodimerization with other members of the EGFR family. ErbB2 potentiates signaling downstream these receptors, hence it acts as an amplifier of other EGFR family members.

and generate numerous combinations of heterodimers which contribute to signal diversification.

5.1 Biological functions

Animals null for ErbB2/neu by gene knockout have identified a role for ErbB2/neu in normal embryonic development, cardiac and Schwann cell development in addition to a role in developing neuromuscular junctions (Andrechek et al., 2002; Chan et al., 2002; Lee et al., 1995; Leu et al., 2003; Lin et al., 2000). Precisely, the catalytic activity of ErbB2 is essential for the development of the cardiac trabecular extensions (Chan et al., 2002). Consistent with this, ErbB2/neu expression is concentrated at the neuromuscular junctions (Huang et al., 2001). It has been suggested that ErbB2/ErbB4 heterodimers regulate the development and maintenance of the nervous system (Murphy et al., 2002). Moreover, ErbB2 is involved in tissue regeneration, expression of an ErbB2 antisense construct delays wound closure following urinary bladder injury (Bindels et al., 2002). ErbB2 localizes to the basolateral membrane in human intestinal epithelial cells and human airway epithelia (Borg et al., 2000; Vermeer et al., 2003).

ErbB-2/neu signaling mediates the growth and differentiation of a variety of cells in culture (Burden and Yarden, 1997). Moreover, ErbB2 is also involved in cell transformation and mammary tumorigenesis in transgenic animals (Dankort et al., 1997; Guy et al., 1996; Guy et al., 1992). Consistently, a constitutively active ErbB2/Neu receptor increases cell proliferation in polarized mouse mammary cell cultures (Janda et al., 2002b). Moreover, heregulin-induced activation of ErbB2 enhances cell migration and invasion of breast cancer cells (Adam et al., 1998; Brandt et al., 1999; Chausovsky et

al., 1998; Meiners et al., 1998). ErbB2/ErbB3 heterodimers promote cell spreading and lamellipodia formation in CHO cells, involving PI3K activation (Chausovsky et al., 2000). However, when N-cadherin is expressed in CHO cells, ErbB2/ErbB3 promotes the reorganization of the cell colonies into ring-shaped structures, this process requires both PI3K and MAPK activities (Chausovsky et al., 2000). Similarly, a previous report has shown that activation of ErbB-2 promotes the remodeling of epithelial cell junctions consistent with a morphogenic response (Chausovsky et al., 1998). ErbB-2 is involved in the morphogenic and functional differentiation of the normal mammary gland epithelium. This role is discussed in section 6.

5.2 Downstream signaling

Structure/function analyses of ErbB2/Neu revealed that five carboxy-terminal tyrosine residues are docking sites for signaling proteins (Figure 8) and mediate

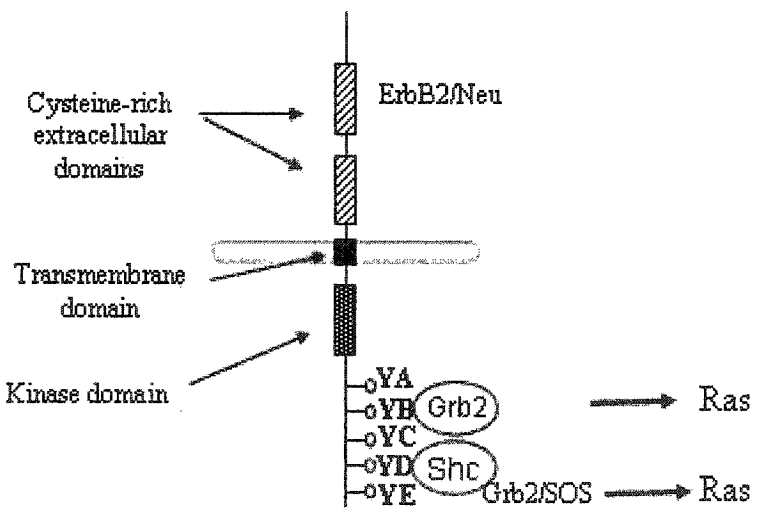

Figure 8: The ErbB2/Neu RTK. The receptor contains two cysteine-rich extracellular domains, a transmembrane domain and a kinase domain. Five C-terminal tyrosine residues (YA, B, C, D, E) act as docking sites for signaling proteins.

ErbB2/neu-induced cell transformation independently (Dankort et al., 2001a; Dankort et al., 1997). The recruitment of Grb2 and Shc through tyrosines Y1144 (YB) and Y1226/7 (YD) respectively, couples the ErbB2/Neu receptor to the Ras signaling pathway in fibroblasts and Drosophila models (Dankort et al., 2001a; Settle et al., 2003).

The recruitment of Shc to ErbB2/Neu is required to enhance VEGF production (Saucier et al., 2004). This is consistent with Shc-induced rapid onset mammary tumor formation (Dankort et al., 2001b). Grb2 mediates the recruitment of the Gab2 docking protein to ErbB2/ErbB3 heterodimers following heregulin stimulation of ErbB2-expressing breast cancer cells (Lynch and Daly, 2002), resulting in increased tyrosine phosphorylation of Gab2. It has been shown that Gab2 coupling to ErbB2 is tightly regulated by PKB/Akt-mediated negative feedback via phosphorylation of the docking protein on a serine residue (Ser159); mutation of this serine residue enhances the transforming potential of Gab2 in fibroblasts and potentiates signaling downstream from ErbB2 (Lynch and Daly, 2002). Interestingly, Gab2 is overexpressed in breast cancer cell lines (Daly et al., 2002), the involvement of this docking protein in ErbB2/Neu-induced tumorigenesis needs further investigation.

We show in this thesis evidence for Gab1 recruitment to ErbB2/neu (Chapter III). A role for Gab1 in ErbB2/Neu induced cell transformation and tumorigenesis has been suggested recently. Gab1-/- fibroblasts exhibit impaired ErbB2-induced colony formation in soft agar and tumor formation in mice (Yamasaki et al., 2003). Similarly, it was suggested that Gab1 activation by the EGFR is required for efficient ErbB2/neu-induced tumor progression (Gillgrass et al., 2003).

C-terminal Y1253 (YE) recruits an unidentified 34kDa protein (p34) that mediates YE transforming potential; p62DOK-related protein (Dok-R) and an unknown p150 are also recruited to YE (Dankort et al., 2001a). A conserved ENPEYL motif is present in YE and YC (Y1201), corresponding to an NPXY consensus for the recruitment of PTB-containing proteins. YC is thought to recruit the adapter protein Crk and Nck (Dankort et al., 2001a; Dankort et al., 1997). It has been suggested that Crk coupling to p130Cas is induced by ErbB2/Neu and mediates cell invasion and migration (Spencer et al., 2000). Elevated Erk activity is observed under these conditions.

In addition to these signals, carboxy-terminal Y1028 (YA) may recruit a negative regulatory protein, restoration of this residue in a tyrosine-phosphorylation deficient ErbB2/Neu mutant suppresses the basal transforming activity (Dankort et al., 1997). Moreover, the Cbl ubiquitin ligase has been shown to target ErbB-2 to a ubiquitin/degradation pathway (Klapper et al., 2000).

The Src tyrosine kinase is activated downstream from ErbB2/Neu and may contribute to ErbB2-induced tumorigenesis (Dankort and Muller, 2000; Muthuswamy and Muller, 1995a). Consistently, elevated Src activity is observed in ErbB2-induced mammary tumors (Muthuswamy and Muller, 1995b).

ErbB2/Neu is the preferred heterodimerization partner for all EGFR family members, this allows it to couple to additional signaling pathways that are activated downstream from its partners. For example, ErbB2 does not recruit PI3K directly but is coupled to the PI3K pathway via its dimerization with ErbB3 (Dankort and Muller, 2000). PI3K-mediated survival signals may contribute to ErbB2/Neu-induced tumorigenesis (Dankort and Muller, 2000).

6. MET AND ERBB2/NEU IN BREAST CANCER

6.1 Met and ErbB2/neu in mammary epithelial remodeling

Because of its function in milk production, the mammary gland undergoes tissue remodeling through the entire reproductive life of mammals, starting *in utero* and reaching the final stage during pregnancy (Russo and Russo, 1987). Development of branching ducts and milk-secreting lobulo-alveolar structures involves extensive tissue remodeling; this is regulated by hormonal factors and local epithelial-mesenchymal inductive signals including transforming growth factor TGFβ and HGF/SF (Pollard, 2001). In addition, the EGFR/ErbB family receptors are differentially expressed during development of the mouse mammary gland, signals downstream from these receptors contribute to this process (Troyer and Lee, 2001).

Genetic ablation of Met and ErbB2 by gene knockout suggests a role for these receptors in epithelial remodeling (Andrechek et al., 2002; Chan et al., 2002; Ebens et al., 1996; Lee et al., 1995; Leu et al., 2003; Schmidt et al., 1995; Uehara et al., 1995). Moreover, Met and ErbB2/neu are expressed in many epithelial tissues, including the breast epithelium (Gonzatti-Haces et al., 1988; Prat et al., 1991a; Rosen et al., 1994a; Vermeer et al., 2003; Yang and Park, 1995a). Met is localized to the ductal epithelium of the human mammary gland and colocalizes with staining to phosphotyrosine (Tsarfaty et al., 1992), whereas HGF is expressed in mesenchymal cells during ductal branching of virgin mice (Yang et al., 1995). This suggests that Met may be activated in lumen structures or ducts. Consistent with this, HGF stimulates the formation of branching duct-like structures in cloned mammary epithelial cells and in primary mammary co-cultures

(Pollard, 2001; Soriano et al., 1995; Zhang et al., 2002) in addition to hyperplastic ductal tree in primary mouse cells transplanted into a recipient cleared fat pad (Yant et al., 1998), HGF blocking antisense abolishes branching differentiation of whole organ cultures (Yang et al., 1995).

ErbB2/neu is expressed in the mouse mammary gland epithelium; ErbB2 might be required for EGFR-mediated ductal morphogenesis (Sebastian et al., 1998; Troyer and Lee, 2001; Wiesen et al., 1999). In addition, neuregulin, a ligand for the ErbB family receptors is expressed during alveolar development in pregnancy and promotes the proliferation and differentiation of mammary epithelia into secretory lobuloalveoli (Jones and Stern, 1999; Yang et al., 1995).

Organ cultures of mammary glands indicate that HGF and neuregulin regulate mammary differentiation in a sequential order (Yang et al., 1995), with a requirement for HGF in branching morphogenesis and ErbB-2 in lobuloalveolar differentiation (Jones and Stern, 1999; Niemann et al., 1998; Yang et al., 1995). Hence, alteration of any of these pathways might affect the outcome of the other signal and disturb mammary epithelial architecture. Altered expression of HGF/Met and ErbB2 is reported in breast carcinomas and will be discussed in the following section.

6.2 Met and ErbB2/neu alterations in breast cancer

The Met and ErbB-2 receptors are important independent prognostic markers for breast cancer (Ghoussoub et al., 1998; Slamon et al., 1987). ErbB2/neu is overexpressed in 20-30% breast cancers (Bieche and Lidereau, 1995; Gusterson et al., 1992; Ross et al., 1999; Slamon et al., 1987). ErbB2 overexpression is the consequence of gene

amplification (17q12) (Kauraniemi et al., 2001). Receptor amplification and overexpression is usually associated with tumor aggressiveness and poor prognosis, ErbB2 expression is rarely observed in benign breast hyperplasia (Allred et al., 1992; Slamon et al., 1987; Zhang et al., 2003). A recent study shows, however, that normal expression levels of ErbB2/Neu detected in 17.5% of a population of 300 tumors was associated with tumor aggressiveness (Camp et al., 2003). Consistent with these results, targeted expression of wild type ErbB2/Neu to the mammary gland of transgenic mice induces metastatic tumor formation; these tumors express normal levels of ErbB2/Neu but exhibit elevated tyrosine phsophorylation of the receptor (Guy et al., 1992).

Transgenic mouse models have provided direct evidence for the involvement of ErbB2/Neu in mammary tumorigenesis. Either wild type ErbB2/Neu or a constitutively activated mutant induces metastatic mammary tumors when expressed under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Bouchard et al., 1989; Guy et al., 1996; Guy et al., 1992; Muller et al., 1988). Constitutively activated ErbB2/Neu is generated by a point mutation in the transmembrane domain (V664E) that results in increased receptor homodimerization and constitutive activation of the kinase domain (Bargmann et al., 1986). This mutation is thought to mimic the effect of ErbB2/Neu overexpression in breast tumors, although it has never been detected in human tumor biopsies (Lemoine et al., 1990; Slamon et al., 1989). However, an alternative spliced form of ErbB2/Neu has been found in human breast tumors (Siegel et al., 1999), this mutant carries a 16 amino acid deletion in the juxtamembrane region allowing the formation of intermolecular disulfide dimers.

The mechanism by which ErbB2/Neu contributes to tumor progression is unclear. The long latency observed in tumor formation downstream from wild type ErbB2/Neu (Guy et al., 1992) suggests the involvement of additional factors. Interestingly, increased expression and activation of EGFR and ErbB3 accompanies ErbB2 overexpression in transgenic mice and human breast tumors (Dankort and Muller, 2000; Siegel et al., 1999), suggesting that they contribute to ErbB2/Neu tumorigenic potential.

Met and HGF have also been identified as prognostic markers for breast cancer (Jin et al., 1997; Nagy et al., 1996; Tuck et al., 1996; Yamashita et al., 1994). High expression levels of Met in human breast carcinomas and high levels of HGF in tumor stroma have been reported (Beviglia et al., 1997; Camp et al., 1999; Jin et al., 1997; Tuck et al., 1996; Yamashita et al., 1994). Tissue microarray analyses of breast tumors show a tight correlation between HGF and Met expression in breast cancer (Kang et al., 2003a). Additionally, evidence for the presence of HGF-Met autocrine loops has been reported in a variety of human and mouse carcinomas including breast cancers, correlating with poor prognosis (Danilkovitch-Miagkova and Zbar, 2002; Edakuni et al., 2001). These observations suggest that Met and HGF are involved in the progression of the malignant phenotype. Consistently, transgenic mice overexpressing either HGF or the oncogenic variant of Met, Tpr-Met, develop multiple malignancies including mammary tumors (Gallego et al., 2003; Liang et al., 1996; Takayama et al., 1997), suggesting a role for Met and HGF in mammary tumorigenesis. Further evidence for Met involvement in breast cancer is provided by the use of a dominant negative approach. Cells expressing dominant negative Met show reduced tumorigenicity and metastasis in Balb/C mice (Firon et al., 2000).

Nevertheless, loss of heterozygosity (LOH) at the Met locus on chromosome 7q31 and loss of Met expression associated with early stages of breast cancer have also been reported (Bieche et al., 1992; Camp et al., 1999; Ghoussoub et al., 1998; Lin et al., 1998; Lin et al., 1996). The LOH suggested that Met might act as a tumor suppressor gene (Bieche et al., 1992; Lin et al., 1996). Although these observations seem to contradict the data discussed in the previous paragraph, they can be explained by a two-stage involvement of Met expression in breast cancer, with Met being lost early in tumorigenesis and then regained at a later stage associated with increased agressiveness (Camp et al., 1999; Ghoussoub et al., 1998). Met expression consistently correlates with the tumorigenic and metastatic potential in a panel of breast cancer cell lines (Lin et al., unpublished). Differentiated, non-metastatic cells have lost Met protein, whereas fibroblast-like highly tumorigenic cells express the Met receptor. Since Met is an important modulator of epithelial differentiation, loss of its expression might be required to promote a dedifferentiated phenotype (EMT), high Met expression in advanced stages might contribute to increased metastatic behavior through Met and HGF-mediated cell migration, cell survival and metalloprotease production (Frisch and Francis, 1994; McCawley *et al.*, 1998). Decreased Met expression by a Met-targeting ribozyme results in reduced migration and *in vitro* invasion through Matrigel (Jiang et al., 2001).

Met expression in breast tumors has been associated with poor outcome independent of ErbB2 (Tolgay Ocal et al., 2003), however the possible implication of Met and HGF in the progression of ErbB2-related malignant disease cannot be excluded. Furthermore, correlation between HGF expression in the stroma and ErbB2/Neu overexpression in breast cancer has not been addressed yet.

7. COOPERATION BETWEEN ONCOGENES

Cooperative induction of epithelial-mesenchymal transition (EMT) has been reported in cultured cell models and transgenic mice. A classical model is TGFβ, which triggers different cellular outcomes depending on the cell context. For example, TGFβ promotes growth arrest in polarized hepatocytes, however in transformed cells, TGFβ promotes EMT with induction of metalloproteases (MMP9), CD44 and Snail, the E-cadherin repressor, in addition to E-cadherin downmodulation (Gotzmann et al., 2002). Other examples of TGFβ cooperation with Ras, Ha-Ras and EGF have been described in primary cultured cells (Grande et al., 2002; Janda et al., 2002a; Janda et al., 2002b; Oft et al., 1996). Interestingly, TGFβ impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis (Siegel et al., 2003), indicating that the importance of TGF-β signaling within the tumor is to specifically enhance the ability of the tumor cells to extravasate into the lung.

Cooperative induction of mammary tumor formation between ErbB2/neu and mutant p53 or TGFα has been reported in transgenic mice, resulting in shortened tumor latency (Li et al., 1997; Muller et al., 1996). Moreover, HGF and Met enhance tumor growth and metastasis of Ras-transformed NIH3T3 cells (Webb et al., 1998).

Crosstalk between EGFR and Met in transformed cells has also been reported, involving TGFα and EGF-induced Met phosphorylation and association with EGFR (Jo et al., 2000). Interestingly, a model for the creation of an autocrine loop involving Met and HGF in breast cancer cells has been proposed (Elliott et al., 2002). According to this model, cooperation between activated Src and sustained STAT3 activity induces the transcription of HGF in breast carcinoma and mammary epithelial cells expressing the

Met receptor, resulting in enhanced EMT with loss of cell-cell contacts. Increased activity of Src and STAT3 is observed in human cancers (Bowman *et al.*, 2000; Dankort and Muller, 2000). Under normal physiological conditions, transient activation of Src and STAT3 contribute to the branching morphogenesis of the breast epithelium (Elliott et al., 2002).

8. GOALS

Although several studies have addressed the molecular mechanisms involved in the transforming and tumorigenic potentials of the ErbB2/Neu receptor, the ability of ErbB2/Neu to promote epithelial remodeling and the underlying mechanisms remain unknown. This thesis sought to assess the effect of deregulated activation of ErbB2/Neu on epithelial organization and to identify potential synergistic factors that contribute to ErbB2/Neu transforming potential.

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Chapter II: Distinct tyrosine autophosphorylation sites mediate induction of epithelial-mesenchymal like transition by an activated ErbB-2/neu receptor

Preface

The development and progression of invasive carcinomas correlates with the disruption of epithelial organization and the acquisition of a motile phenotype. This process is known as epithelial-mesenchymal transition (EMT). EMT is also a common event in embryogenesis where a transient gain of cell motility is required for tissue remodeling. This process can be initiated by different growth factors (HGF, EGF, TGFβ) or extracellular matrix components. Moreover, the elevated expression of signaling molecules involved in this process, for example the MEK dual specificity kinase or ILK promotes the loss of epithelial organization in non-transformed epithelial cells in culture.

The ErbB-2/neu receptor tyrosine kinase is involved in normal tissue development. However, this receptor has been implicated in the genesis of human breast and renal carcinomas, where the ErbB2 receptor is amplified in 20-30 % of human breast cancers and correlates with poor prognosis. Moreover therapeutic strategies directed at the HER2 receptor have shown some promise as combination therapies in clinical trials Although several studies have addressed the molecular mechanisms involved in the transforming and tumorigenic potentials of the receptor, the mechanisms through which ErbB2/neu contributes to loss of tissue architecture in tumors remains poorly understood. Using the non-transformed MDCK epithelial cell model, we sought to address the functional significance of specific ErbB-2/neu initiated signals in epithelial cell dispersal and remodeling.

Abstract

Tight control of cell proliferation and morphogenesis is required to ensure normal tissue patterning and prevent cancer formation. Overexpression of the ErbB-2/Neu receptor tyrosine kinase is associated with increased progression in human breast cancer, yet in breast explant cultures, the ErbB-2/Neu receptor contributes to alveolar differentiation. To examine the consequence of deregulated ErbB-2/Neu activation on epithelial morphogenesis, we have expressed a constitutively activated mutant of ErbB-2/Neu in a Madin-Darby canine kidney (MDCK) epithelial cell model. Using two-dimensional cultures we demonstrate that activated ErbB-2/Neu induces breakdown of cell-cell junctions, increased cell motility and dispersal of epithelial colonies. This correlates with reorganization of the actin cytoskeleton and focal adhesions and loss of insoluble cell-cell junction complexes involving E-cadherin. Interestingly, a constitutively activated ErbB-2/Neu receptor promotes an invasive morphogenic program in MDCK cells in a three-dimensional matrix. We show that two tyrosines in the carboxy-terminal tail of ErbB-2/Neu, involved in the phosphorylation of the Shc adapter protein, are each sufficient to promote epithelial-mesenchymal like transition and enhanced cell motility in two-dimensional culture and cell invasion rather than a morphogenic response in matrix culture. This provides a model system to investigate ErbB-2/Neu induced signaling pathways required for epithelial cell dispersal and invasion versus morphogenesis.

Keywords: ErbB-2/Neu; epithelial-mesenchymal transition; morphogenesis; MDCK epithelial cells; Shc

Introduction

Breast cancer, the most frequently diagnosed cancer, is a leading cause of death in women. The development and progression of this disease to an invasive phenotype is mediated by the deregulation of different modulators of growth and differentiation. Notably, several receptor tyrosine kinases that regulate growth, morphogenesis and differentiation of the breast epithelium are associated with the development and progression of breast cancer. Among these tyrosine kinases, ErbB-2/HER-2/Neu (Pegram et al., 1998) and Met, the Hepatocyte growth factor/Scatter factor (HGF/SF) receptor (Ghoussoub et al., 1998; Yamashita et al., 1994) are prognostic markers for breast cancer. The amplification and overexpression of ErbB-2 is associated with increased progression and metastasis of 25 per cent of human breast carcinomas and is indicative of poor prognosis in breast, ovarian, and renal collecting duct carcinomas (Pegram et al., 1998; Selli et al., 1997; Zhang et al., 1997). Consistent with this, overexpression of a wild type or constitutively activated ErbB-2/neu transgene in the mammary epithelia of mice induces the formation of metastatic mammary tumors (Muller et al., 1988). In contrast, the Met and ErbB-2 receptor tyrosine kinases are also associated with the morphogenic and functional differentiation of the normal mammary gland epithelium. Whole organ cultures and cell culture models suggest a requirement for Met for tubulogenesis of the breast epithelium and ErbB-2 for lobuloalveolar differentiation (Yang et al., 1995).

The development of invasive cancers correlates with a shift from an organized epithelia towards cells with a fibroblast-like mesenchymal phenotype, referred to as epithelial-mesenchymal transition (EMT) (Boyer et al., 1996). In some breast tumor cell lines, ErbB-2 enhances cell migration and invasion in response to neuregulins or heregulin, ligands that

indirectly activate ErbB-2 (Brandt et al., 1999; Meiners et al., 1998), whereas in others, activation of ErbB-2 promotes a remodeling of epithelial cell junctions consistent with a morphogenic response (Chausovsky et al., 1998). Hence the biological consequence of an ErbB-2 activation signal to promote epithelial cell dispersal versus reorganization, may depend on signals altered in the various tumor cells assayed. Although several studies have addressed the molecular mechanism through which this receptor transforms fibroblasts and induces tumors in transgenic animals (Dankort et al., 1997; Guy et al., 1996; Guy et al., 1992), no studies have examined the ErbB-2 initiated signal transduction events required for epithelial cell dispersal versus morphogenesis.

Activation of receptor tyrosine kinases by their ligand promotes receptor homo- or heterodimerization and transphosphorylation of the receptor on tyrosine residues that provide specific binding sites for signaling proteins (Pawson and Nash, 2000; Weiss and Schlessinger, 1998). Although this event is normally tightly regulated, receptor overexpression can promote receptor clustering and activation in the absence of ligand. To address the functional significance of specific ErbB-2/Neu initiated signals in epithelial cell dispersal and morphogenesis we have utilized a series of activated Neu receptor mutants. Mutant receptors either lack all known carboxy-terminal tyrosine phosphorylation sites, where five tyrosine residues are substituted for nonphosphorylated phenylalanine residues, or possess only one of these tyrosine phosphorylation sites in isolation (Dankort et al., 1997).

To date HGF and its receptor Met are one of the most potent inducers of EMT known. This has been extensively studied using a Madin-Darby Canine Kidney (MDCK) renal epithelial cell model, where HGF activation of the Met tyrosine kinase receptor promotes

EMT in two-dimensional culture and branching morphogenesis in three-dimensional matrix culture (Royal and Park, 1995; Weidner et al., 1993; Zhu et al., 1994). We have used the MDCK epithelial cell model for structure-function studies to examine the role of ErbB-2/Neu signals involved in epithelial cell dispersal versus a morphogenic program, and the consequence of deregulated ErbB-2 activation. We show that a constitutively activated Neu receptor induces EM-like transition of MDCK cells in two-dimensional culture but promotes a morphogenic invasive program in three-dimensional matrix culture. Using a series of activated Neu substrate binding mutants, we show that three tyrosines in the carboxyterminal tail of Neu are each sufficient to promote EM-like transition and enhanced cell motility. Two of these, involved in the phosphorylation of the Shc adapter protein, also promote a partial morphogenic program. This provides a model system to investigate ErbB-2/Neu induced signaling pathways required for epithelial cell dispersal and morphogenesis.

Materials and methods

Antibodies and reagents. Antibodies used in this paper are: mouse anti-Neu antibodies Ab-3 and Ab-4 (Oncogene Research Products, Cambridge, MA, USA), mouse anti-phosphotyrosine (4G10) (Upstate Biotechnology Inc., Lake Placid, NY, USA), mouse anti-E-cadherin and mouse anti-β-catenin (Transduction Laboratories, Mississauga, ON, USA), CY3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West-Point, DA, USA), TRITC (tetramethyl rhodamine isothio-cyanate)-labeled phalloidin and mouse anti-vinculin (Sigma, St-Louis, MO, USA) Alexa 488-labeled anti-mouse (Molecular Probes). Rabbit anti-She antibody was generously provided by Dr JM Bergeron, Department of Anatomy and Cell Biology, McGill University. Mouse anti-E-cadherin used for immunofluorescence experiments was kindly provided by Dr M Pasdar, University of Alberta. HGF was generously provided by Dr G Vande Woude, National Institutes of Health (Frederick, MD, USA). EGF, heregulins αl and βl were from Boehringer Mannheim (West Germany) and Neo-markers (Fremont, CA, USA), respectively. The MEK inhibitor (PD98059) and the PI3K inhibitor LY294002 were purchased from New England Biolabs (Nepean, ON, USA) and Biomol (Plymouth Meeting, PA, USA), respectively.

Cell culture. MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50 μg/ml gentamycin (Gibco-BRL). Neu mutants (Dankort et al., 1997)were transfected into MDCK cells by the calcium phosphate method, stable cell lines were selected in G418 (400 ug/ml) (Geneticin, Gibco-BRL). 5 x 10^3 cells were seeded in 24-well dishes (Nunc) for scatter and

immunofluorescence studies. After 48 h, HGF, LY294002 and PD98059 were added to the medium at 5 U/ ml, 20-50 uM and 20-50 uM respectively, for 24 h.

Motility assay. Cells (5 x 10^5/well) were plated on Transwell filters (Costar). Twenty-four hours later, filters were submerged in formalin phosphate buffer (Fisher) 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. Non-migrating cells were scraped off the upper layer of the filter using a cotton swab. Filters were then air-dried and photographs were taken. To quantitate the rate of migration for each cell line, filters were cut, solubilized in 10% acetic acid, and absorbance readings taken at 596 nm. The results plotted are average numbers of four experiments for each cell line.

Tubulogenesis assay. MDCK cells were suspended in a collagen matrix as described previously (Maroun et al., 1999). HGF (5 U/ml), EGF (20 ng/ml) and heregulin (20 and 100 ng/ml) were added to the medium at day 5 where applicable. Quantitation of the morphogenic response was performed as described in (Maroun et al., 1999).

Immunoprecipitation and Western blotting

Cells were seeded at 10^6/100-mm dish (Nunc). The next day, cells were washed once with DMEM and serum-starved for 24 h in DMEM containing 0.02% FBS. Cells were then washed once with PBS before they were lysed in Triton X-100 lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.5% Triton, 1 mM

phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin and aprotinin, 1 mM Na3VO4). Alternatively, RIPA lysis buffer was used (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin and aprotinin, 1 mM Na_3VO_4). Equal amounts of total protein were immunoprecipitated, separated by electrophoresis on 8% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane and immunoblotted as described previously (Maroun et al., 1999). NP-40 soluble and insoluble fractions were prepared and electrophoresed as described previously (Potempa and Ridley, 1998).

Immunofluorescence microscopy. Cells grown on glass coverslips (Bellco Glass, Vineland, NJ, USA) were fixed in 3.7% formaldehyde for 10 min and permeabilized in PBS containing 0.2% Triton X-100 and 5% FBS for 5 min at room temperature. Cells were then incubated with anti-E-cadherin (1:200) for 30 min, washed in PBS, then incubated in the presence of Alexa 488-labeled anti-mouse (1:1000) and TRITC-labeled phalloidin (1:1000) for 15 min. Phalloidin was used to visualize polymerized actin. For β-catenin and vinculin staining, cells were treated for 5 min with CSK lysis buffer (10 mM PIPES buffer, pH 7.0, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) prior to fixation. Coverslips were mounted onto slides in Immuno-Fluore medium (ICN, St-Laurent, QC), and images were photographed using a BioRad confocal microscope (Hercules, CA, USA).

Thin layer microscopy. Collagen cultures were fixed and sectioned as described earlier (Fournier et al., 1996). Photographs were taken using a digital image

processing system (Northern Eclipse, Empix Imaging Inc., Toronto, ON) at a magnification of 32x.

Results

Activated ErbB-2/Neu induces dispersal of MDCK cells. To examine the consequence of deregulated ErbB-2/Neu activation in epithelial tissues, we have generated MDCK epithelial cell lines stably expressing wild type (WTNeu) as well as mutant Neu receptors (Figure 1). A constitutively activated Neu mutant (NT) carries a point mutation in the transmembrane domain (V664E) resulting in increased receptor homodimerization and constitutive activation of the kinase domain in the absence of ligand (Bargmann et al., 1986). Mutation of the five carboxy-terminal tyrosine residues of the NT mutant into phenylalanine results in a *Neu* Tyrosine Phosphorylation-Deficient (NYPD) mutant, which is kinase active but has an impaired ability to transform Rat1 fibroblasts (Dankort et al., 1997). Add-back mutants were generated by reverting individual phenylalanine substitutions to tyrosine residues, allowing association with distinct signaling molecules (Figure 1a). The resulting mutants YB, YC, YD and YE exhibit transformation of fibroblasts to a comparable level as the activated NT receptor whereas it is believed that site A acts as a negative regulatory site for Neu (Dankort et al., 1997). All of these mutant receptors are tyrosine phosphorylated and catalytically active (Dankort et al., 1997); Figure 1b).

We show that expression of NT in MDCK cells induces morphological changes, breakdown of cellular junctions and cell dispersal, in a similar manner to HGF-stimulated cell scatter (Figure 2). The observed morphological changes are characteristic of epithelial-mesenchymal transition (EMT). In contrast, MDCK cells expressing WTNeu exhibit an epithelial morphology, comparable to the parental MDCK cell line (tight colonies), whereas the NYPD, YA and YB-expressing cells grow as colonies of flattened cells (Figure 2). In

contrast, add-back mutants YC, YD and YE induce dispersed cells that have a similar morphology, albeit less scattered, to cells expressing NT, suggesting that signaling pathways downstream from these tyrosine residues are sufficient for the NT-induced morphological changes.

Activated ErbB-2/Neu induces breakdown of adherens junctions and cytoskeletal reorganisation of MDCK epithelial cells. E-cadherin is involved in promoting and maintaining cell-cell adhesion through the formation of adherens junctions that contain *β*-catenin. Breakdown of these contacts is a prerequisite for cell dissociation. We and others have shown that HGF stimulation of MDCK cells disrupts adherens junctions and induces the redistribution of E-cadherin into a cytoplasmic soluble compartment (Royal and Park, 1995); Figure 3). To examine the consequence of Neu expression on cell-cell junctional complexes we examined the localization of E-cadherin and *β*-catenin by indirect immunohis-tochemistry. Whereas E-cadherin and *β*-catenin are localised at the membrane at cell-cell contacts in parental MDCK cells, or in cells expressing WT, NYPD, YA or YB mutants (Figure 3a and data not shown), MDCK cells expressing NT, YC, YD and YE show loss of insoluble E-cadherin staining at cell-cell contacts but retain β-catenin staining when cells are in contact. This is consistent with reduced levels of E-cadherin but not *β*-catenin in an NP40-insoluble fraction (Figure 3a, 3d and data not shown), although the steady-state levels of E-cadherin and *β*-catenin are not altered (Figure 3e and data not shown).

To establish the nature of the cytoskeletal reorganisation induced by the expression of Neu-NT, we have used indirect immunofluorescence with TRITC-labeled

phalloidin to detect polymerized actin and vinculin to visualize focal adhesion complexes. We show by immunofluorescence that NT induces the loss of cortical actin at cell-cell junctions and the formation of actin stress fibers localized in lamellipodial-like structures (Figure 3c). In addition, vinculin staining reveals the redistribution of focal adhesions from the periphery of the colony into larger complexes throughout the surface of NT-expressing cells (Figure 3c). Mutants YC, YD and YE show a similar pattern (data not shown), whereas cells expressing WTNeu resemble MDCK cells (Figure 3c). The NYPD, YA and YB mutants show increased large vinculin-containing complexes and actin stress fibers, consistent with the increased spreading of cells expressing these mutants (Figure 3c and data not shown).

Activated ErbB-2/Neu increases spontaneous motility of epithelial cells. Cell dispersal involves the disruption of cellular adhesion complexes, reorganization of the actin cytoskeleton and formation of membrane extensions. Therefore, the observed cytoskeletal changes in NT-expressing cells suggest that cells have adopted a fibroblastic-like morphology with increased motility following NT expression. To establish this we have used modified Boyden chambers where the ability of cells to migrate across the filter can be quantitated. We show that expression of NT increases spontaneous cell motility by 10-fold when compared to parental MDCK cells, and is comparable to the HGF-induced increase in MDCK cell motility (Figure 4a, 4b). These results were confirmed by time-lapse video microscopy (data not shown). Consistent with their scattered phenotype, cells expressing the YC, YD and YE mutants show enhanced cell motility (Figure 4). Although cells expressing the YA, YB

or the NYPD mutants grow as colonies and are not dispersed, when seeded as single cells into transwell chambers all cell lines exhibited increased motility when compared to control cells albeit less than cells expressing the YC, D or E mutants (Figure 4b). This suggests that an activated Neu kinase can enhance cell motility through a mechanism independent from substrate association with the YA-E tyrosine residues.

Activated ErbB-2/Neu induces branching tubulogenesis in MDCK epithelial cells. We have shown previously that the tyrosine kinase activity of the Met receptor is required to induce branching morphogenesis, and that EGF fails to induce tubulogenesis of MDCK cells cultured in a three-dimensional collagen matrix, although these cells express the EGF receptor (Maroun et al., 1999; Zhu et al., 1994); Figure 5d). Interestingly, NeuNT-expressing MDCK cells spontaneously form tubules when seeded in a collagen matrix (Figure 5a, 5b). Tubules induced by NeuNT are composed of a single layer of epithelial cells, surrounding a hollow interior (Figure 5c), and resemble HGF-induced branching tubules (Figure 5; (Fournier et al., 1996). In contrast the expression of the NYPD, YA, YB and YC mutants promoted the formation of large cysts that contain a single layer of epithelium with a hollow interior (Figure 5a,b,c and data not shown). Expression of the YD and YE mutants induced the formation of various structures, ranging from spikes to unbranched hollow tubules containing organized epithelium, but at reduced efficiency when compared with cells expressing the NT mutant (Figure 5a, 5c, Table 1). Overexpression of WTNeu did not induce a morphogenic program (Figure 5a), neither did stimulation of corresponding cells with either EGF or heregulin, a ligand for ErbB family receptors, whereas in these cells HGF induced efficient branching tubules (Figure 5d). Instead, the formation of large cysts was observed in response to heregulin when compared

to control cells (Figure 5d). These results suggest that signaling downstream from a constitutively activated ErbB-2/Neu is sufficient for the induction of a morphogenic invasive response, but that the Neu YD and YE mutants are insufficient for a full morphogenic response.

MEK dependent signaling pathways are crucial for ErbB-2/Neu induced epithelial dispersal. We and others have previously shown that the breakdown of epithelial cell-cell junctions in response to HGF requires PI3K, MEK, Ras and Rho family dependent signals (Potempa and Ridley, 1998; Royal et al., 2000; Royal and Park, 1995); Figure 6a). Pharmacological inhibitors of PI3K (LY294002, 20-50*uM)* and the MAPK Kinase, MEK (PD98059, 50*UM*) were used to establish the requirements of these pathways in the Neu-NT-induced dispersal of epithelial cells. Treatment of cells with the PI3K inhibitor, LY294002, partially reverses Neu-NT-induced cell dispersal. In the presence of LY294002 NT-expressing cells still show extensive spreading, lack cortical actin and show a partial redistribution of E-cadherin and β-catenin to cell-cell junctions, although under similar conditions LY294002 blocks HGF-induced cell dispersal (Figure 6). A similar pattern of inhibition was obtained with the YC, YD and YE mutants (data not shown). In contrast treatment of Neu-NT-expressing cells with PD98059 reverses this process. In the presence of PD98059, cells reform extensive cortical actin and show reorganization of focal adhesion complexes and relocalisation of insoluble E-cadherin and β-catenin to cell-cell junctions in a similar manner to parental MDCK cells (Figures 3a, 3b and 6b). The YB and YD mutants were previously shown to bind Grb2 and Shc, an upstream activator of Ras and MEK (Dankort et al., 1997). Immunopreci-pitation of Shc and immunoblotting with anti-phosphotyrosine sera, shows an increase in Shc phosphorylation predominantly

in NT, YD and YE-expressing cells and to a lesser extent in YC-expressing cells (Figure 7). Previous data have suggested that when compared with other receptor tyrosine kinases only the Met receptor promotes tubulogenesis of MDCK cells in collagen (Maroun et al., 1999; Sachs et al., 1996). To establish whether NT-induced scatter and tubulogenesis of MDCK cells is mediated through an enhancement of Met activation by an activated Neu receptor, we have examined Met phosphorylation. Although a baseline level of Met phosphorylation was detected, no detectable changes in Met phosphorylation levels were observed in Neu-expressing MDCK cells (Figure 8, data not shown). Moreover, conditioned medium collected from NT expressing cells was unable to induce scatter of MDCK cells (data not shown), whereas conditioned medium from MRC5 cells that secrete HGF, induced efficient cell scatter. Taken together, our data suggest that activated Neu induces tubulogenesis and cell dispersal through a Met independent mechanism.

Discussion

Epithelial-mesenchymal transition (EMT) is characterized by the conversion of epithelial cells into fibroblastlike motile cells. This occurs during specific physiological processes of embryonic and adult life and in tumor dissemination and metastasis (Boyer et al., 1996; Meiners et al., 1998). ErbB-2/Neu amplification and overexpression has been implicated in the etiology of breast and ovarian carcinomas and correlates with poor clinical prognosis (Pegram et al., 1998). Several studies have addressed the requirement for specific ErbB-2 dependent signals in cell transformation and tumorigenesis in animal models, however no studies have addressed these requirements for EMT. Here we demonstrate that activated ErbB-2/Neu promotes an EM-like transition with breakdown of cell-cell junctions, dispersal of epithelial colonies and enhanced cell motility, but promotes a morphogenic program when these cells are placed in a three-dimensional matrix. Using receptor mutants we show that three independent tyrosine autophosphorylation sites on the ErbB-2/ Neu protein, YC, YD and YE, induce an EM-like transition and cell dispersal, two of these, YD and YE, promote invasive growth but are insufficient for the full morphogenic response.

Many processes are involved in epithelial cell dispersal and cell motility. These include reorganization of the actin cytoskeleton and turnover of focal contacts, breakdown of cellular junctions to allow cell dissociation, and the formation of membrane extensions allowing cells to attach to the extracellular matrix. In a similar manner to HGF-induced EMT, we show that activated ErbB-2/Neu promotes epithelial cell dispersal. This supports observations that a chimeric Trk-ErbB-2 receptor can induce partial dispersal of MDCK

cells, although these cells were not analysed in detail (Sachs et al., 1996). By indirect immunofluorescence, cells expressing activated ErbB-2/Neu NT show loss of cortical actin and the formation of bundled actin stress fibers, as well as loss of peripheral vinculin-containing focal complexes and the appearance of large intracellular focal contacts (Figure 3). Consistent with their dispersed phenotype, extensive membrane extensions and lamellipodia are visible in NT-expressing cells (Figure 3) and these cells show enhanced cell motility in Transwell assays, comparable to cells stimulated with HGF (Figure 4). These data are similar to the cytoskeletal changes and enhanced cell motility observed following stimulation of some breast cancer cell lines with heregulin, a ligand that transactivates ErbB-2/Neu (Adam et al., 1998; Chausovsky et al., 1998; Meiners et al., 1998). However many breast tumor cell lines lack functional adherens junctions through genetic alterations that lead to loss of expression of E-cadherin (Ji et al., 1997) and a role for Her2/Neu in the breakdown of epithelial junctions had not been addressed.

Importantly, the expression of NT promotes the disruption of stable adherens junctions in MDCK cells, as shown by the loss of insoluble E-cadherin at cell-cell junctions and a redistribution of insoluble β-catenin in NT-expressing cells (Figure 3). This is similar to HGF stimulation of MDCK cells, which induces the relocalization of E-cadherin into a cytoplasmic soluble compartment (Potempa and Ridley, 1998; Royal and Park, 1995). However, in contrast to (D'Souza and Taylor-Papadimitriou, 1994)), who reported the transcriptional downmodulation of E-cadherin in an immortalized human mammary epithelial cell line overexpressing wild type ErbB-2, the steady-state levels of E-cadherin and β-catenin remain unaltered following expression of activated ErbB-2/ Neu in MDCK cells (Figure 3e). Moreover, when NeuNT-expressing cells are in contact, E-cadherin and β-

catenin localize at cell-cell contacts in insoluble complexes indicating that junctional complexes can form and that an irreversible epithelial -mesenchymal transition has not occurred (Figure 3a, 3b).

Our structure - function analyses using add-back mutants of ErbB-2/Neu demonstrated that the YC, YD or YE sites were each sufficient to promote EM-like transition as well as enhanced cell motility (Figures 2 and 4). The YD and YE sites were previously shown to interact with the Shc adapter protein (Dankort et al., 1997; Ricci et al., 1995). Consistent with this, Shc phosphorylation was elevated in cells expressing the activated NT or the YD or YE mutants and to a lesser extent in cells expressing the YC mutant, but was not elevated in cells expressing mutants that fail to induce cell dispersal (YA and YB, Figures 2 and 7). Similarly the recruitment of Shc to the Met/HGF receptor is required for full cell dispersal in response to HGF (Fournier et al., 1996)and Shc overexpression enhances motility of MDCK cells (Pelicci et al., 1995). Moreover, using pharmacological inhibitors, we demonstrated that MEK activation is required for the maintenance of Neu-induced cell dispersal and related cytoskeletal and junctional alterations (Figure 6). Importantly, treatment of cells with a MEK inhibitor (PD98059), reverses the phenotypic EM-like transition induced by activated Neu, and promotes the reformation of cortical actin and the redistribution of E-cadherin and β-catenin to cellular junctions (Figure 6b). This is consistent with a requirement for MEK activity for HGF-induced breakdown of epithelial cell junctions (Potempa and Ridley, 1998), but indicates in addition that MEK activity is required to maintain loss of E-cadherin dependent junctional complexes. Importantly, many breast cancers exhibit elevated activity of ERK 1 and 2, downstream targets of MEK, and role for MAPK was implicated in cell motility through

its ability to phosphorylate myosin light chain kinase and enhance cell contractility (Klemke et al., 1997). The Neu YB add-back mutant, which recruits Grb2, and was shown to promote transformation of fibro-blasts in a Ras-dependent manner (Dankort et al., 1997) is unable to promote the breakdown of cell-cell junctions, although it enhances cell motility when a single cell suspension is seeded in a Transwell migration assay (Figures 2 and 4). This may reflect that signals from Shc, distinct from Grb2 (Rozakis-Adcock et al., 1992) are important for breakdown of cell junctions or that additional unknown signaling proteins are recruited to the YD, YE or YC sites.

In contrast to the MEK inhibitor, the PI3K inhibitor, LY294002, does not promote a reversal of the EMT-like phenotype of NT-expressing cells (Figure 6). Although treatment of NT-expressing cells with LY294002 inhibited the formation of membrane extensions, cells failed to form extensive E-cadherin containing cell-cell junctions or cortical actin (Figure 6). Hence, although PI3K activity is required for the breakdown of cell-cell junctions in response to HGF (Khwaja et al., 1998; Potempa and Ridley, 1998; Royal et al., 2000; Royal and Park, 1995), once epithelial cells are dispersed in response to activated Neu, a PI3K-dependent signal is not required to inhibit junction formation. However a PI3K-dependent signal is required for the formation of lamellipodia in the dispersed NT-expressing cells. This is consistent with a requirement for PI3K activity for the activation of the small GTPase Rac and for PAK1, a direct target for activated Rac, both of which are involved in HGF-induced lamellipodia formation (Royal et al., 2000). (Adam et al., 1998) have shown that PAK1 is activated in a PI3K-dependent manner downstream of ErbB-2 in MCF7 cells, suggesting a role for PAK in the cytoskeletal actin reorganization and increased cell motility.

Whereas MDCK cells expressing activated Neu scatter in two-dimensional culture, when seeded in a collagen matrix these cells undergo an invasive morphogenic program and form tubule-like structures with organised epithelia and a lumen in a similar manner to HGF-stimulated MDCK cells (Figure 5). Cells expressing the YD and YE mutants that couple with and/or induce phosphorylation of Shc, form some tubular structures, however many dissociated cells are observed, consistent with loss of epithelial organization and invasion of single cells into the surrounding matrix (Figure 5). This may reflect the inability of the YD and YE mutants to activate Neu-dependent signaling pathways required for a full morphogenic response. Consistent with this, transgenic mice expressing the Shc binding NeuYD receptor to mammary epithelium, induce mammary tumors with rapid onset (Dankort and Muller, unpublished). However, transgenic mice expressing elevated levels of Shc in the mammary epithelium, exhibit extensive branching and alveolar development of the breast epithelium (Rauh et al., 1999), which supports a role for Shc dependent signals in epithelial morphogenesis. In addition, in MDCK cells and in murine 'knock in' models, Grb2-dependent signaling pathways are required for a morphogenic response downstream from the Met receptor (Fournier et al., 1996; Maina et al., 1996; Maroun et al., 1999). Interestingly the YB mutant, which couples with Grb2, is insufficient to induce an invasive morphogenic response required for the formation of branching tubules, and instead maintains an organized epithelium and promotes cyst expansion, consistent with its inability to promote dispersal of epithelia in two-dimensional cultures (Figure 2 and 5).

To date Met was the only receptor capable of inducing a branching tubulogenic program in MDCK cells, and previous studies using a chimeric Trk-ErbB2 receptor failed to induce tubulogenesis in response to stimulation (Sachs et al., 1996). This may reflect the

difference of a steady-state signal rather than a transient signal, where a steady-state signal initiated by a constitutively activated Neu initiates an invasive morphogenic response required for tubulogenesis, rather than cyst expansion. In this regard, EGF stimulation of MDCK cells induces a transient signal and cyst formation, whereas HGF promotes a sustained signal and an invasive morphogenic response (Figure 5 and (Maroun et al., 1999). Consistent with this, heregulin is unable to promote tubule formation in MDCK cells overexpressing WTNeu (Figure 5), but rather promotes an enlargement of the cyst in a similar manner to cells expressing the YB mutant. This may be consistent with the ability of heregulin to promote alveologenesis in breast epithelium (Niemann et al., 1998; Yang et al., 1995) and is not inconsistent with a requirement for Grb2-dependent signals for a morphogenic response. In this respect the observation that an activated Neu receptor promotes a tubulogenic response whereas the YD and YE mutants promote a more invasive response may reflect the possibility that the YB-Grb2 binding site may enhance or stabilize a morphogenic response induced by the YD or YE add-back mutants. Alternatively, an NT receptor may also recruit negative regulatory proteins that modulate the signal in response to receptor activation. For example Cbl, a ubiquitin ligase targets ErbB-2 to a ubiquitin/degradation pathway (Klapper et al., 2000). Together these data demonstrate that a constitutive ErbB-2/Neu signal is sufficient to induce the breakdown of epithelial junctions and cell dispersal of highly differentiated and nontumorigenic epithelial cells supporting a role for ErbB2 in the epithelial mesenchymal transition and dispersal of human cancers

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Figure 1

a) Schematic representation of ErbB2/Neu receptor tyrosine kinase mutants. The Neu receptor contains two cysteine-rich domains (dark striped box), a transmembrane domain (solid box), and a tyrosine kinase domain (striped box). Carboxy-terminal tyrosine residues A, B, C, D and E represent autophosphorylation sites. Wild type (WTNeu), activated (NT) and tyrosine phosphorylation deficient (NYPD) ErbB2/Neu receptors are illustrated.

b) Stable expression of Neu mutant receptors in MDCK cells. Neu protein was immunoprecipitated from RIPA cell lysates (500μg of protein) with mouse Ab-4. Proteins were separated by SDS-PAGE (8%) and immunoblotted with Ab-3 (anti-Neu) or 4G10 (anti-phosphotyrosine) antibodies.

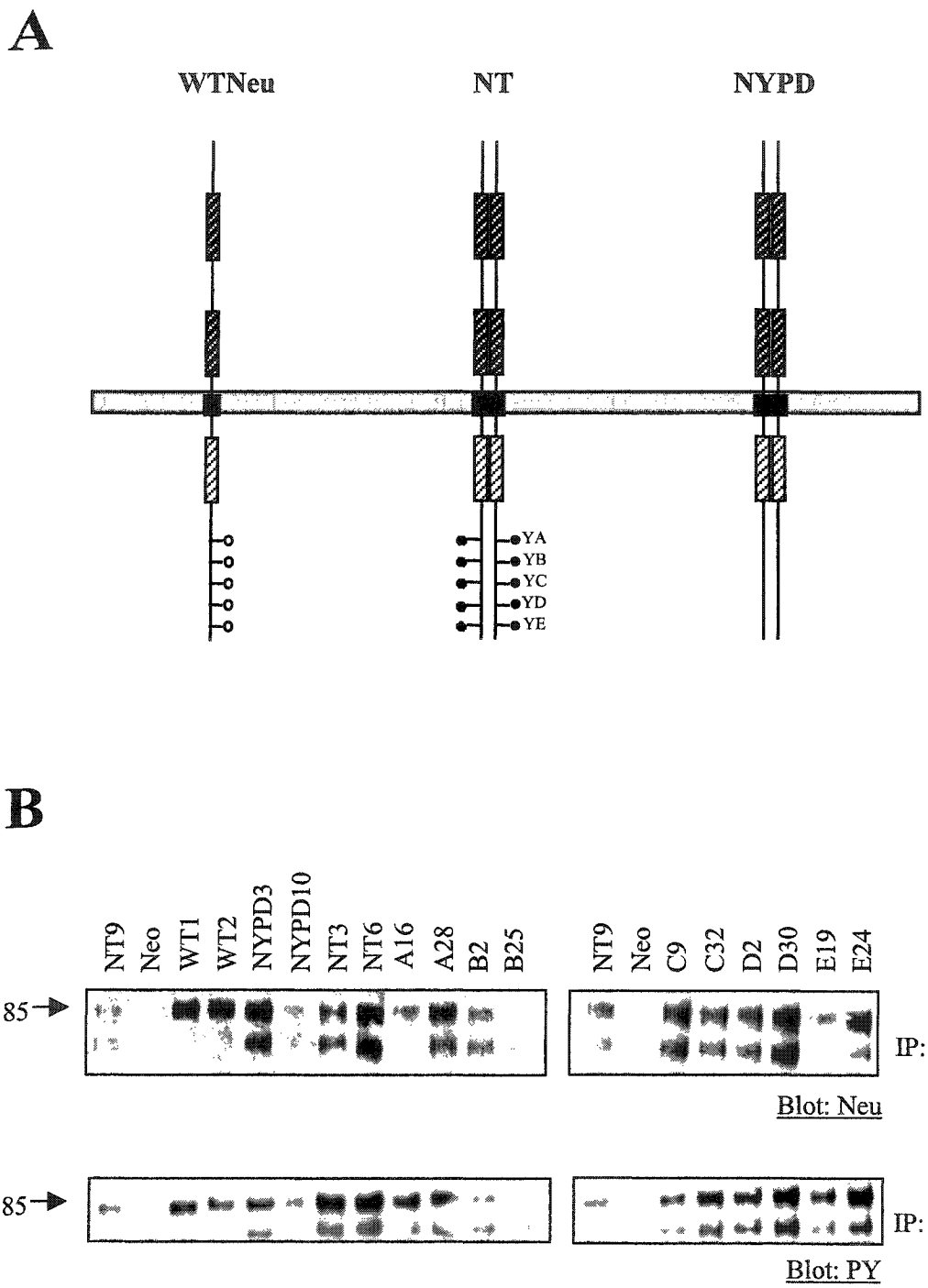

A
WTNeu
NT
NYPD
YA
YB
YC
YD
YE
B
NT9
Neo
WT1
WT2
NYPD3
NYPD10
NT3
NT6
A16
A28
B2
B25
NT9
Neo
C9
C32
D2
D30
E19
E24
p185
IP: Neu
Blot: Neu
p185
IP: Neu
Blot: PY

Figure 1

Figure 2 Activated ErbB2/Neu induces MDCK cell dispersal. Stable lines and MDCK cells expressing Neu add-back mutants were grown in DMEM with 10% FBS. Representative clones were photographed using the Northern Eclipse image processing system (10x magnification)

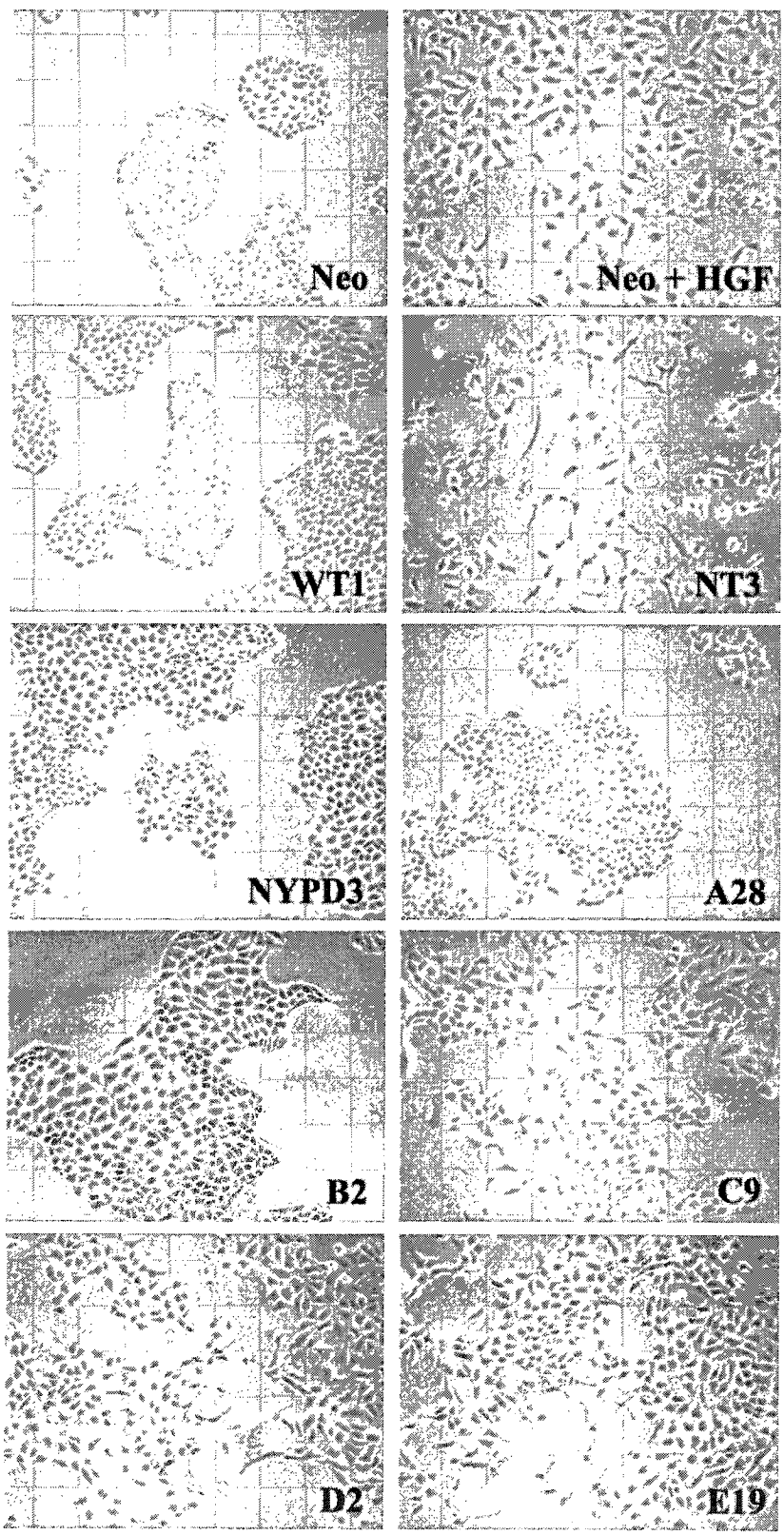


Figure 2

Figure 3 Activated ErbB2/Neu induces loss of adherens junctions and reorganization of the actin cytoskeleton and focal complexes. a) MDCK cells and cells expressing Neu were grown on glass coverslips, fixed and stained for E-cadherin and actin (TRITC-phalloidin) as described in Materials and methods. Pictures were taken on a confocal microscope at a magnification of 63x. **b)** Cells treated with CSK lysis buffer were labeled with anti-β-catenin in the presence of TRITC-phalloidin. **c)** Cells treated with CSK lysis buffer were labeled with anti-vinculin in the presence of TRITC-phalloidin. **d)** Proteins from equal volumes of NP40-soluble (S) and insoluble (I) cell fractions were separated by SDS ± PAGE, transferred to a nitrocellulose membrane and probed with anti-E-cadherin or anti-b-catenin. Control MDCK cells were stimulated with HGF (3rd and 4th lanes) for 24 h. **e)** Stable lines of MDCK cells were lysed and 50 mg of whole cell lysate were separated on an 8% SDS± PAGE, transferred to a nitrocellulose membrane and probed with anti-E-cadherin or anti-b-catenin antibodies.

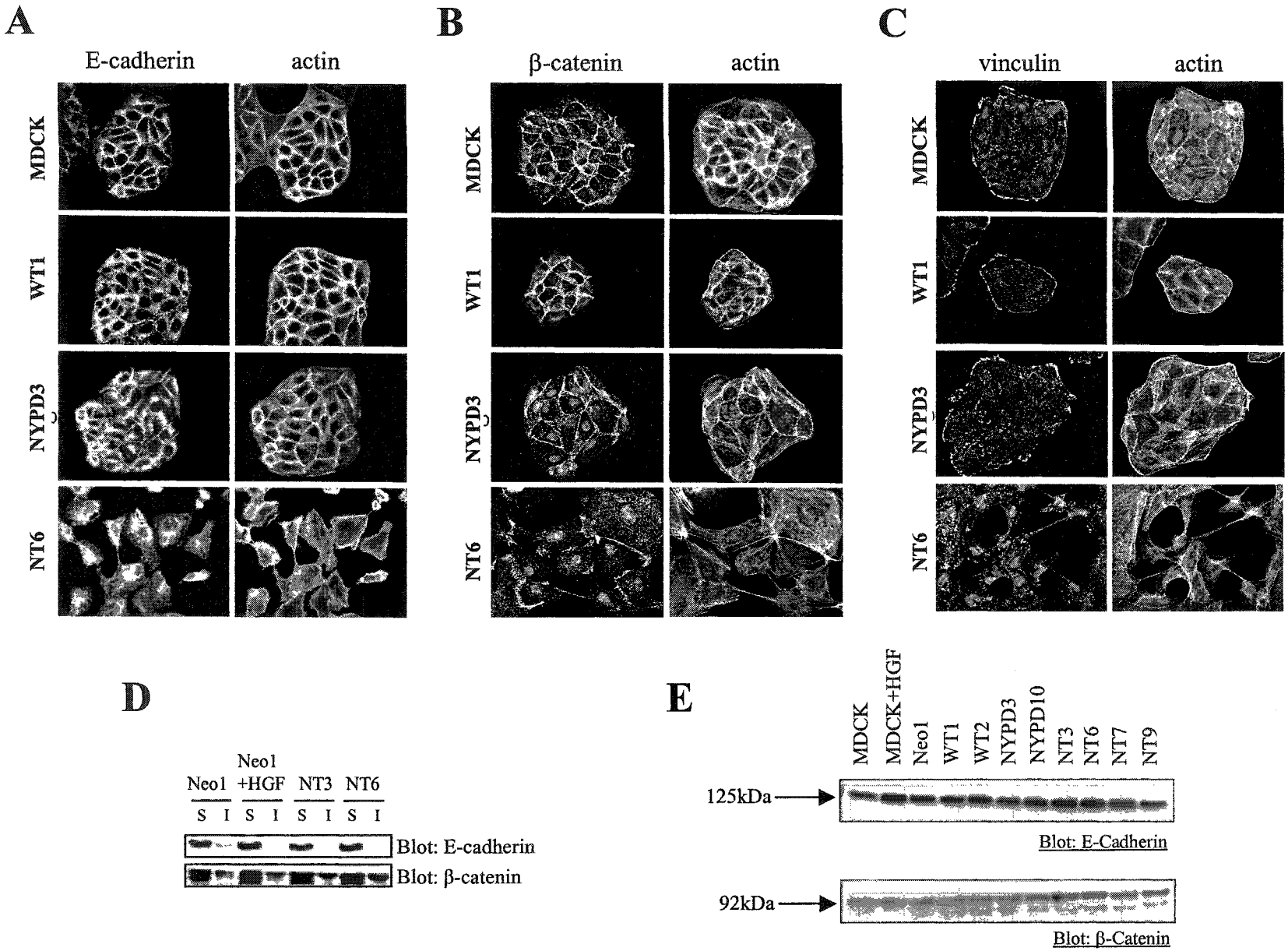
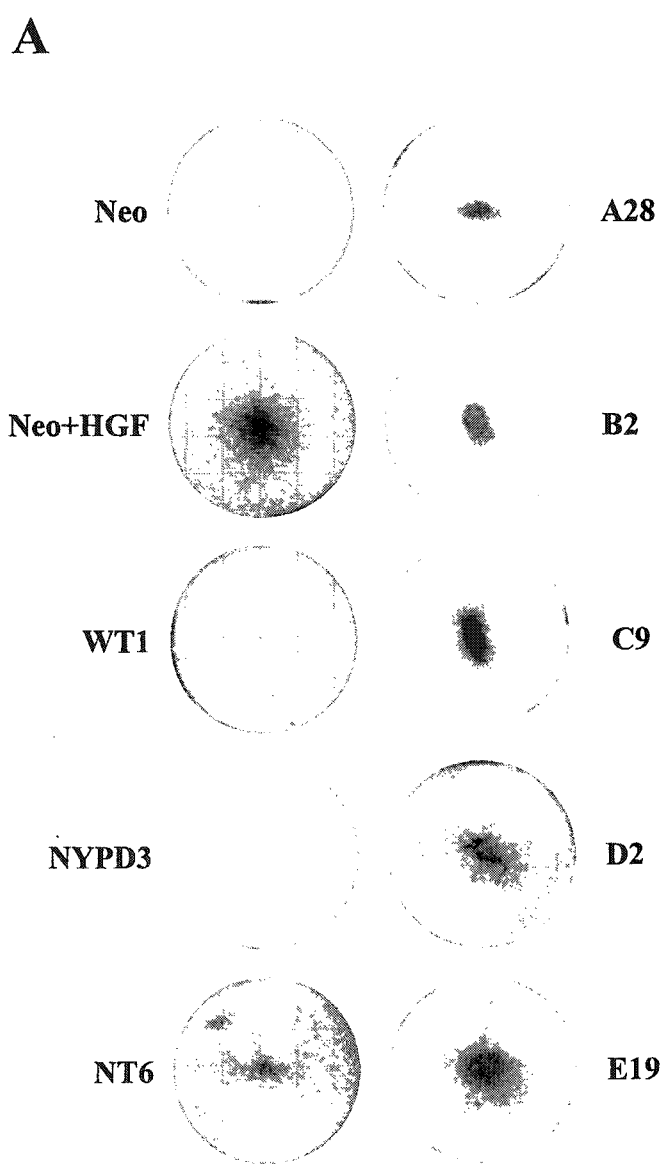

A
E-cadherin
actin
MDCK
WT1
NYPD3
NT6
B
β-catenin
actin
MDCK
WT1
NYPD3
NT6
C
vinculin
actin
MDCK
WT1
NYPD3
NT6
D
Neo1
Neo1 +HGF NT3 NT6
S I S I S I S I
Blot: E-cadherin
Blot: β-catenin
E
MDCK
MDCK+HGF
Neo1
WT1
WT2
NYPD3
NYPD10
NT3
NT6
NT7
NT9
125kDa
Blot: E-Cadherin
92kDa
Blot: β-Catenin

Figure 3

Figure 4 Activated ErbB-2/Neu increases spontaneous MDCK cell motility. Motility assays were performed using Transwell filters, as described in Materials and methods. **a)** Representative photographs of crystal violet-stained filters. **b)** Filters were solubilized in 10% acetic acid, the migration rate of Neu mutant-expressing cells was determined by measuring the absorbance at a wavelength of 596 nm. Each value represents the average of four filters.


A
Neo
A28
Neo+HGF
B2
WT1
C9
NYPD3
D2
NT6
E19

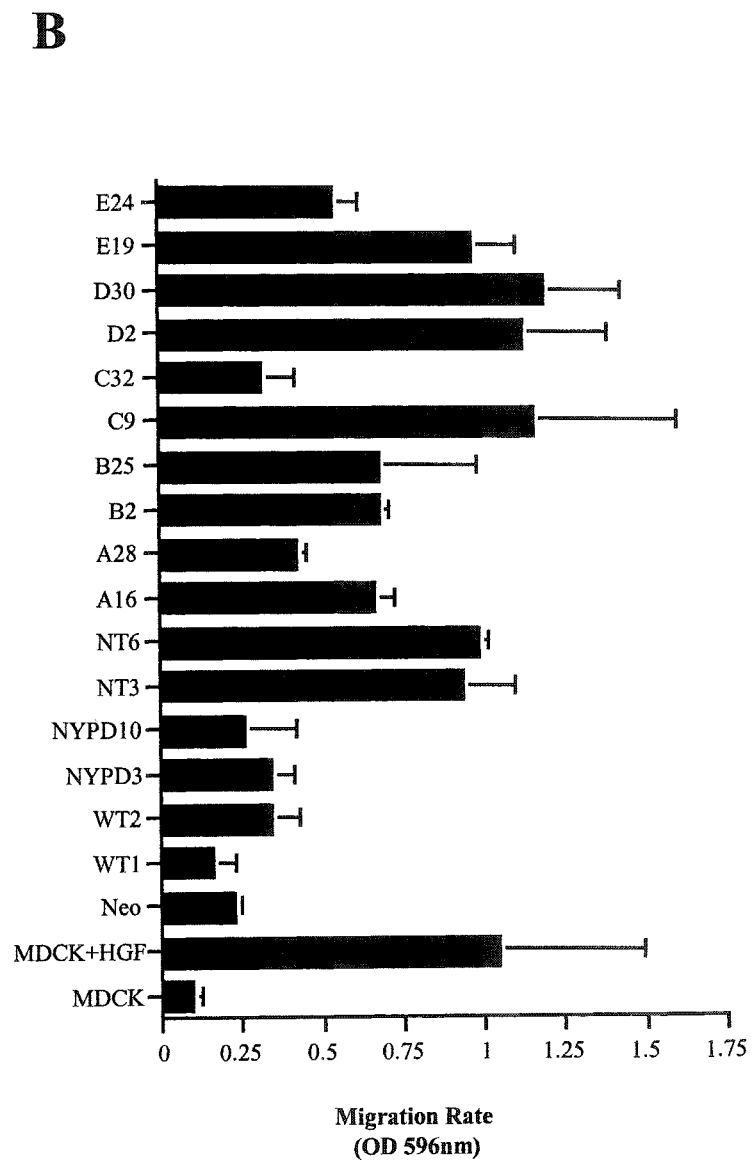

B
E24
E19
D30
D2
C32
C9
B25
B2
A28
A16
NT6
NT3
NYPD10
NYPD3
WT2
WT1
Neo
MDCK+HGF
MDCK
0
0.25
0.5
0.75
1
1.25
1.5
1.75
Migration Rate
(OD 596nm)

Figure 4

Figure 5 Activated ErbB-2/Neu induces branching tubulogenesis of MDCK cells in collagen. **a)** Stable lines of MDCK cells were grown in collagen I. Structures were allowed to develop and representative pictures were taken at a magnification of 10x. **b)** Quantitation of the morphogenic response to Neu expression in MDCK cells. Columns represent cysts (solid), tubules (blank) 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)** Thin layer microscopy of tubules (NT, YD, YE) and cysts (Neo, NYPD). Picture magnification: 32x. **d)** MDCK cells grown in a collagen matrix were stimulated with EGF and heregulin α1 at 20 ng/ml and 100 ng/ml respectively. Picture magnification: 10x. *`Tubules' are structures whose length is five times their diameter. `Structures' represents any structure that does not belong to the `Cysts' or `Tubules' categories, it includes spikes, unbranched tubules, protrusions as well as dispersed structures

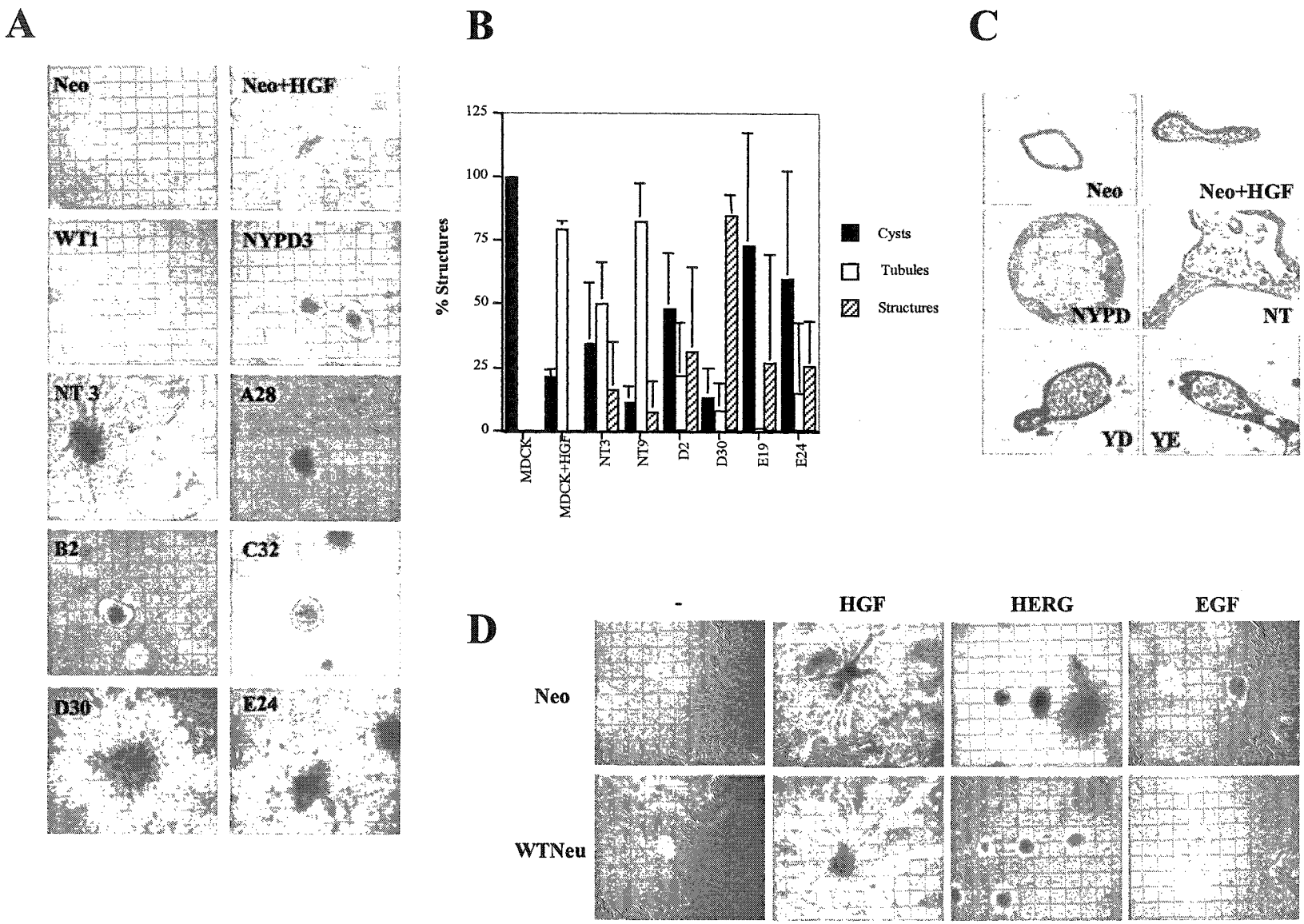

A
Neo
Neo+HGF
WT1
NYPD3
NT 3
A28
B2
C32
D30
E24
B
% Structures
125
100
75
50
25
0
MDCK
MDCK+HGF
NT3
NT9
D2
D30
E19
E24
Cysts
Tubules
Structures
C
Neo
Neo+HGF
NYPD
NT
YD
YE
D
-
HGF
HERG
EGF
Neo
WTNeu

Figure 5

Table 1 Quantitation of the morphogenic response to ErbB-2/Neu expression in MDCK cells. Each value represents the mean average from at least four different assays. Numbers in brackets represent standard deviation values.

	Cysts	*Tubules*	*Various structures*
Neo	100 (0)	0(0)	0(0)
Neo + HGF	4.4 (5.7)	15.2(24.2)	80.4(21.9)
WT1	100 (0)	0(0)	0(0)
NYPD3	100 (0)	0(0)	0(0)
NT3	29.3(21.1)	43.7 (17)	27.2 (25.8)
NT6	14(11.5)	51.6(28.4)	30.6 (35.9)
NT9	7.8 (7)	64 (34.8)	27.8 (38.8)
A16	100 (0)	0(0)	0(0)
A28	100 (0)	0(0)	0(0)
B2	99.8 (0.4)	0 (0.2)	0.2 (0.4)
B25	100 (0)	0(0)	0(0)
C9	97.3 (30.5)	11.6(11.5)	2.3 (5.6)
C32	100 (0)	0(0)	0(0)
D2	56.8 (20.5)	15 (20.1)	34.6 (29.4)
D30	30.2 (29.8)	5.2 (12.4)	67.7 (28)
E19	65.3 (48)	0.3(18)	18.2 (35.7)
E24	70.7 (36.9)	9.3 (23.3)	19.8 (17.7)

'Tubules' are structures whose length is five times their diameter. 'Various structures' include spikes, unbranched tubules, protrusions as well as dispersed cells or any structure that is not a cyst or a tubule.

Figure 6 NT-induced cell scatter is blocked by the MEK inhibitor. a) Cells were grown in 24-well dishes. HGF, LY294002 and PD98059 were added to the medium at 5 U/ml, 50 uM and 50 uM, respectively, overnight. Picture magnification, 10x. **b)** NT-induced cytoskeleton reorganization and alteration of adherens junctions are reversed by the MEK inhibitor, PD98059 (50 uM). Cells were grown on glass coverslips and treated as in a. Confocal picture magnification, 63x.

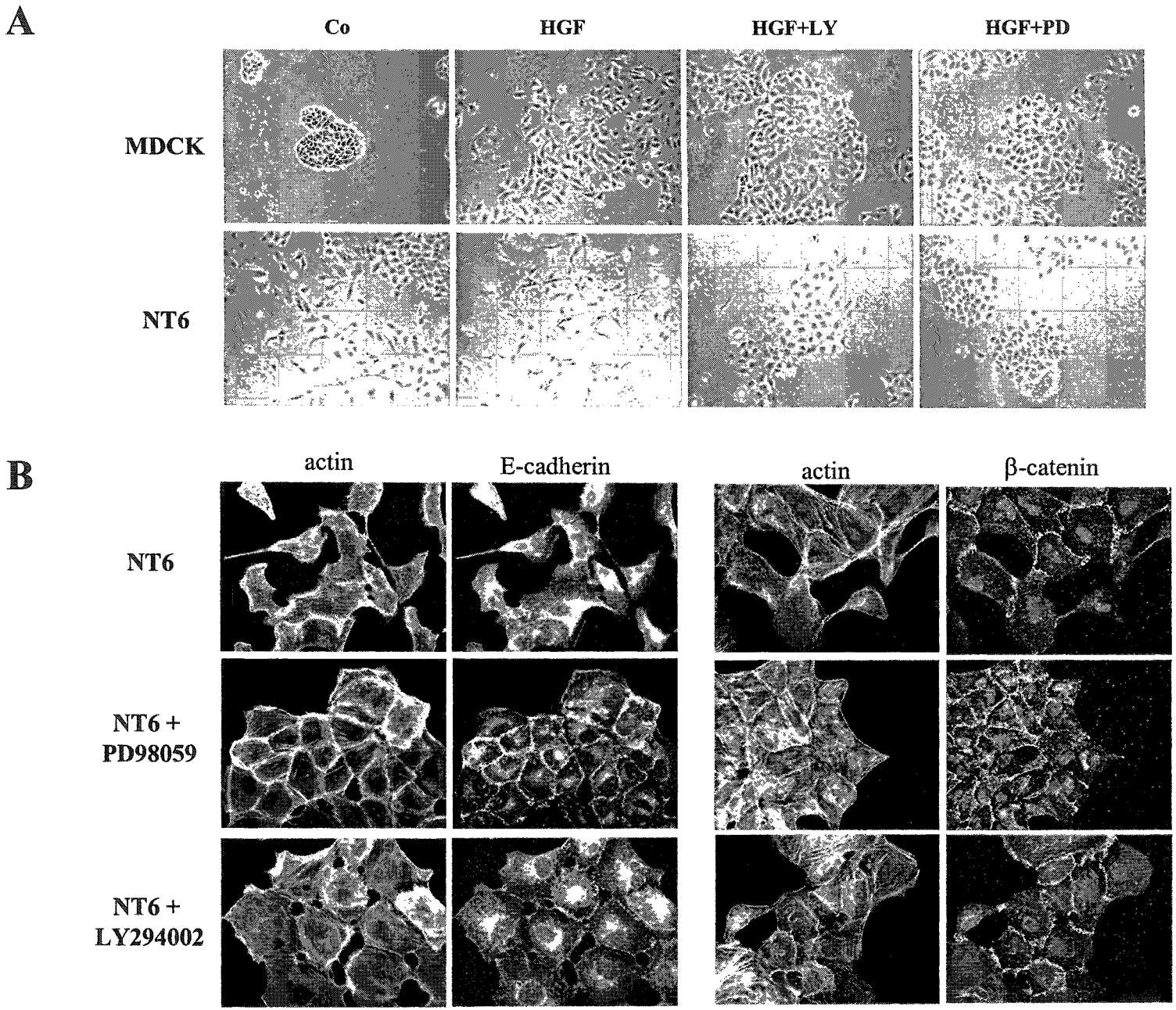

A
Co
HGF
HGF+LY
HGF+PD
MDCK
NT6
B
actin
E-cadherin
actin
β-catenin
NT6
NT6 +
PD98059
NT6 +
LY294002

Figure 6

Figure 7 Neu-induced cell scatter and tubulogenesis correlate with increased phosphorylation of the Shc adaptor protein. Proteins were immunoprecipitated from Triton X-100 cell lysates (1 mg) with rabbit anti-Shc, separated on a 10% SDS-poly-acrylamide gel and immunoblotted with anti-phosphorytosine (4G10), stripped then reprobed with anti-Shc.

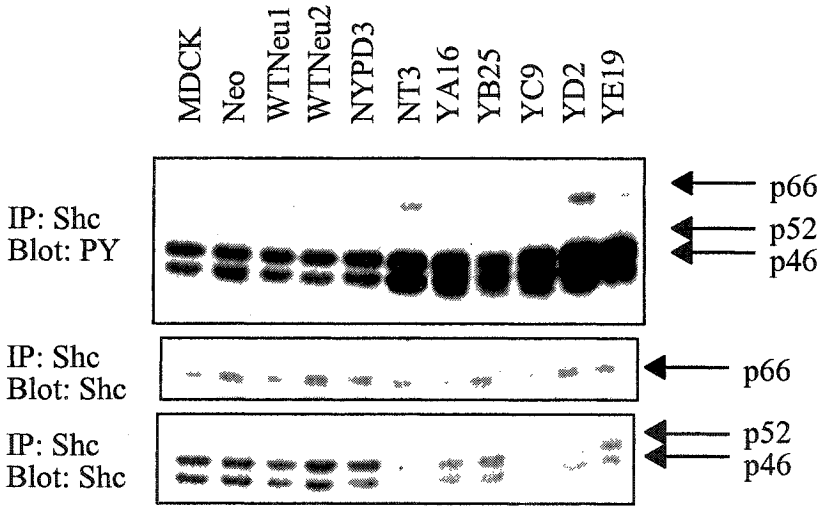

MDCK
Neo
WTNeu1
WTNeu2
NYPD3
NT3
YA16
YB25
YC9
YD2
YE19
IP: Shc
Blot: PY
p66
p52
p46
IP: Shc
Blot: Shc
p66
IP: Shc
Blot: Shc
p52
p46

Figure 7

Figure 8 ErbB2/Neu expression does not induce phosphorylation of the Met receptor in MDCK cells. Triton X-100 cell lysates (1 mg) were immunoprecipitated by various antibodies as indicated and the proteins separated on an 8% SDS-polyacrylamide gel. Following transfer to nitrocellulose, membranes were probed with anti-phosphotyrosine (4G10), anti-Neu (Ab-3) or rabbit anti-Met (Ab144).

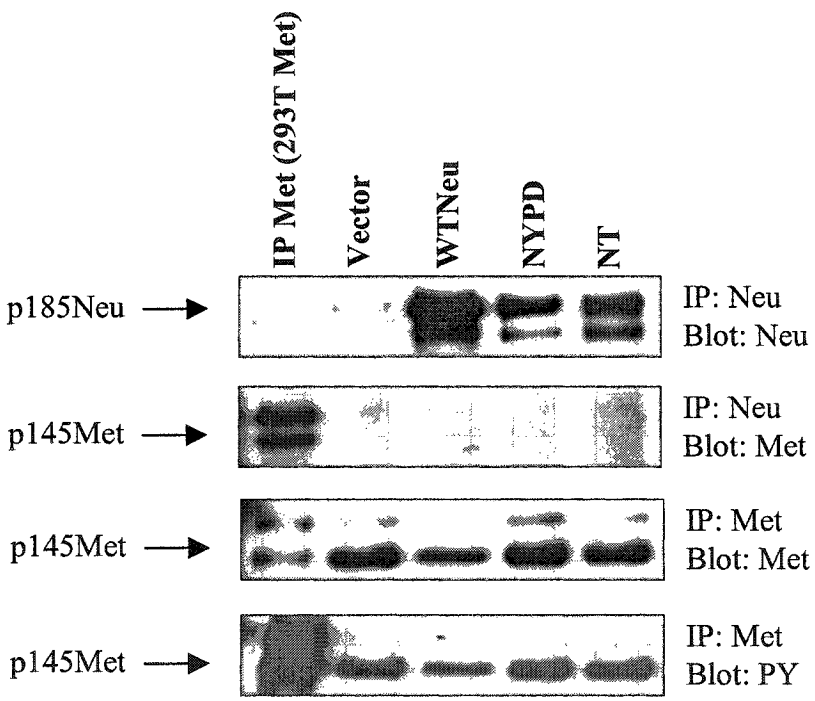

IP Met (293T Met)
Vector
WTNeu
NYPD
NT
p185Neu
IP: Neu
Blot: Neu
p145Met
IP: Neu
Blot: Met
p145Met
IP: Met
Blot: Met
p145Met
IP: Met
Blot: PY

Figure 8

Chapter III: Invasive morphogenesis downstream from a constitutively activated ErbB-2/Neu correlates with increased phosphorylation of the Gab1 docking protein and Grb2-dependent recruitment of Gab1.

Preface

The Gab1 docking protein is a common substrate for receptor tyrosine kinases and interleukins. It is thought to be recruited to the activated receptors through the Grb2 adapter protein. In addition, Gab1 can be recruited to the HGF receptor Met directly, through its Met binding site. This unique mode of interaction is thought to be essential but not sufficient for Met-mediated morphogenesis in MDCK epithelial cells. In addition, the Met receptor promotes prolonged phosphorylation of Gab1 in this cell system whereas the EGF receptor induces transient phosphorylation kinetics of Gab1 and recruits Gab1 in a Grb2-dependent manner. Consequently, EGF fails to induce a morphogenic response in MDCK cells, implicating a requirement for a sustained phosphorylation of Gab1 for the morphogenic response.

Until recently, Met was the only receptor capable of triggering the inherent morphogenic program of MDCK cells. We have shown in the previous chapter that deregulated ErbB2/neu induces a similar response, while the wild type receptor fails to do so. We performed this study to assess the involvement of Gab1 in deregulated ErbB2/neu-induced morphogenesis.

Abstract

Tight control of cell proliferation and morphogenesis is required to ensure normal tissue patterning and prevent cancer formation. Overexpression and deregulation of the ErbB2/Neu receptor tyrosine kinase is associated with increased progression in human breast cancer, yet in breast explant cultures, the ErbB2/Neu receptor contributes to alveolar differentiation. We have previously shown that a constitutively activated ErbB2/Neu (NT) receptor but not the wild type (WTNeu) receptor promotes an invasive morphogenic program in MDCK cells in a three-dimensional matrix. Previous studies in our lab have indicated that downstream from the Met receptor, invasive morphogenesis correlates with a sustained phosphorylation of the Gab1 docking protein and of downstream kinase (Erk), whereas a transient phosphorylation of Gab1 and Erk induced by EGF is not sufficient to promote a mophogenic response. We show that cell lines expressing activated ErbB2/Neu but not WTNeu display elevated levels of Gab1 and Erk phosphorylation which correlates with their ability to form an invasive morphogenic response. Using a pharmacological inhibitor to MEK, an upstream regulator of Erk, demonstrates a requirement for Erk activity for the morphogenic response. In addition, we report evidence for Gab1 recruitment to ErbB2/Neu in a Grb2-dependent as well as Grb2 independent manner. This provides a model system to dissect ErbB2-dependent signaling pathways that contribute to increased invasiveness of organised epithelial cells.

Introduction

Receptor tyrosine kinases (RTK) regulate diverse biological processes including epithelial cell differentiation and proliferation. Following ligand-induced dimerization and phosphorylation on tyrosine residues, RTKs recruit signaling molecules and activate downstream signaling pathways, resulting in changes in gene expression (Pawson and Nash, 2000; Weiss and Schlessinger, 1998). The biological outcome downstream from each receptor is dictated by the repertoire of signaling molecules recruited to the receptor. Upon recruitment, these molecules become phosphorylated on tyrosine residues providing binding sites for multiple proteins involved in signal transduction, allowing the assembly of multiprotein complexes and consequent signal diversification downstream from RTKs.

Gab1 (Grb2-associated binder 1) is a member of the IRS-1 multisubstrate docking protein family which includes IRS-1, IRS-2, p62dok and DOS (Carpino et al., 1997; Yenush and White, 1997). Gab1 contains several tyrosine residues that may be potential binding sites for SH2 and PTB domain containing proteins, as well as several proline-rich regions which could interact with SH3 domain-containing proteins such as Grb2 (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997). In addition, Gab1 contains an amino-terminal pleckstrin homology (PH) domain, involved in membrane phospholipid binding and recruitment of Gab1 to the membrane (Maroun et al., 1999). Gab1 was identified as the major phosphorylated protein in MDCK epithelial cells that undergo a morphogenic response following HGF stimulation (Fixman et al., 1997;

Nguyen et al., 1997). In these cells, Gab1 recruits p85-PI3K, PLCγ, SHP2 and the Crk adaptor to Met in response to HGF (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Lamorte et al., 2000; Lamorte et al., 2002; Maroun et al., 1999; Maroun et al., 2000) .

In addition to Met, Gab1 is phosphorylated downstream from the EGFR, the insulin receptor, the TrkA receptor, as well as interleukins (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Takahashi-Tezuka et al., 1998). The mechanism of recruitment of Gab1 to these receptors is not well known. It is believed that EGFR recruits Gab1 indirectly through Grb2. We have reported an additional mechanism for the Met receptor, where Gab1 is recruited both indirectly through Grb2 and directly to tyrosine 1349 of Met via the Gab1 proline-rich Met binding domain (MBD) (Lock et al., 2000).

HER2/Neu, also called ErbB2, is a member of the EGF receptor (EGFR) family which includes EGFR (ErbB1), ErbB3 and ErbB4. A ligand for ErbB2/Neu has not been identified yet, the receptor is activated through heterodimerization with other EGFR family members following stimulation with their corresponding ligands such as EGF and heregulin. EGF and TGFα bind to the EGFR; betacellulin, heregulin or NDF are ligands for ErbB3 and ErbB4. ErbB2/Neu is the preferred heterodimerization partner for all the EGFR family members (Graus-Porta et al., 1997; Muthuswamy et al., 1999; Pinkas-Kramarski et al., 1996; Qian et al., 1999), it has been shown to potentiate signaling downstream from these receptors by preventing receptor degradation and increasing

recycling to the membrane (Wang et al., 1999; Waterman et al., 1998; Worthylake et al., 1999). Structure/function studies revealed that ErbB2/Neu is able to recruit several signaling molecules including Grb2, Shc, Crk, Nck, Dok-R, in addition to PLCγ and Src (Dankort et al., 2001a; Dankort et al., 1997). Grb2 and Shc mediate ErbB2/Neu-induced cell transformation and tumor formation (Dankort et al., 2001b; Dankort et al., 1997). Recently, a role for Gab1 downstream from ErbB2/Neu has been proposed using Gab1-/- fibroblasts (Yamasaki et al., 2003). It has been shown that ErbB2-induced colony formation in soft agar and tumor formation in mice was impaired in Gab1-/- fibroblasts (Yamasaki et al., 2003).

ErbB2/Neu signaling mediates the growth and differentiation of a variety of cultured cells and contributes to the proper development of cardiac and neural tissues, as well as differentiation of the breast epithelium. It is also involved in neuronal migration. Deregulation of ErbB2/Neu by receptor amplification/overexpression has been reported in many cancer types including human breast cancer, where HER2/ErbB2 overexpression correlates with poor prognosis in 25% of breast carcinomas. Consistent with this, overexpression of ErbB2/Neu in the mammary epithelia of transgenic mice induces metastatic mammary tumors (Muller et al., 1988). We have shown previously that constitutively activated ErbB2/Neu promotes epithelial cell dispersal in a PI3K and MEK-dependent manner (Khoury et al., 2001). In addition, we reported that activated ErbB2/Neu induces invasive morphogenesis in collagen, correlating with increased phosphorylation of the Shc adaptor protein (Khoury et al., 2001), while transactivation of wild type ErbB2/Neu by heregulin or EGF induced cyst enlargement. To date, Met was

the only receptor capable of promoting invasive morphogenesis in normal MDCK kidney epithelial cells in collagen (Maroun et al., 1999; Sachs et al., 1996). Met, the HGF/SF (Hepatocyte Growth Factor/Scatter Factor) receptor, initiates the inherent morphogenic programs of kidney, breast and lung epithelium in matrix culture (Brinkmann et al., 1995). Other receptors such as EGFR and a chimeric TrkA-Neu receptor fail to induce morphogenesis in MDCK cells (Sachs et al., 1996). We have attributed this difference to the duration of the signal downstream from each receptor, Met induces a prolonged and sustained phosphorylation of Gab1 and downstream kinases Erk1/2, whereas EGF induces transient phosphorylation of these proteins (Maroun et al., 1999).

In the present study, we investigated the phosphorylation kinetics of Gab1 and MAPK downstream from heregulin and constitutively activated ErbB2/Neu. Moreover, we have studied the interaction of Gab1 with ErbB2/Neu and its requirements.

Materials and Methods

Antibodies and reagents. Antibodies used in this paper are: mouse anti-Neu antibodies Ab-3 and Ab-4 (Oncogene Research Products, Cambridge, MA), mouse anti-phosphotyrosine (4G10) (Upstate Biotechnology Inc., Lake Placid, NY), anti-phosphotyrosine (RC20H) (Transduction Laboratories, Mississauga, ON), P-MAPK (New England Biolabs, Nepean, ON), total MAPK (generously provided by Dr J. Blenis, Harvard, Boston), HA.11. HGF was generously provided by Dr G. Van de Woude, National Institutes of Health, Frederick, Maryland. EGF and heregulin β1 were from Boehringer Mannheim (West Germany) and Neomarkers (Fremont, CA), respectively. The MEK inhibitor (UO126) was purchased from Promega.

Cell culture. MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50μg/ml gentamycin (Gibco BRL). ErbB2/Neu mutants (Dankort et al., 1997) were transfected into MDCK cells by the calcium phosphate method, stable cell lines were selected in G418 (400μg/ml) (Geneticin, Gibco BRL). Transient transfections in 293T fibroblasts were performed using the calcium phosphate method.

Tubulogenesis assay. MDCK cells were suspended in a collagen matrix as described previously (Maroun et al., 1999). HGF (5U/ml), EGF (20ng/ml) or β-heregulin (100 ng/ml) were added to the cultures for ten days, then fixed and pictures taken. Where indicated, UO126 (20uM) was added to the medium as indicated.

Immunoprecipitation and Western blotting. Cells were seeded at 10^6/100-mm dish (Nunc). The next day, cells were washed once with DMEM and serum-starved for 48h in

DMEM containing 0.1% FBS. Growth factors (HGF 5U/ml, EGF 100ng/ml and heregulin 1nM) were then added to the cells and incubated for the indicated times, cells were then lysed in Triton X-100 lysis buffer (50mM Hepes pH 7.4, 150mM NaCl, 10% glycerol, 0.5% Triton, 1mM phenylmethylsulfonyl fluoride, 1μg/ml of leupeptin and aprotinin, 1mM Na_3VO_4). Equal amounts of total protein were immunoprecipitated, separated by electrophoresis on 8% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane and immunoblotted as described previously (Maroun et al., 1999).

Light microscopy. Collagen cultures were fixed in 4% paraformaldehyde and photographs were taken using a digital image processing system (Northern Eclipse, Empix Imaging Inc., Toronto, ON) at a magnification of 2.5x (bright field objective) and 10x (phase contrast).

Results

Kinetic analysis of Gab1 phosphorylation and downstream MAPK. We have shown recently that a constitutively activated ErbB2/Neu receptor promotes invasive morphogenesis in MDCK epithelial cells similar to HGF, whereas activation of the EGFR family by EGF or heregulin (HRG) fails to induce an invasive response (Khoury et al., 2001). Moreover, previous studies conducted in our lab suggested that the morphogenic response downstream from the Met receptor correlates with the ability of HGF to induce a prolonged phosphorylation of the Gab1 adaptor protein and downstream Erk1/2 phosphorylation (Maroun et al., 1999) (Fig.1A and B). Consequently, the inability of EGF to promote the morphogenic response may be explained by its inability to induce a sustained Gab1 phosphorylation, EGF induces a transient phosphorylation of Gab1 (Maroun et al., 1999) (Fig.1A). We show here that HRG induces a weak and transient phosphorylation of Gab1 in MDCK cells (Fig.1A), correlating with its inability to induce invasive morphogenesis (Fig.1). HRG induces phosphorylation of Erk1/2 in these cells (Fig.1B), indicating that MDCK cells express endogenous ErbB family receptors that are activated by HRG. It has been suggested previously that sustained Erk phosphorylation was required for a morphogenic response downstream from HGF (Maroun et al., 1999). Taken together, these data suggest that ligand-induced activation of the ErbB family receptors fails to induce a sustained signal required to promote invasive morphogenesis in epithelial cells.

Activated Neu promotes an increase in the steady-state phosphorylation levels of Gab1. Oncogenic activation of receptor tyrosine kinases correlates with constitutive activation of the receptor. This occurs via different mechanisms including receptor overexpression and amplification as well as point mutations. A point mutation in the transmembrane domain of ErbB2/Neu (V664E) results in increased receptor homodimerization and constitutive activation of the kinase domain in the absence of ligand, mimicking ErbB2 overexpression observed in breast tumors (Bargmann et al., 1986). We have shown previously that this mutant receptor (NT) promotes a morphogenic program in MDCK epithelial cells, similar to HGF (Khoury et al., 2001). In order to assess the involvement of Gab1 in the morphogenic response downstream from activated ErbB2/Neu, we have examined the phosphorylation status of Gab1 in NT-expressing cells. Consistent with the results described in Fig.1, we show that a constitutively activated ErbB2/Neu (NT) induces an increase in the steady-state phosphorylation level of HA-tagged Gab1 in MDCK cells when compared to parental and wild type Neu-expressing cells (Fig.2A). Increased phosphorylation of endogenous Gab1 is also observed (data not shown). Similarly, steady state phosphorylation levels of Erk1/2 are elevated in MDCK cells expressing NeuNT (Fig.2B). These data indicate that the ability of activated ErbB2/Neu to induce invasive morphogenesis correlates with its ability to increase phosphorylation of Gab1 and downstream Erk, suggesting a role for Gab1 and Erk in NT-induced morphogenic response. In order to further assess the involvement of the MAPK (Erk) pathway in the morphogenic phenotype, we have used a pharmacological inhibitor for the MAPK kinase MEK (UO126) in three-dimensional collagen cultures. We show that UO126 reverses the morphogenic response downstream

from activated NeuNT (Fig.2C), indicating that MEK is involved in NT-induced invasive morphogenesis.

Gab1 interacts with activated Neu in a Grb2-dependent manner. Gab1 is phosphorylated downstream from RTK (including EGFR and Met) following recruitment to the receptors, generally indirectly through the Grb2 adapter protein. In addition, we have previously reported a direct Grb2-independent mode of interaction of Gab1 with Met. No reports have described the interaction of Gab1 with ErbB2/Neu. Therefore, to assess whether Gab1 is recruited to ErbB2/Neu, we have transiently co-expressed HA-tagged Gab1 mutants with activated NeuNT as shown in Fig.3, and followed protein interaction by co-immunoprecipitation. Transfected Gab1 constructs include wild type Gab1 as well as deletion mutants lacking the Grb2 binding site (ΔGrb2), the Met binding domain or MBD (ΔMet), Gab1 PH domain (ΔPH), and a triple mutant lacking all three above mentioned domains (Lock et al., 2000). Wild-type HA-Gab1 (WTGab1) is phosphorylated and co-immunoprecipitates with activated NeuNT (Fig.3, lane 2). Similarly a Gab1 mutant lacking the Met binding domain MBD coimmunoprecipitates with NeuNT to similar levels as the WT Gab1 protein, suggesting that Gab1 recruitment to NeuNT is independent of the MBD. In contrast, a Grb2-deficient Gab1 mutant shows significantly reduced co-immunoprecipitation and phosphorylation (Fig.3, lane 3), suggesting that Gab1 interaction with activated NeuNT occurs in a Grb2-dependent manner. This was further confirmed by an in vitro association assay (data not shown). In addition, a mutant lacking the PH domain shows reduced co-immunoprecipitation, however its phosphorylation level is not affected. The triple mutant that fails to bind

Grb2 and lacks the PH domain behaves in a similar manner to the ΔGrb2 mutant, with reduced co-immunoprecipitation and phosphorylation levels. Taken together, these data suggest that Gab1 is recruited to an ErbB2/Neu dependent signaling complex in a Grb2 dependent manner and this is required for full phosphorylation of Gab1 downstream from ErbB2.

Specific Neu C-terminal tyrosine residues are required for Gab1 interaction. To further identify domain requirements for Gab1 interaction with NeuNT, Neu add-back mutants that couple to single signaling pathways have been used in a transient transfection assay (Fig.4). The Neu Tyrosine Phosphorylation-Deficient (NYPD) mutant results from the mutation of the five carboxy-terminal tyrosine residues of the activated NT mutant into phenylalanine; NYPD is kinase active but has an impaired ability to transform Rat1 fibroblasts, due to its inability to couple to downstream signaling pathways (Dankort et al., 1997). Add-back mutants were generated by reverting individual phenylalanine substitutions to tyrosine residues in the NYPD mutant, allowing association with distinct signaling molecules (Dankort et al., 1997). The resulting mutants YB, YC, YD and YE are tyrosine phosphorylated and catalytically active, and are thought to recruit Grb2, Crk, Shc and Dok-R respectively (Dankort et al., 2001a; Dankort et al., 1997). The YA site is thought to recruit a negative regulator of ErbB2/Neu (Dankort et al., 1997). To further identify domain requirements for Gab1 interaction with NeuNT, Neu add-back mutants that couple to single signaling pathways have been used in a transient transfection assay (Fig.4). YA and NYPD mutants fail to recruit Gab1 in this assay (Fig.4, lanes 3 and 5), suggesting that Gab1 recruitment to Neu contributes to

the positive signaling events downstream from ErbB2/Neu. When compared with the WT or consitutivelty activated NeuNT Gab1 co-immunoprecipitates to similar levels with YB, a Grb2 binding mutant (Fig.4, lane 6). In addition, Gab1 co-immunoprecipitates with YC and YE mutants. However, mutant YD, a major Shc binding site fails to recruit Gab1 in this assay. These data suggest that while Gab1 requires a functional Grb2 binding site to be recruited to Neu, other binding sites may also be involved.

Discussion

Increasing evidence supports a significant biological role for the Gab1 docking protein downstream from RTKs, including a role in neuronal cell survival, neurite outgrowth, DNA synthesis and epithelial cell morphogenesis (Holgado-Madruga et al., 1997; Weidner et al., 1996). Recently, Gab1 was shown to be required for ErbB2/Neu-induced transformation and tumorigenesis. Gab1-/- fibroblasts exhibit impaired ErbB2-induced colony formation in soft agar and tumor formation in mice (Yamasaki et al., 2003). Similarly, it was suggested that Gab1 activation by the EGFR is required for efficient ErbB2/neu-induced tumor progression (Gillgrass et al., 2003)

Gab1 is a common substrate for RTKs including EGFR and Met (Fixman et al., 1997; Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Nguyen et al., 1997; Takahashi-Tezuka et al., 1998). However, Gab1 recruitment to these different receptors does not promote the same biological response. For example, while Gab1 is involved in the morphogenic response downstream from the Met receptor, an activated EGFR which recruits and phosphorylates Gab1 does not exhibit a morphogenic response but rather promotes a mitogenic response in epithelial cells (Maroun et al., 1999; Sachs et al., 1996). We have shown that the duration of the signal plays a critical role in this issue, where a sustained signal is required for the morphogenic response to occur. More precisely, we have shown that HGF induces a prolonged Gab1 phosphorylation which correlates with invasive morphogenesis, whereas EGF-induced transient Gab1 phosphorylation correlates with a mitogenic response (Maroun et al., 1999) (Fig.1). Similarly, we show in this study that activation of the ErbB family receptors (ErbB3 and

ErbB4) by heregulin promotes a transient and weak phosphorylation of Gab1 and fails to promote invasion in MDCK epithelial cells (Khoury et al., 2001) (Fig.1). Heregulin and EGF also fail to promote invasive morphogenesis in cells overexpressing wild type ErbB2/Neu, indicating that receptor transactivation by ErbB family ligands is not sufficient to trigger the invasive response. However, constitutive activation of ErbB2/Neu by a point mutation in its transmembrane domain promotes spontaneous invasive morphogenesis and cell dispersal (Khoury et al., 2001), and this correlates with the ability of activated ErbB2/Neu to induce an increase in the steady-state phosphorylation levels of Gab1 and downstream kinase Erk1/2 (Fig.2). This provides further evidence that the duration of the signal is a critical factor that determines the biological outcome.

Our data show a different cellular response to ligand-induced activation of ErbB2/Neu compared to ligand-independent constitutive activation of the receptor. This could be explained by the fact that the ligand-activated receptor is subject to normal regulation mechanisms including receptor internalization and downmodulation. Conversely, constitutive dimerization and subsequent activation of ErbB2/Neu results in a signal imbalance that might be responsible for the sustained Gab1 and Erk phosphorylation (Fig.2). Moreover, it has been suggested that ErbB2/Neu potentiates signaling downstream from other EGFR family members. ErbB2/Neu was reported to retard EGFR internalization and degradation and increase recycling of the receptor to the cell surface, resulting in increased signaling through the EGFR system (Wang et al., 1999; Waterman et al., 1998; Worthylake et al., 1999). This factor might contribute to the observed Gab1 constitutive phosphorylation. Also, ErbB2/Neu forms hetero-oligomers with other members of the EGFR family, allowing signal diversification (Graus-Porta et

al., 1997; Muthuswamy et al., 1999; Pinkas-Kramarski et al., 1996; Qian et al., 1999). The involvement of other EGFR family members in deregulated Neu-induced invasive morphogenesis has not been investigated in this study.

Several lines of evidence for the involvement of MAPK downstream from Gab1 have been reported, Gab1 knockout fibroblasts show reduced Erk activation (Itoh et al., 2000; Yart et al., 2001). Moreover, it has been suggested that sustained ERK phosphorylation downstream from HGF and EGF in a carcinoma cell line promotes changes in gene expression including expression of metalloproteases required for the degradation of the extracellular matrix during the invasive process (McCawley et al., 1998). Our data suggest that sustained activation of Erk1/2 correlates with the ability of deregulated NeuNT to promote invasion (Fig.2). Erk activity is required at different steps of the invasive morphogenic process, including matrix degradation, cell proliferation and remodeling of cell-cell junctions. We have recently reported that NT-induced loss of adherens junctions is MEK-dependent, and that a MEK inhibitor reverses this phenotype and restores cellular junctions (Khoury et al., 2001). Similarly, we show that the MEK inhibitor UO126 reverses the invasive morphogenic process in a three-dimensional culture model in a dose-dependent manner, indicating that MEK activity is required for this process (Fig.2).

Gab1 is a common substrate for receptor tyrosine kinases. Based on interaction studies with the EGFR (Lock et al., 2000; Rodrigues et al., 2000; Saxton et al., 2001), it has been suggested that Gab1 is generally recruited to RTK in a Grb2-dependent manner. In addition, a direct recruitment mode to the Met receptor has been described (Lock et al.,

2000), involving a stretch of 13 amino acids within a Met binding domain (MBD) (Lock et al., 2002). We show here that Gab1 phosphorylation and co-immunoprecipitation with ErbB2/Neu requires a functional Grb2 binding site (Fig.3), the MBD domain does not seem to be involved in the putative interaction. In addition, Gab1 PH domain seems to be required for Gab1 association but does not affect Gab1 phosphorylation downstream from deregulated ErbB2/Neu (Fig.3). We have previously shown that the association and phosphorylation of Gab1 with Met is not dependent on the Gab1 PH domain (Maroun et al., 1999).

Using ErbB2/Neu add back mutants, we confirm that Gab1 associates with ErbB2/Neu in a Grb2-dependent manner (Fig.4), since WTGab1 immunoprecipitates with the Grb2 binding mutant receptor YB. Moreover, Gab1 associates with YC, a Crk and Nck binding mutant (Dankort et al., 2001a), and YE, a Dok-R and p34 binding mutant, suggesting that other mechanisms for recruitment of Gab1 to ErbB2/Neu might also be involved (Fig.4). These findings correlate with recent data from Neu add-back transgenes in Drosophila (Settle et al., 2003), showing that mutations in *dos* (the drosophila Gab1 homolog) attenuated signaling most potently from the Grb2 binding site YB (Y1144), followed by suppression of YC signals, and to a lesser extent, YD and YE. Interestingly, we find that the sequence upstream of YC and YE in ErbB2/Neu, ENPEYL, is highly conserved (Dankort et al., 1997). This corresponds to a NPXY consensus binding motif for PTB-containing proteins. Alteration of this sequence results in reduced transforming potential of YC and YE add-back mutants (Dankort et al., 2001a). The involvement of this sequence as a consensus motif for Gab1 recruitment to Neu is currently under investigation.

Previous studies have suggested that Grb2-dependent signaling pathways involving Gab1 association and phosphorylation downstream from the Met receptor are required for the morphogenic activities of Met (Fournier et al., 1996; Maina et al., 1996; Maroun et al., 1999). Similarly, we show that Gab1 constitutive phosphorylation and association with deregulated ErbB2/Neu receptor correlates with its ability to promote invasive morphogenesis in epithelial cell lines, compared to wild type ErbB2/Neu or a signaling-deficient mutant (NYPD) (Fig.2). Nevertheless, Gab1 association pattern obtained from transient expression assays does not correlate with the ability of the add-back mutants to promote an invasive response in MDCK cells (Fig4) (Khoury et al., 2001). We have previously reported that YD and YE, two add back mutants thought to bind the Shc adaptor protein induce a partial invasive morphogenic response, while the other mutants failed to promote a similar response (Khoury et al., 2001). YD mutant is unable to co-immunoprecipitate with wild type Gab1 (Fig.4), while YE mutant does. Moreover, the Grb2 binding mutant YB recruits Gab1 but fails to promote invasive morphogenesis (Fig.4). One possible explanation is that Gab1 recruitment to ErbB2/Neu through Grb2 or any other single site (YC or YE) might be required but not sufficient for a full morphogenic response. A full morphogenic response requires the presence of all active binding sites, as in the deregulated ErbB2/Neu. The additional sites might then enhance or stabilize the morphogenic response, alternatively they could act by recruiting negative regulatory proteins that modulate the signal in response to receptor activation.

Taken together, our data suggest that Gab1 associates with ErbB2/Neu in a Grb2-dependent manner, and that other mechanisms might be involved. In addition, deregulated ErbB2/Neu triggers a sustained signal leading to the constitutive

phosphorylation of Gab1 and downstream kinases Erk1/2, promoting invasive morphogenesis in epithelial cells.

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Figure 1: Heregulin induces transient phosphorylation of Gab1 in MDCK epithelial cells. A) MDCK epithelial cells overexpressing HA-tagged Gab1 were stimulated for the indicated times with HGF (200U), EGF (100ng) and β-heregulin (1nM) respectively. HA-Gab1 protein was immunoprecipitated from triton cell lysates with mouse anti-HA antibody. Proteins were separated by SDS-PAGE and probed with RC20 (anti-phosphotyrosine), stripped then reprobed with HA.11 (anti-HA). Bottom panel: MDCK cells expressing HA-Gab1 were grown in Collagen I. Five days later, cultures were stimulated with HGF (5U/ml), EGF (20ng/ml) or β-heregulin (100 ng/ml) for ten days, then fixed and pictures taken. Magnification 10x. **B)** MDCK epithelial cells were stimulated as in A. Whole cell lysates (25ug) were probed with rabbit anti-phospho-Erk or rabbit total Erk.

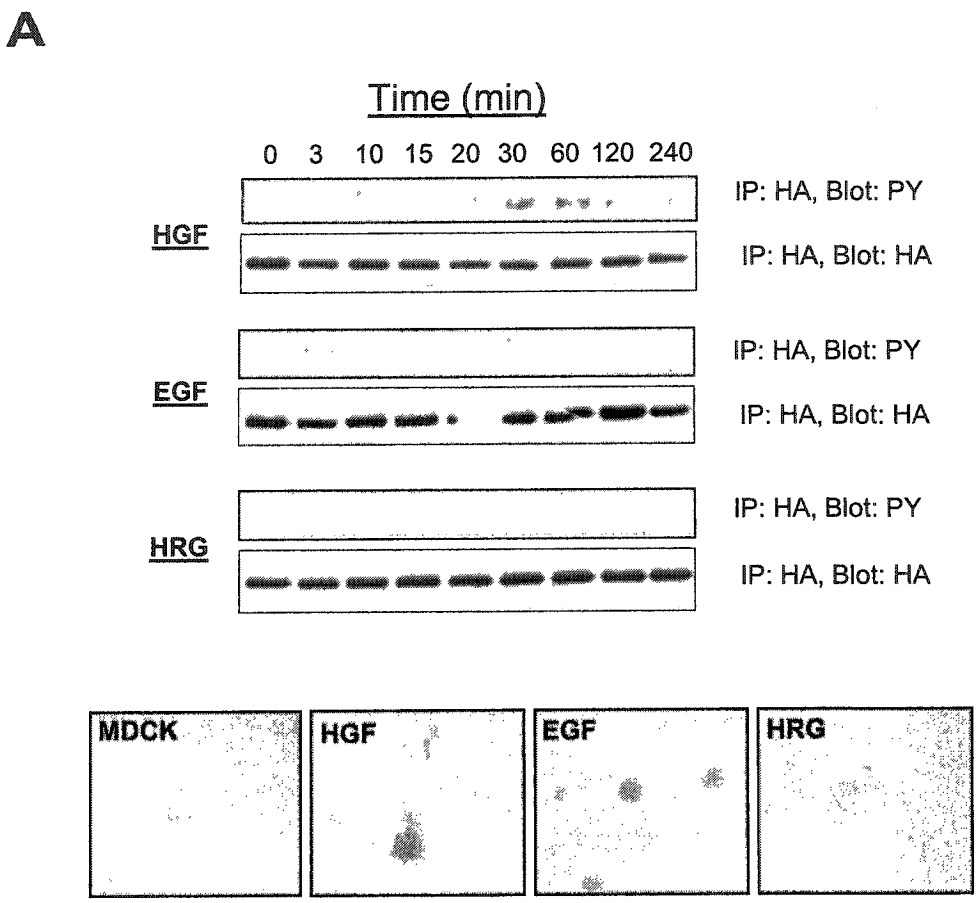

A
Time (min)
0 3 10 15 20 30 60 120 240
HGF
IP: HA, Blot: PY
IP: HA, Blot: HA
EGF
IP: HA, Blot: PY
IP: HA, Blot: HA
HRG
IP: HA, Blot: PY
IP: HA, Blot: HA
MDCK
HGF
EGF
HRG
B
0 3 10 15 20 30 60 120
HGF
p44
p42
WCL, Blot: P-Erk
WCL, Blot: total Erk
EGF
WCL, Blot: P-Erk
WCL, Blot: total Erk
HRG
WCL, Blot: P-Erk
WCL, Blot: total Erk

Figure 1

Figure 2: Constitutively activated NeuNT induces an increase in the steady-state phosphorylation levels of Gab1 and Erk in MDCK epithelial cells. A) Different ErbB2/Neu mutant receptors (WT wild type, NYPD tyrosine phosphorylation deficient mutant, NT constitutively activated) were transfected into HA-Gab1 expressing MDCK cells and populations were selected using hygromycin. Proteins from triton lysates were immunoprecipitated and probed using anti-ErbB2, anti-HA and anti-phosphotyrosine (4G10) antibodies as indicated. **B)** MDCK stable cell lines expressing wild type and constitutively activated Neu mutants were lysed in triton lysis buffer and whole cell lysates (25ug) separated by SDS-PAGE and probed with phospho-Erk and total Erk antibodies as indicated. **C)** MEK inhibitor blocks NT-induced invasive morphogenesis. Stable lines of MDCK cells were grown in collagen I. Structures were allowed to develop for one week and then treated or not with the MEK inhibitor UO126 for another week at which they were fixed in 4% paraformaldehyde. Both bright field (2.5x) and phase contrast (10x) pictures were taken on representative fixed cultures.

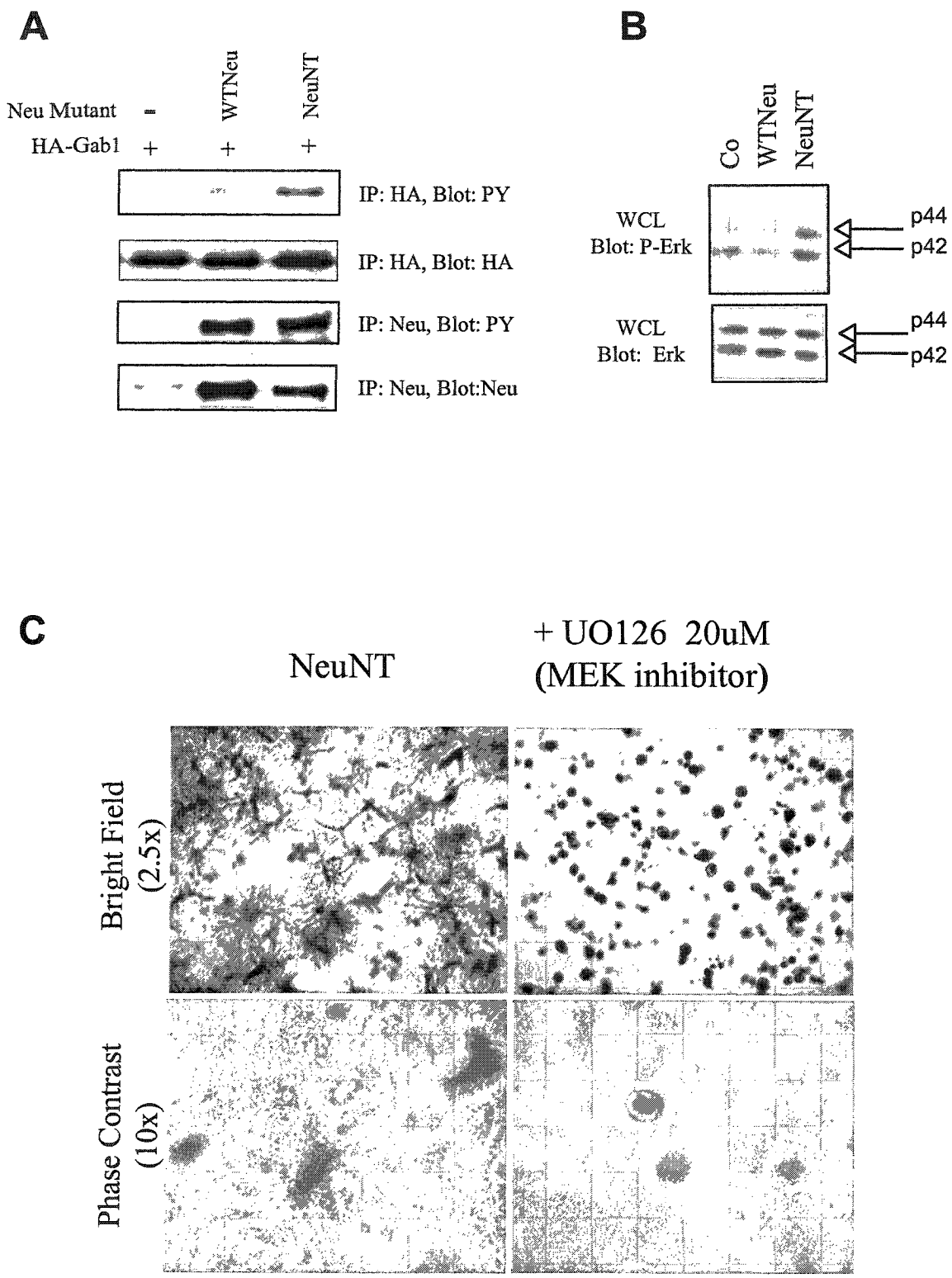
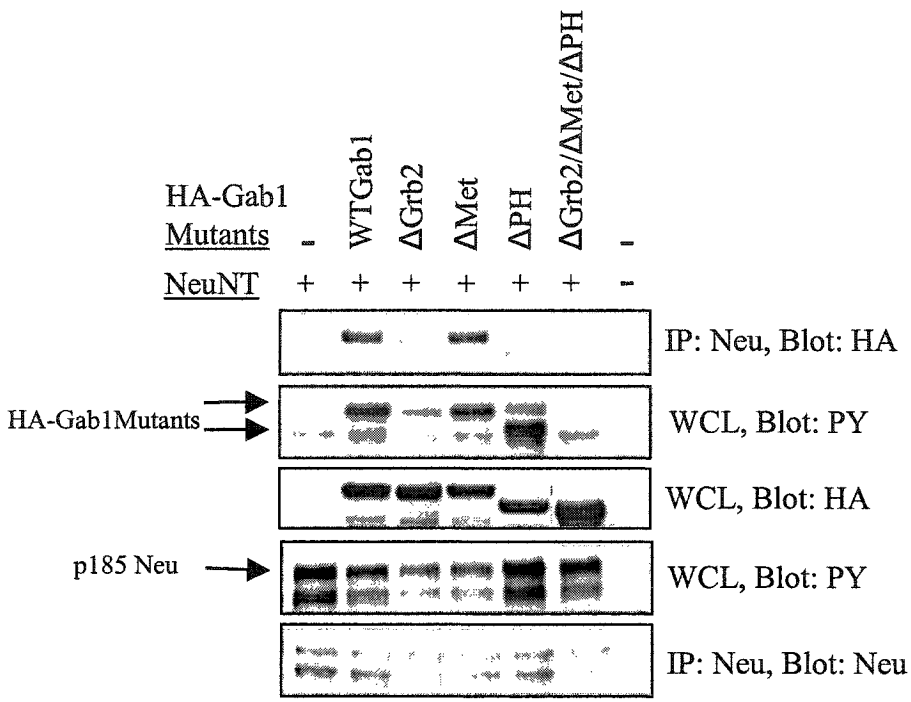

A
Neu Mutant -
HA-Gab1 +
WTNeu
NeuNT
+ +
IP: HA, Blot: PY
IP: HA, Blot: HA
IP: Neu, Blot: PY
IP: Neu, Blot:Neu
B
Co
WTNeu
NeuNT
WCL
Blot: P-Erk
p44
p42
WCL
Blot: Erk
p44
p42
C
NeuNT
+ UO126 20uM
(MEK inhibitor)
Bright Field
(2.5x)
Phase Contrast
(10x)

Figure 2

Figure 3: **Gab1 interacts with activated ErbB2/Neu in a Grb2 dependent manner.** Constitutively activated NeuNT was transiently cotransfected into 293T fibroblasts with HA-Gab1 deletion mutants. Triton lysates were immunoprecipitated, separated on 8% SDS-Polyacrylamide gels and immunoblotted as indicated. Bottom panel: structure of the Gab1 docking protein.


HA-Gab1 Mutants
NeuNT
-
WTGab1
ΔGrb2
ΔMet
ΔPH
ΔGrb2/ΔMet/ΔPH
-
+ + + + + + -
IP: Neu, Blot: HA
HA-Gab1Mutants
WCL, Blot: PY
WCL, Blot: HA
p185 Neu
WCL, Blot: PY
IP: Neu, Blot: Neu

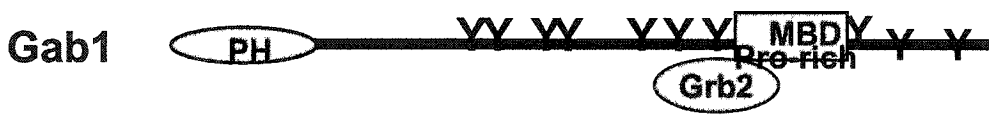
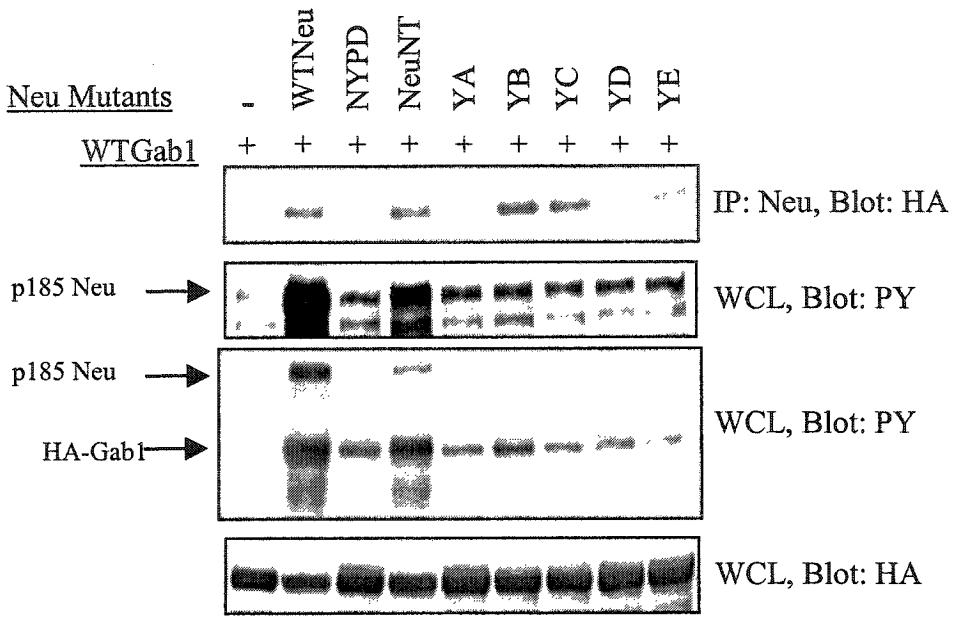

Gab1
PH
MBD
Pro-rich
Grb2

Figure 3

Figure 4: **Gab1 interaction with Neu mutants involves specific tyrosine residues in the ErbB2/Neu C-terminal**. Wild type HA-Gab1 was transiently cotransfected into 293T fibroblasts with different ErbB2/Neu mutants (illustrated in the bottom). Triton lysates were immunoprecipitated, separated on 8% SDS-Polyacrylamide gels and immunoblotted as indicate. Bottom: illustration of the ErbB2/Neu mutant receptors.


Neu Mutants
-
WTNeu
NYPD
NeuNT
YA
YB
YC
YD
YE
WTGab1
+ + + + + + + + +
IP: Neu, Blot: HA
p185 Neu
WCL, Blot: PY
p185 Neu
HA-Gab1
WCL, Blot: PY
WCL, Blot: HA

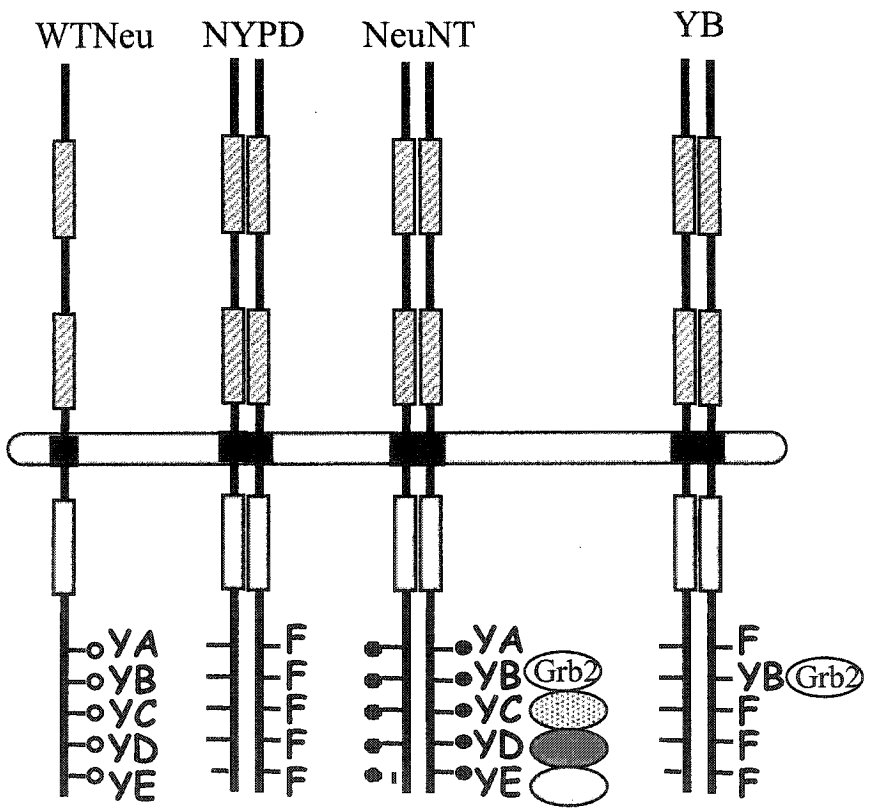

WTNeu
NYPD
NeuNT
YB
YA
YB
YC
YD
YE
F
F
F
F
F
YA
YB
YC
YD
YE
Grb2
F
YB
Grb2
F
F
F

Figure 4

Chapter IV: HGF/Met and deregulated ErbB2/Neu cooperate to promote breakdown of tubular epithelia and cell invasion

Preface

The use of three-dimensional culture models has revealed increasing evidence for the contribution of the tumor stroma to the progression of the malignant phenotype. In human breast cancer, increased levels of stromal HGF have been associated with poor prognosis. Moreover, HER2/ErbB2/neu overexpression is a prognostic marker in 20-30% breast tumors. Consistently, ErbB2/neu promotes mammary tumors in transgenic mice, and the tumor latency can be decreased by cooperating oncogenes.

We show in chapter II that expression of deregulated ErbB2/neu induces the inherent morphogenic program of MDCK cells in a three-dimensional collagen matrix, namely the formation of organized epithelial structures. This involves a transient epithelial-mesenchymal transition (EMT), suggesting that additional factors might be required to trigger a more potent EMT characteristic of the malignant phenotype. Using the MDCK cell model, we have investigated the involvement of HGF and Met in this process in the context of deregulated activation of the ErbB2/neu receptor.

Abstract

Overexpression and deregulation of the HER2/ErbB-2/Neu receptor tyrosine kinase is associated with increased progression in human breast cancer. However, in breast explant cultures the ErbB2/Neu receptor contributes to alveolar differentiation. Met, the hepatocyte growth factor (HGF) receptor is involved in branching morphogenesis of the breast epithelium. We have previously shown that constitutively activated ErbB2/Neu (called NeuNT) promotes an invasive morphogenic program in MDCK cells in a three-dimensional collagen matrix. This involves the transient loss of epithelial organization and remodeling of E-cadherin junctions. In this paper, we show that HGF promotes the disruption of NeuNT-induced epithelial structures. This phenotype correlates with a decrease in E-cadherin protein in HGF-treated NT-expressing cells and enhanced NeuNT-invasive potential. HGF-induced invasion can be abrogated by pretreatment with the MEK inhibitor, UO126, establishing the involvement of MEK-dependent pathways in this process. These results demonstrate that physiological signals from HGF/Met synergize with deregulated HER2/Neu to enhance the malignant phenotype promoting a more stable epithelial-mesenchymal transition. This is particularly important for human breast cancer where ErbB2/Neu is overexpressed in 20-30% tumors and HGF is a physiological growth factor for the mammary epithelium.

Introduction

Breast cancer is a leading cause of death in women. The development and progression of this disease to an invasive phenotype is mediated by the deregulation of different modulators of growth and differentiation. Among these are receptor tyrosine kinases (RTK). Under normal conditions, activation of RTKs by their ligand promotes receptor dimerization and phosphorylation on tyrosine residues that provide specific binding sites for signaling proteins (Pawson and Nash, 2000; Weiss and Schlessinger, 1998). This event is tightly regulated. However, receptor overexpression can promote clustering and deregulated ligand-independent activation (Schlessinger, 2000). This alteration is frequently reported in different types of human tumors.

HER2/ErbB2/Neu, a member of the EGFR family (Pegram et al., 1998) and Met, the Hepatocyte growth factor/Scatter factor (HGF/SF) receptor tyrosine kinases (Ghoussoub et al., 1998; Yamashita et al., 1994) are prognostic markers for breast cancer. The amplification and overexpression of ErbB-2 is associated with increased progression and metastasis in 25% human breast carcinomas and is indicative of poor prognosis in breast, ovarian, and renal collecting duct carcinomas (Pegram et al., 1998; Selli et al., 1997; Zhang et al., 1997). Consistent with this, overexpression of a wild-type or constitutively activated ErbB-2/Neu (NeuNT) transgene in the mammary epithelia of mice induces the formation of metastatic mammary tumors (Muller et al., 1988). In contrast, the Met and ErbB-2 receptor tyrosine kinases are associated with the morphogenic and functional differentiation of the normal mammary gland epithelium. Whole organ cultures and cell culture models suggest a requirement for Met in branching

morphogenesis and ErbB-2 in lobuloalveolar differentiation of the breast epithelium (Jones and Stern, 1999; Niemann et al., 1998; Yang et al., 1995).

The branching process involves degradation of the extracellular matrix and epithelial reorganization of extending cells (O'Brien et al., 2002; Pollack et al., 1998). This change, involving the remodeling of cell-cell junctions and increased cell motility, is only transient in normal cells and occurs during specific physiological processes of embryonic and adult life. It is referred to as epithelial-mesenchymal transition (EMT) (Boyer et al., 1996; Meiners et al., 1998; Savagner, 2001). However, EMT also occurs in tumor dissemination and metastasis, where tumor cells undergo loss of differentiation accompanied by increased cell invasion (Thiery, 2002). Repression of E-cadherin is usually associated with malignant progression (Thiery, 2002).

Using a non-transformed kidney epithelial cell model (MDCK), we have shown that activation of the Met receptor by ligand and deregulated NeuNT each promote the inherent morphogenic program of MDCK cells, namely the formation of branching tubules in a three-dimensional collagen matrix (Khoury et al., 2001; Maroun et al., 1999). These tubular structures consist of organized sheets of epithelial cells. However, constitutively activated NeuNT has been shown to transform fibroblasts and induce tumors in transgenic animals (Dankort et al., 1997; Guy et al., 1996; Guy et al., 1992), as previously mentioned. Moreover, heregulin-activated ErbB-2/Neu enhances cell invasion in some breast tumor cell lines (Brandt et al., 1999; Meiners et al., 1998), whereas in other lines, it promotes a remodelling of epithelial cell junctions consistent with a morphogenic response (Chausovsky et al., 1998) . Taken together, these data suggest that the biological consequence of ErbB-2/Neu activation depends on other signals that may

differ among cells. Deregulated activation of ErbB2/Neu may predispose normal cells to malignant transformation, and additional factors might be required to trigger the progression of invasive disease. To test this hypothesis, we have used stable MDCK cell lines expressing NeuNT treated with HGF. We report that the HGF/Met pathway synergizes with deregulated ErbB2/Neu to induce permanent loss of epithelial organization.

Materials and Methods

Antibodies and reagents. Antibodies used in this paper are: rabbit anti-phosphoErk from New England Biolabs (Nepean, ON), mouse anti-E-cadherin from the Hybridoma Bank (Iowa university, USA), rabbit anti-ZEB1 from Santa Cruz and CY3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West-point, DA, USA). Rabbit anti-Erk was a generous gift from Dr J. Blenis, Harvard, Boston. HGF was generously provided by Dr G. Van de Woude, National Institutes of Health, Frederick, Maryland. Heregulin β1 was purchased from Neomarkers (Fremont, CA). The MEK inhibitor UO126 was purchased from Promega.

Cell culture and dispersal assay. MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50μg/ml gentamycin (Gibco BRL). Neu mutants (Dankort et al., 1997) were transfected into MDCK cells by the calcium phosphate method as described in Khoury et al., 2001.

$5x10^3$ cells were seeded in 24-well dishes (Nunc) for cell dispersal studies. After 48 hours, HGF was added to the medium at 5U/ml for 24 hours.

Motility assay. Cells ($5x10^5$/well) were plated on Transwell filters (6-well, Costar) in the presence or absence of HGF (5U/ml). 24 hours later, filters were submerged in formalin phosphate buffer (Fisher) for 15 minutes, washed twice with distilled water, and stained with crystal violet for 15 minutes at room temperature followed by several washes with water. Non-migrating cells were scraped off the upper layer of the filter using a cotton swab. Filters were then air-dried and photographs were taken. To quantitate the rate of migration for each cell line, filters were cut, solubilised in 10% acetic acid, and

absorbance readings taken at 596 nm. The results plotted are average numbers of 4 experiments for each cell line.

Matrigel invasion assay. Transwell filters (24-well, Costar) were coated with Matrigel (50-100 $\mu g/cm^2$). Cells ($8x10^4$/well) were plated and processed as above (motility assay). Where indicated, cells have been grown in the presence of HGF for 15 days before plating them on Matrigel. For quantitation, filters were photographed and invading cells counted.

Tubulogenesis assay. MDCK cells were suspended in a collagen matrix as described previously (Maroun et al., 1999). HGF (5U/ml) and UO126 (5 and 20uM) were added to the medium at five-seven days later either simultaneously or at two days interval, as indicated.

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

Immunoprecipitation and Western blotting. Cells were seeded at 10^6/100-mm dish (Nunc). The next day, cells were washed once with DMEM and serum-starved for 48h in DMEM containing 0.1% FBS. Growth factors (HGF and Heregulin) were then added to the cells and incubated for the indicated times, then cells were lysed in Triton X-100 lysis buffer (50mM Hepes pH 7.4, 150mM NaCl, 10% glycerol, 0.5% Triton, 1mM phenylmethylsulfonyl fluoride, 1μg/ml of leupeptin and aprotinin, 1mM Na_3VO_4). Equal amounts of total protein separated by electrophoresis on 8% SDS-polyacrylamide gels,

transferred to a nitrocellulose membrane and immunoblotted as described previously (Maroun et al., 1999).

For E-cadherin protein levels, cells were seeded in the presence of HGF for the indicated times. Triton lysates were separated on 8% gels and blotted.

Light microscopy. Photographs of fixed collagen cultures and dispersed cells were taken using a digital image processing system (Northern Eclipse, Empix Imaging Inc., Toronto, ON) at a magnification of 2.5x (bright field objective) and 10x (phase contrast).

Immunofluorescence microscopy. Collagen cultures were stained as previously described (Pollack et al., 1998). Live/dead staining was performed using Calcein and Ethidium bromide (Molecular probes) as previously described (Debnath et al., 2002). Images were photographed using confocal LSM510 (Zeiss).

Results

Cooperation between HGF and NeuNT promotes the disruption of organized epithelia in a three-dimensional collagen matrix

We have shown previously that a constitutively activated ErbB2/Neu receptor (NeuNT) induces a morphogenic response in normal kidney epithelial MDCK cells in a three-dimensional collagen matrix, resulting in the formation of tubular structures (Khoury et al., 2001) (Fig.1). This response is similar to the previously reported HGF-induced invasive tubulogenesis in MDCK cells. In order to establish whether HGF and NeuNT cooperate to enhance the morphogenic response in normal epithelia, we have grown NeuNT-induced tubular structures in the presence of HGF. We show that HGF promotes the disruption of tubules into single cells that invade the collagen matrix (Fig.1). This response occurs in a time-dependent manner leading to a complete loss of differentiated structures within 10 days (Fig.1B). Heregulin and EGF, ligands for the EGFR family receptors, fail to induce a similar phenotype (data not shown). In contrast, HGF promotes a morphogenic response in cells expressing wild type Neu or a mutant receptor with impaired ability to couple signaling molecules (NYPD).

Three-dimensional epithelial structures consist of polarized cells surrounding a hollow lumen formed through apoptosis of centrally located cells (Fig.2) (O'Brien et al., 2002; Ojakian et al., 1997). This process can be monitored by immunofluorescence using calcein/ethidium homodimer reagents (Debnath et al., 2002), where dead cells appear in the lumen (red staining) surrounded by live cells (green staining) (Fig.2). Using this assay, we show that HGF-induced disruption of epithelial structures produces live single

cells invading the collagen matrix (Fig.2). These data suggest that activation of the Met receptor with HGF cooperates with a deregulated activated NeuNT to promote the loss of epithelial integrity.

HGF and NeuNT synergize enhance cell invasion

The development of invasive cancers correlates with the loss of epithelial organization, referred to as epithelial-mesenchymal transition (EMT) (Boyer et al., 1996; Thiery, 2002). This process involves the loss of cell-cell junctions and cell dispersal, in addition to increased cell invasion and motility. HGF is one of the most potent inducers of EMT known.

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, cells expressing NeuNT show a low level of invasion whereas stimulation of these cells with HGF greatly enhances their invasive capacity (Fig.3A). The most dramatic effect is observed when NT-expressing MDCK cells are pretreated with HGF for 15 days prior to the assay as shown by the intense staining. Control MDCK epithelial cells are not able to invade the matrigel matrix, whereas a prolonged treatment of these cells with HGF induces a modest invasive response (Fig.3A).

It has previously been reported that HGF increases cell survival by protecting epithelial cells from anoikis or cell death induced by detachment from the extracellular matrix. Anoikis can be monitored by increased genomic DNA degradation (Frisch, 2000). In order to assess whether deregulated NeuNT protects MDCK cells from anoikis, we

have isolated genomic DNA from NeuNT-expressing cells grown in suspension, and followed DNA degradation on agarose gels. We show that under these conditions, WTNeu fails to prevent DNA degradation, whereas deregulated NeuNT prevents DNA degradation, comparable to HGF (Fig.3B and data not shown). This suggests that deregulated ErbB2/Neu confers a cell survival advantage in an anchorage independent manner.

Deregulated NeuNT enhances cell migration

We have recently shown that deregulated ErbB2/Neu (NeuNT) induces an EM-like transition, including loss of cell-cell junctions and epithelial cell dispersal, as well as enhanced cell migration (Khoury et al., 2001), comparable to the HGF-induced cell scatter. When assayed for their migratory capacity using modified Boyden chambers NeuNT expressing cells show elevated migration when compared to control MDCK cells however this is still less that the level of migration stimulated by HGF treatment of MDCK cells (Fig.4A). Noteably, HGF treatment of MDCK NeuNT expressing cells is not elevated over that of MDCK cells, demonstrating that HGF and ErbB2 signals do not synergize for cell migration.

HGF promotes downmodulation of E-cadherin levels in NeuNT-expressing cells

Loss of epithelial differentiation is associated with the disruption of cell-cell junctions. E-cadherin, a component of adherens junctions, has been reported to be downregulated in many human cancers and in established cancer cell lines (Ji et al., 1997). Using immunofluorescence staining, we show that E-cadherin localizes at cell-cell

contacts in NeuNT tubular structures (Fig5B), similar to HGF-treated MDCK cells (data not shown). Upon HGF treatment, reduced E-cadherin staining is observed in the detaching cells, although few cells (marked with an arrow) show E-cadherin fluorescence at the membrane (Fig.5B). In addition, we have assessed E-cadherin protein levels in NeuNT cells grown in the presence of HGF for different time points (Fig.5A and data not shown). We find that E-cadherin levels are reduced in a time-dependent manner, prolonged treatment (five days to two weeks) reduces E-cadherin levels considerably (Fig.5A, lane 4). In Src-transformed MDCK cells, E-cadherin levels are maintained (Fig.5A, last lane).

Transcriptional repression of E-cadherin is associated with epithelial-mesenchymal transition (Thiery, 2002). Several E-cadherin repressors have been identified, these include the transcription factor Snail which is upregulated in tumors and carcinoma cell lines (Batlle et al., 2000; Cano et al., 2000), Slug (Hajra et al., 2002), Snail-induced □EF1/ZEB1 transcriptional repressor (Sekido et al., 1994) and SIP1/ZEB2 (Comijn et al., 2001). ZEB1 expression was monitored by indirect immunofluorescence. We find that ZEB1 is expressed in MDCK cells independent of NeuNT expression and HGF treatment (Fig.6). However, the pattern of ZEB1 expression is different among these conditions, with a nuclear punctuate staining in the presence of NeuNT (Fig6c and d), suggesting a subnuclear localization of ZEB1. This pattern is also found in Src-transformed MDCK cells (data not shown). Untreated MDCK cells exhibit a perinuclear ZEB1 staining (Fig.6a).

To examine if E-cadherin was transcriptionally repressed in our system, real-time PCR analyses using a 300 base pair E-cadherin probe were performed. Results did not

show any alterations of E-cadherin mRNA levels following long-term treatment with HGF (data not shown), suggesting that post-translational mechanisms might be involved in the observed reduction of E-cadherin protein levels.

Taken together, these results suggest that HGF promotes the downmodulation of cell-cell junctions in epithelial cells expressing a deregulated ErbB2/Neu receptor, involving mechanisms other than transcriptional repression.

The MEK/MAPK pathway is involved in HGF-induced loss of differentiation of epithelial cells overexpressing deregulated ErbB2/Neu

The breakdown of epithelial cell-cell junctions in response to HGF requires PI3K, MEK, Ras and Rho family dependent signals (Potempa and Ridley, 1998; Royal et al., 2000; Royal and Park, 1995). Similarly, we have recently shown that a pharmacological inhibitor for MEK, the MAPK kinase, reverses the dispersed phenotype and restores adherens junctions in MDCK cells expressing constitutively activated NeuNT, suggesting that the MEK signaling pathway is involved in NeuNT-induced cell dispersal and loss of adherens junctions (Khoury et al., 2001). We show here that UO126 prevents HGF-induced disruption of tubular structures in NT-expressing cells in a dose-dependent manner (Fig.7A). Pretreatment of cells with a low dose of the inhibitor (5uM) prior to adding HGF prevents tubule disruption without blocking the formation of these structures (Fig.7B). Under these conditions, tubules regain E-cadherin expression at cell-cell contacts, as revealed by immunofluorescence (Fig.7C). However, when these cells are pretreated with HGF, epithelial disorganization is triggered and UO126 fails to block it (Fig.7B, bottom), suggesting that it is an irreversible process. Moreover, kinetic studies

of Erk phosphorylation show that HGF enhances NT-induced steady-state phosphorylation (data not shown), suggesting that HGF and NeuNT synergize to enhance Erk phosphorylation. Taken together, these results suggest that MEK is involved in HGF-induced loss of differentiation in NeuNT-expressing cells.

Discussion

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 is called epithelial-mesenchymal transition (EMT). This process can be initiated by different growth factors (HGF, EGF, TGF□...) or extracellular matrix components such as collagen. We and others have shown that HGF induces EMT in normal MDCK epithelial cells (Khoury et al., 2001; Weidner et al., 1993; Zhu et al., 1994), however these changes are reversible after HGF withdrawal. EMT also occurs in tumor dissemination and metastasis, where the phenotypic changes are more potent. We have shown previously that a deregulated activated ErbB2/Neu receptor (NeuNT) induces an EM-like transition in MDCK cells (Khoury et al., 2001). NeuNT also promotes the inherent branching morphogenesis of these cells in a collagen matrix, similar to HGF. However, the same mutant induces metastatic mammary tumors in transgenic mice (Muller et al., 1988). Deregulation of ErbB2/Neu is also involved in the progression of various human cancers where 25% of breast tumors overexpress the receptor (Pegram et al., 1998; Selli et al., 1997; Zhang et al., 1997).

We show in this paper that HGF-induced activation of the Met receptor in MDCK epithelial cells expressing a deregulated ErbB2/Neu receptor results in the complete disruption of epithelial organization and enhanced cell invasion (Figures 1, 2 and 3). This observation correlates with metastatic behavior, and highlights the importance of cross-talk between normal and deregulated signals in the progression of invasive malignancies.

HGF-induced loss of epithelial organization is particularly relevant for breast cancer where ErbB2/Neu deregulation is reported in 20-30% mammary tumors and elevated HGF and Met expression levels have been shown to correlate with poor prognosis (Kang et al., 2003; Tuck et al., 1996). Similarly, TGF□has been shown to promote pulmonary metastasis in transgenic mouse models with ErbB2/Neu-induced mammary tumorigenesis (Siegel et al., 2003). Moreover, synergy between TGF□ and Ras or EGF has been reported to induce EMT in primary cultured cell models (Grande et al., 2002; Janda et al., 2002). Hence, our results present an experimental model system that mimicks the physiological context, where we can study the involvement of specific signaling pathways in cancer progression.

Loss of differentiation during cancer progression is accompanied by the breakdown of cell-cell junctions, a prerequisite for cell dissociation. E-cadherin, a major component of adherens junctions is downregulated in many human cancers and in established cancer cell lines (Birchmeier and Behrens, 1994; Ji et al., 1997). It has been suggested that E-cadherin gene expression is repressed by the transcription factor Snail which is upregulated in tumors and carcinoma cell lines (Batlle et al., 2000; Cano et al., 2000). Snail upregulates ZEB1 expression which has been shown to repress E-cadherin independently (Sekido et al., 1994).

In MDCK cells, the scatter factor HGF induces the redistribution of E-cadherin from the cell membrane into a cytoplasmic soluble compartment (Khoury et al., 2001; Royal and Park, 1995), without affecting E-cadherin expression levels (Khoury et al., 2001). Similarly, NeuNT-expressing cells lose membrane-localized E-cadherin during EM-like transition but are able to restore it at high density (Khoury et al., 2001). We

show here that E-cadherin protein levels are considerably reduced when NeuNT-expressing cells are maintained in HGF-containing medium (Fig.5). However, this reduction is not due to transcriptional repression as revealed by real-time PCR (data not shown), suggesting that post-translational mechanisms might be involved. These include proteolytic cleavage or protein internalization and degradation. It has been shown that E-cadherin is cleaved by matrilysin and stromelysin-1 resulting in cellular changes associated with epithelial-mesenchymal transition (Lochter et al., 1997; Noel et al., 2000). In our system, sustained Erk activation may upregulate metalloprotease expression and hence promote cleavage of E-cadherin (McCawley et al., 1998). Alternatively, ErbB2/Neu induced Src activation might promote tyrosine phosphorylation of E-cadherin leading to the association of E-cadherin with a ubiquitin-ligase and subsequent endocytosis and degradation of E-cadherin (Fujita et al., 2002). Importantly, Src-transformed MDCK cells do not show altered E-cadherin protein levels (Fig5), suggesting that other pathways downstream from HGF and deregulated ErbB2/Neu might be required to reduce E-cadherin.

Expression of the ZEB1 transcriptional repressor exhibits a distinct subcellular pattern in MDCK cells expressing deregulated ErbB2/Neu (Fig.6). This pattern does not seem to be directly related to reduced E-cadherin protein levels since mRNA levels are not altered (data not shown). Furthermore, the observed pattern is similar to ZEB1 staining in Src-transformed MDCK cells which do not lose E-cadherin protein (Fig.5 and data not shown). The same pattern is also observed in breast cancer cell lines expressing moderate to high levels of ErbB2, but not in ErbB2-negative cells (data not shown). These observations suggest that ErbB2/Neu signals involving Src activation might

modulate ZEB1 targeting to subnuclear compartments, however additional signals will be required to enhance ZEB1 transcriptional activity. The involvement of HGF in this process needs further investigation. ZEB1 is involved in TGF-□ induced growth arrest and transcriptional gene regulation (Postigo et al., 2003). Potential targets for ZEB1 downstream from deregulated ErbB2/Neu and HGF need to be identified.

We have recently shown that the invasive morphogenic response correlates with the ability of deregulated NeuNT and HGF/Met to induce a sustained signal involving the adaptor protein Gab1 and downstream kinase Erk (manuscript in preparation). Erk activity is required for different cellular events involved in the morphogenic process, such as cell proliferation and remodeling of epithelial cell-cell junctions (Khoury et al., 2001; Maroun et al., 1999; Potempa and Ridley, 1998). Erk is also implicated in cell motility through its ability to phosphorylate myosin light chain kinase and enhance cell contractility (Klemke et al., 1997). Moreover, a sustained Erk activation might be required to induce changes in gene expression resulting in the secretion of matrix degrading metalloproteases (Liang and Chen, 2001; McCawley et al., 1998; Tanimura et al., 2002). However, deregulated activation of the MAPK pathway induces permanent loss of epithelial structures in MDCK cells in collagen, as revealed by the overexpression of constitutively activated forms of Ras and MEK, upstream effectors of MAPK (Khwaja et al., 1998). Consistent with this, we show that MEK activity is required for HGF-induced disruption of epithelial structures in NeuNT-expressing MDCK cells (Fig.7). Furthermore, HGF synergizes with deregulated ErbB2/Neu to enhance Erk phosphorylation (data not shown), suggesting that Erk activation may contribute to

increased cell invasion and loss of differentiation downstream from HGF and deregulated ErbB2.

In conclusion, we provide evidence that the synergy between HGF/Met and ErbB2/Neu is involved in the progression of the malignant phenotype of epithelial cells, and propose an experimental model system to address similar questions and dissect signaling pathways contributing to increased invasiveness of cancer cells. This will ultimately lead to the design of specific chemotherapeutic drugs to block progression of the disease.

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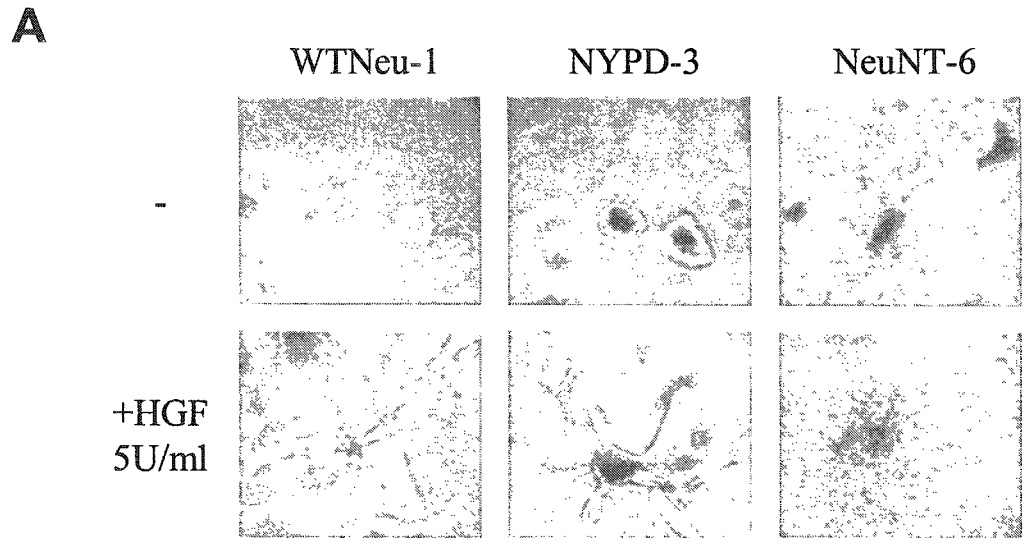
Figure 1: **HGF promotes the disruption of organized epithelia in MDCK cells overexpressing deregulated NeuNT.** **A)** Stable lines of MDCK cells expressing wild type, mutant and deregulated Neu receptors were grown in collagen I. Five days later, cultures were stimulated with HGF (5U/ml) for ten days, then fixed and pictures taken. **B)** Cultures were fixed at different time points following HGF addition to follow the progression of epithelial disruption. Time points indicated refer to the time when HGF was added (*ie* Day 4 corresponds to four days of HGF treatment). Magnification, 10x


A
WTNeu-1
NYPD-3
NeuNT-6
-
+HGF
5U/ml

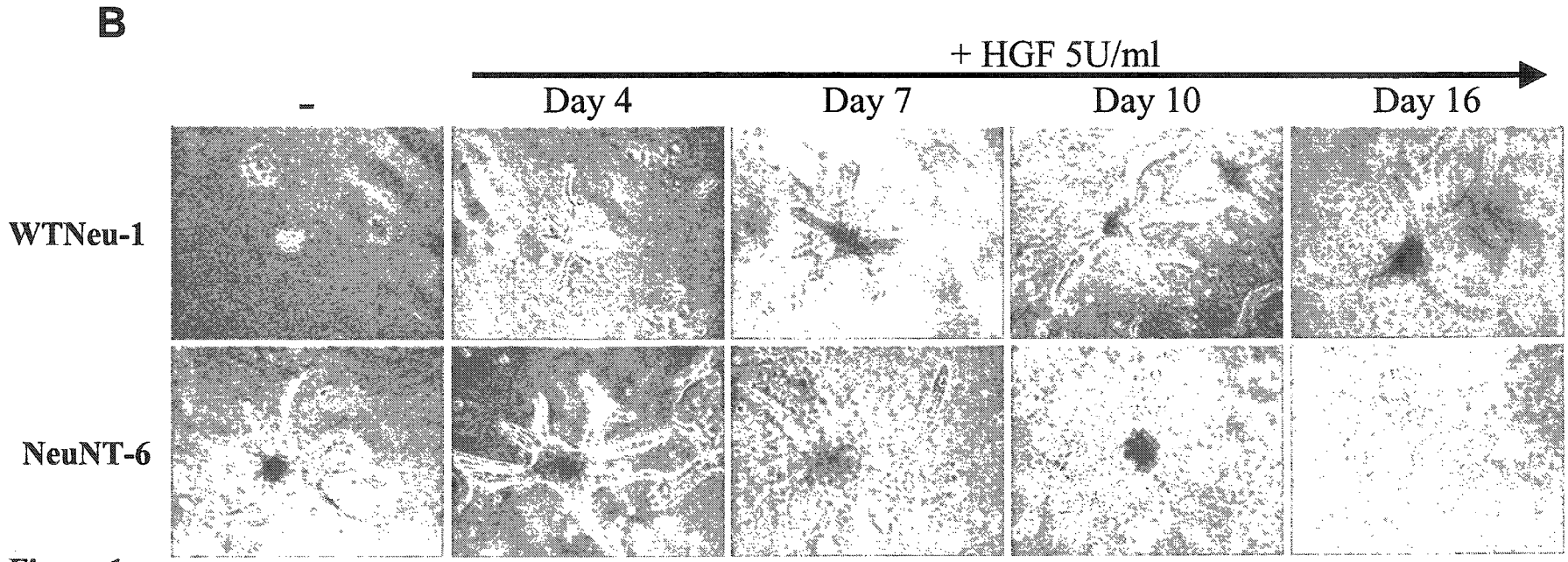

B
+ HGF 5U/ml
-
Day 4
Day 7
Day 10
Day 16
WTNeu-1
NeuNT-6

Figure 1

Figure 2: **HGF and deregulated NeuNT synergize to promote collagen invasion.** Live 3D collagen cultures of MDCK cells and NeuNT-expressing cells were treated with HGF for 10 days and stained with Calcein for live cells (green; arrowhead) and Ethidium bromide for dead cells (red; white arrow). LSM510, Magnification 25x.

MDCK
NeuNT
-
+ HGF 5U/ml
10 days
-
+ HGF 5U/ml
10 days
3D collagen
Calcein/EtBr
Lumen with apoptotic cells

Figure 2

Figure 3: Synergy between HGF and deregulated ErbB2/Neu promotes matrigel invasion. A) Matrigel assay was performed in the presence or absence of HGF (5U/ml), as described in Materials and Methods. In one case, NeuNT lines were maintained in HGF-containing medium for 15 days before seeding them on Matrigel. Representative filters were scanned using a digital scanner. **B)** NeuNT 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 hours and genomic DNA was extracted and separated on a 1.5% agarose gel.

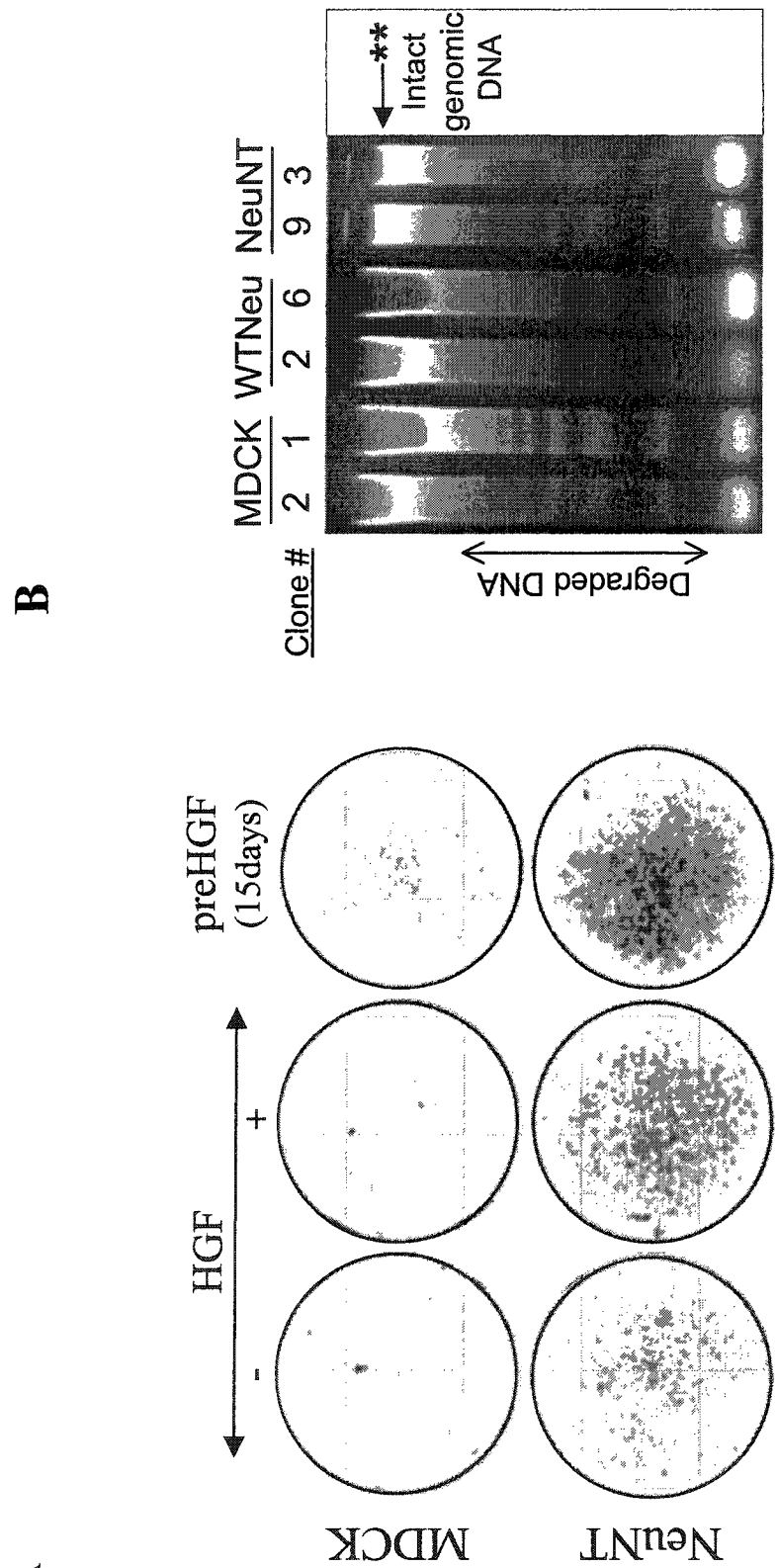
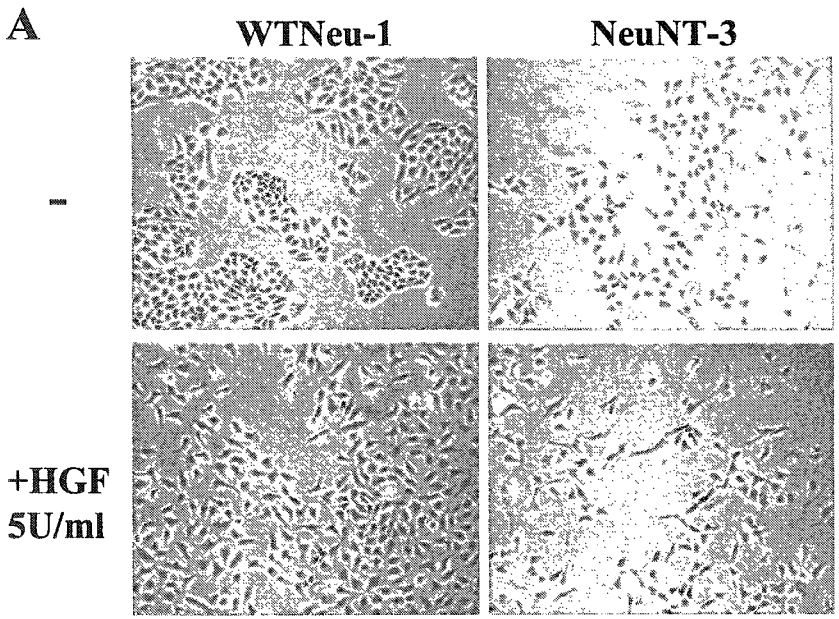
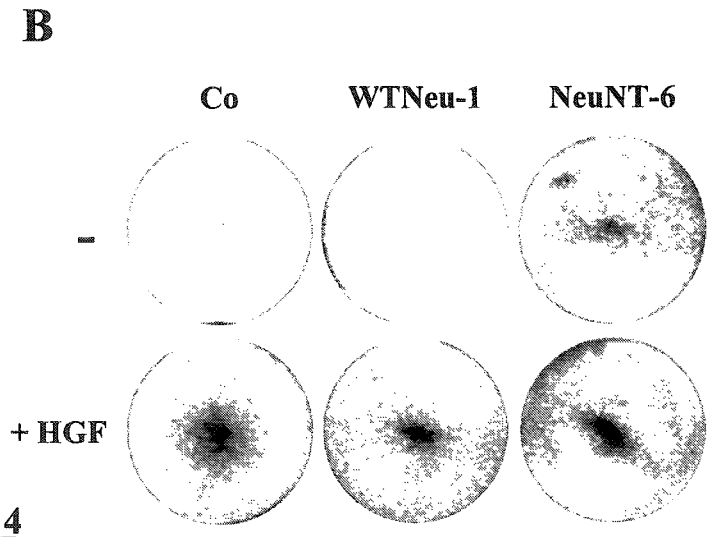

A
B
MDCK
NeuNT
HGF
-
+
preHGF
(15days)
Clone #
MDCK
2 1
WTNeu
2 6
NeuNT
9 3
Degraded DNA
**
Intact
genomic
DNA

Figure 3

Figure 4: Additive effects of HGF and deregulated ErbB2/Neu enhance cell dispersal and motility. **A)** Stable MDCK cells expressing deregulated ErbB2/Neu were grown in DMEM with 10% FBS in 6-well dishes, in the presence or absence of HGF. Representative clones were photographed using the Northern Eclipse image processing system (10x magnification). **B)** Motility assays were performed using Transwell filters, as described in Materials and Methods. Representative photographs of crystal violet-stained filters were taken. **C)** Filters were solubilised in 10% acetic acid, the migration rate of Neu mutant-expressing cells was determined by measuring the absorbance at a wavelength of 596nm. Each value represents the average of four filters.


A
WTNeu-1
NeuNT-3
-
+HGF
5U/ml


B
Co
WTNeu-1
NeuNT-6
-
+ HGF

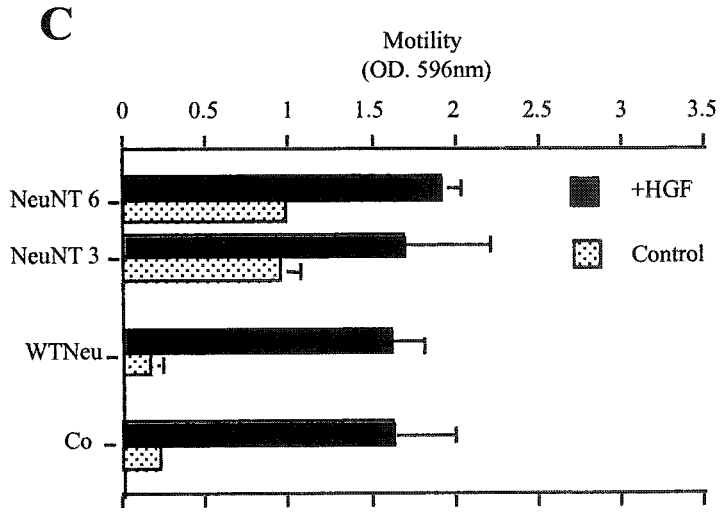

C
Motility
(OD. 596nm)
0
0.5
1
1.5
2
2.5
3
3.5
NeuNT 6
NeuNT 3
WTNeu
Co
+HGF
Control

Figure 4

Figure 5: **HGF induces downmodulation of E-cadherin in MDCK cells expressing deregulated NeuNT. A)** Cells were maintained in the presence or absence of HGF for 5 days as indicated. Triton cell lysates (25ug) were separated on 8% polyacrylamide gels, transferred to nitrocellulose membranes and probed with mouse anti E-cadherin. **B)** Collagen cultures of NeuNT-expressing MDCK cells treated or not with HGF (5U/ml) were labeled with anti E-cadherin. Confocal picture magnification 63x.

A
MDCK
NT
Activated Src
HGF (days)
-
+5
-
+5
-
p125
E-cadherin
p97
ß-catenin
p44
p42
Erk
B
NeuNT
NeuNT + HGF
E-cadherin
Immunofluorescence (63x)

Figure 5

Figure 6: **MDCK cells express the ZEB1 transcriptional repressor.** Cells grown on glass coverslips were fixed in 3.7% formaldehyde and stained for ZEB-1 and actin (phalloidin). Confocal picture magnification 63x.

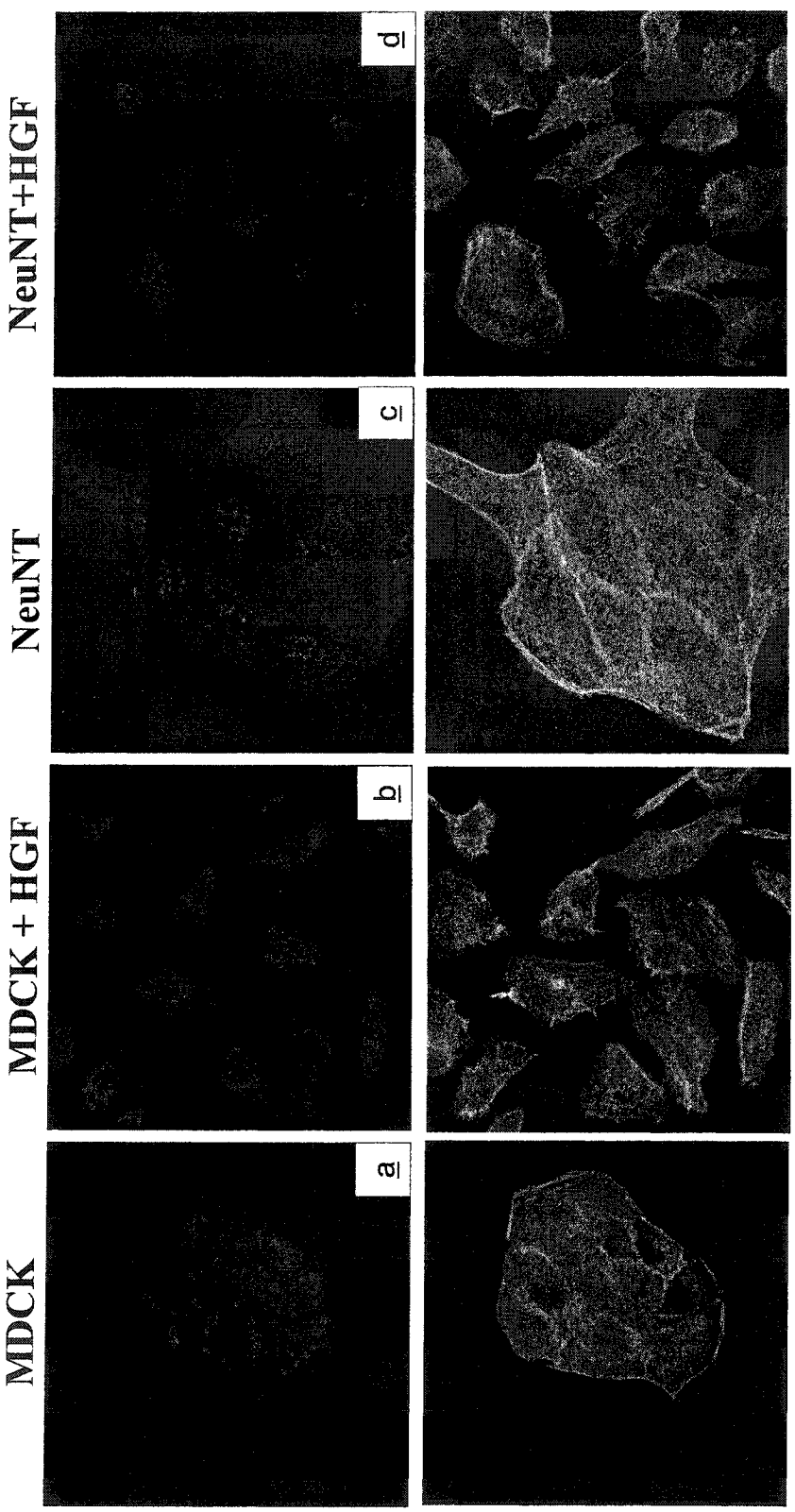


Figure 6

Figure 7: 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 (5U/ml) and UO126 (5uM and 20uM) were added either simultaneously as in **A)**, or at a two-day interval where pretreatment was required as in **B)**. Seven days later, cultures were fixed in 4% paraformaldehyde. Representative pictures were taken using bright field (2.5x) and phase contrast (10x) objectives. **C)** Representative collagen cultures from **B)** stained for E-cadherin show restored epithelial structures with adherens junctions. Confocal picture magnification, 63x

Figure 7

A
Phase Contrast (10x)
Bright Field (2.5x)
-
HGF 5U/ml
HGF+ UO126 5uM
HGF+ UO126 20uM
B
PreHGF + UO126 5uM
PreU0126 5uM + HGF
Bright Field (2.5x)
C
E-cadherin (63x)
HGF
PreU0126 (5uM) + HGF

Discussion

Epithelial tissue architecture is modulated by different factors from the microenvironment, including interactions with extracellular matrix components through integrins, as well as released growth factors from the stroma. Deregulation of these interactions perturbs tissue homeostasis and contributes to the loss of epithelial differentiation observed in cancer. Under these conditions, the cell response to unaltered signals differs, contributing further to the malignant phenotype, as has been reported for TGFβ (Lehmann and Schleicher, 2000).

The dedifferentiation process involves the conversion of polarized epithelial cells into fibroblast-like motile cells, accompanied by the loss of epithelial markers such as E-cadherin and the upregulation of mesenchymal markers (Thiery, 2002). This process, known as epithelial-mesenchymal transition (EMT), is also required for tissue remodeling in embryonic and adult life (Boyer et al., 2000; Meiners et al., 1998; Thiery, 2002). EMT is promoted by growth factor-induced activation of receptor tyrosine kinases (RTK) in tissue culture models. HGF, the Met receptor ligand, promotes cell dispersal and branching morphogenesis in a non-transformed kidney epithelial cell model (Brinkmann et al., 1995; Montesano et al., 1991; Stoker et al., 1987; Zhu et al., 1994).

The Met and ErbB2/neu receptor tyrosine kinases contribute to the differentiation of epithelial tissues including breast (Jones and Stern, 1999; Niemann et al., 1998; Schmidt et al., 1995; Yang et al., 1995). Altered expression of Met and ErbB2/neu is thought to be involved in the genesis of mammary and renal carcinomas (Danilkovitch-Miagkova and Zbar, 2002; Schmidt et al., 1997; Selli et al., 1997; Zhang et al., 1997a;

Zhang et al., 1997b). In addition, stroma-derived HGF was identified as a prognostic marker for breast cancer. The involvement of Met or ErbB2 in cell transformation and tumor induction in mice has been well studied, however the mechanisms through which these receptors contribute to loss of tissue architecture in tumors is poorly understood.

1. The MDCK epithelial cell model

Epithelial tissue architecture involves a complex three-dimensional organization modulated by the cells' interactions with each other and with their environment. Therefore, the dissection of signals involved in tissue organization and eventually loss of organization in carcinomas requires the use of appropriate experimental models that preserve properties of the tissue of origin. In this context, MDCK epithelial cells represent a common model for these studies. Derived from renal collecting duct cells, this normal non-transformed cell model has been extensively used to study the structure and regulation of epithelial cell junctions and epithelial polarity, and by us to examine Met-dependent signals involved in cell dispersal and differentiation (Stoker et al., 1987; Weidner et al., 1993a; Weidner et al., 1993b; Weidner et al., 1993c; Zhu et al., 1994). MDCK cells grow on plastic as polarized epithelial sheets with mature cellular junctions. Embedded in a collagen I matrix, they organize into three-dimensional epithelial structures called cysts. HGF added to the culture medium promotes branching morphogenesis of these structures, which is the inherent morphogenic program of renal collecting duct cells (Zhu et al., 1994). These branching tubules construct a basal lamina by producing laminin and fibronectin (Jiang et al., 2000). Therefore, MDCK cells have preserved the pattern of normal cell behavior. This confers an advantage over carcinoma

cell lines, which carry many genetic alterations that would modify the cellular context, and hence the cell response to a specific signal may no longer be representative of the in vivo situation. Primary cultures have been used, however their usage is limited by their sensitive growth conditions.

Using the MDCK cell model we have examined the consequence of expression of an activated oncogenic ErbB2/neu RTK on epithelial cell integrity, and have demonstrated the synergistic induction of cell invasion between HGF and ErbB2/neu.

2. Deregulated ErbB2/neu in normal epithelial cells

2.1 Partial EMT

ErbB2/neu amplification and overexpression is implicated in the etiology of breast cancer. Overexpression results in ligand-independent receptor clustering and deregulated activation, leading to signal imbalance (Schlessinger, 2000). Several reports have addressed the consequence of deregulated ErbB2/neu activation on tumor formation in addition to fibroblast cell transformation and cancer cell invasion, however the mechanisms through which ErbB2/neu contributes to loss of tissue architecture in tumors remain poorly understood.

Using non-transformed epithelial cells, I have shown that deregulation of the ErbB2/neu receptor promotes a panel of morphological changes that favor cell migration and support a role for ErbB2/neu in tumorigenesis (Khoury et al., 2001). This conversion involves the breakdown of adherens junctions and dispersal of epithelial colonies, with internalization rather than downmodulation of E-cadherin and β-catenin. Moreover, in three-dimensional collagen cultures, I have established that deregulated ErbB2/neu

promotes the inherent morphogenic program of kidney epithelial cells, branching tubulogenesis consistent with its ability to induce an E-M like transition in two dimensional cultures (Khoury et al., 2001).

The described phenotypes are similar to HGF-induced responses in MDCK cells, they correlate with a partial epithelial-mesenchymal transition involved in tissue remodeling (Thiery, 2002). Ligand-induced activation of wild type ErbB2 receptor fails to promote a similar transition (Khoury et al., 2001; Sachs et al., 1996). This indicates that deregulation of ErbB2/neu is required to initiate molecular changes that predispose cells to the malignant phenotype, however it is not sufficient to promote the loss of epithelial integrity that characterizes the malignant phenotype. Additional factors might be required for this progression. Consistent with this, transgenic mice overexpressing ErbB2/neu develop mammary tumors with a relatively long latency of 200 days (Guy et al., 1992), suggesting that other oncogenic events are necessary. Indeed, cooperative induction of mammary tumor formation between ErbB2/neu and mutant p53 or TGFα has been reported in transgenic mice (Li et al., 1997; Muller et al., 1996). Moreover, TGFβ impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis (Siegel et al., 2003), indicating that the importance of TGF-β signaling within the tumor is to specifically enhance the ability of the tumor cells to extravasate into the lung.

2.2 Sustained signal

Ligand-induced transactivation of a wild type ErbB2 receptor fails to promote partial EMT and tissue remodeling induced by the deregulated receptor, although both receptors activate similar downstream signaling pathways (Chapter II) (Khoury et al.,

2001). Similarly, a chimeric Trk-ErbB2 receptor failed to induce tubulogenesis in response to stimulation (Sachs et al., 1996). This may reflect a difference in signal duration, where a steady-state deregulated ErbB2/neu triggers a prolonged signal required for the morphogenic response. Conversely, ligand-activated ErbB2 promotes a transient signal and cyst formation. In this regard, EGF stimulation of MDCK cells induces transient phosphorylation of Gab1 and Erk insufficient to induce morphological changes, whereas HGF promotes a sustained Gab1 and Erk phosphorylation and a morphogenic response (Khoury et al., 2001; Maroun et al., 1999). Consistently, HGF was shown to induce prolonged nuclear retention of activated Erk and subsequent increased expression of matrix metalloprotease MMP9, resulting in the induction of cell dispersal and motility (Liang and Chen, 2001; Tanimura et al., 2002).

I show that heregulin, a ligand for ErbB receptors, fails to promote tubulogenesis in MDCK cells overexpressing wild type ErbB2/neu (Khoury et al., 2001), correlating with its ability to induce a transient and weak phosphorylation of Gab1 (Chapter III, Fig.1). Cells expressing deregulated ErbB2/neu, however, exhibit increases in the steady-state levels of phosphorylation of Shc, Erk and Gab1 (Chapter II, Fig.7; Chapter III, Fig.2) (Khoury et al., 2001).

The difference in signal duration could be explained by the fact that the ligand-activated receptor is subject to normal regulation mechanisms including receptor internalization and degradation. Receptor deregulation by constitutive dimerization and subsequent activation would result in a signal imbalance that could overcome the negative feedback regulation (Harari and Yarden, 2000). In support of this, it has been suggested that ErbB2/neu potentiates signaling downstream from other EGFR family

members. ErbB2/neu was reported to retard EGFR internalization and degradation and increase recycling of the receptor to the cell surface, resulting in increased signaling through the EGFR system (Gulliford et al., 1997; Huang et al., 1999; Wang et al., 1999; Waterman et al., 1998; Worthylake et al., 1999). Moreover, ErbB2/neu forms hetero-oligomers with other members of the EGFR family, allowing signal diversification (Graus-Porta et al., 1997; Muthuswamy et al., 1999; Pinkas-Kramarski et al., 1996a; Pinkas-Kramarski et al., 1996b; Qian et al., 1999). Although overexpression of wild type ErbB2/neu does not seem to potentiate EGFR family-induced signals, the involvement of other EGFR family members in deregulated ErbB2/neu-induced invasive morphogenesis has not been investigated in this study.

2.3 Receptor add back mutants as tools to dissect signals for dispersal and morphogenesis

Using receptor add-back mutants, I demonstrated that mutant receptors YD and YE which recruit the Shc adapter protein (Dankort et al., 1997; Ricci et al., 1995) promote cell dispersal and a partial morphogenic response in collagen. In addition, I demonstrated that the YC mutant induces cell dispersal. Although tyrosine C has not been identified as a Shc PTB domain binding site, cells expressing this mutant, demonstrate increased Shc phosphorylation (Khoury et al., 2001). This suggests that Shc dependent signals are involved in ErbB2/neu induced cell dispersal and morphogenesis.

A role for Shc in HGF-mediated epithelial cell dispersal has been reported (Fournier et al., 1996). Moreover, Shc overexpression enhances the motility of MDCK cells (Pelicci et al., 1995). Transgenic mice expressing elevated levels of Shc in the

mammary epithelium, exhibit extensive branching and alveolar development of the breast epithelium (Rauh et al., 1999), providing further support for a role for Shc-dependent signals in epithelial remodeling.

The MEK/Erk pathway regulates the breakdown of epithelial cell junctions and cell migration (Potempa and Ridley, 1998). Moreover, Erk activity is required for cellular processes during tissue remodeling, such as cell proliferation, matrix degradation, and remodeling of cell junctions and polarity (Reddy et al., 2003). Downstream from HGF, Erk phosphorylation is required for the morphogenic response, cell dispersal and breakdown of junctions (Khoury et al., 2001; Maroun et al., 1999; Potempa and Ridley, 1998). Prolonged Erk phosphorylation is considered to be required for enhanced transcription of metalloprotease MMP9 downstream of HGF (Liang and Chen, 2001; McCawley et al., 1998; Tanimura et al., 2002). Importantly, many breast cancers exhibit elevated Erk activity (Sivaraman et al., 1997), Erk is implicated in cell motility through its ability to phosphorylate myosin light chain kinase and enhance cell contractility (Klemke et al., 1997). Consistent with these results, I have shown that deregulated ErbB2/neu increases the steady-state phosphorylation of Erk1/2 and promotes epithelial cell dispersal (Khoury et al., 2001). Inhibition of MEK, an activator of Erk, by a pharmacological inhibitor (PD98059) reverses the partial EMT induced by deregulated ErbB2/neu and restores adherens junctions. This demonstrates that MEK activity is required to maintain the loss of E-cadherin dependent junctional complexes.

Gab1 is involved in many cellular outcomes downstream from RTKs, including a role in neuronal cell survival, neurite outgrowth, DNA synthesis and epithelial cell morphogenesis (Holgado-Madruga et al., 1997; Weidner et al., 1996). HGF-induced cell dispersal and morphogenesis require Gab1 recruitment and phosphorylation whereas Gab1 is involved in EGFR-induced mitogenic response (Maroun et al., 1999; Sachs et al., 1996). Elevated steady-state phosphorylation levels of Gab1 are observed in MDCK cells overexpressing deregulated ErbB2/neu, supporting a role for Gab1 in the morphogenic process from ErbB2/neu (Fig.2, Chapitre III), although futher experiments are required to establish if Gab1 regulates ErbB2-induced cell dispersal and morphogenesis, since phosphorylation and recruitment to the ErbB2/neu mutant add-back receptors did not parallel the morphogenic response (Fig.4, Chapitre III). One possible explanation is that Gab1 recruitment to YC or YE in ErbB2 is required yet not sufficient for a full morphogenic response. A full morphogenic response might require the coordinated presence of all active binding sites as in deregulated ErbB2/Neu, to stabilize the interaction with Gab1 and invasive morphogenesis.

Evidence for the involvement of Gab1 in ErbB2-induced tumorigenesis has been shown recently where Gab1-/- fibroblasts exhibit impaired ErbB2-induced colony formation in soft agar and tumor formation in mice (Yamasaki et al., 2003). Similarly, it was suggested that Gab1 activation by the EGFR is required for efficient ErbB2/neu-induced tumor progression (Gillgrass et al., 2003).

3. Gab1 recruitment to ErbB2/neu

Gab1 is a common substrate for RTKs including EGFR and Met (Fixman et al., 1997; Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Nguyen et al., 1997; Takahashi-Tezuka et al., 1998). It is generally recruited to these receptors in a Grb2-dependent manner, as shown from Gab1 interaction with the EGFR (Lock et al., 2000; Rodrigues et al., 2000). In addition, direct recruitment to the HGF receptor through a Met binding site has been demonstrated recently (Lock et al., 2002; Lock et al., 2000). This direct recruitment mode is required for the morphogenic response downstream from Met (Lock et al., 2002).

I show that Gab1 phosphorylation and co-immunoprecipitation with ErbB2/neu requires a functional Grb2 binding site (Fig.3, Chapter III). A recent analysis of Neu add-back transgenes in Drosophila (Settle et al., 2003) supports this data, showing that mutations in *dos* (the Drosophila Gab1 homolog) attenuated signaling most potently from the Grb2 binding site YB (Y1144), followed by suppression of YC signals, and to a lesser extent, YD and YE. Consistently, we report that Gab1 associates with YC and YE (Fig.4, Chapter III). Interestingly, a conserved ENPEYL motif is present upstream of YC (Y1201) and YE (Y1253) in ErbB2/neu (Dankort et al., 2001a; Dankort et al., 2001b; Dankort et al., 1997). A NPXY sequence forms a β-turn motif that recruits PTB-containing proteins. Alteration of this sequence results in reduced transforming potential of YC and YE add-back mutants (Dankort et al., 2001a). The involvement of this sequence as a consensus motif for Gab1 recruitment to ErbB2/neu needs to be investigated.

Direct recruitment of Gab1 to Met involves a stretch of 13 amino acids within a Met binding domain (MBD) (Lock et al., 2002). The MBD domain is not involved in the putative interaction of Gab1 with ErbB2/neu, as a Gab1 construct lacking this domain still co-immunoprecipitates with ErbB2/neu (Fig.3, Chapter III).

4. Synergy between HGF and ErbB2/neu

4.1 HGF and deregulated ErbB2/neu

Data obtained in chapter II concluded that deregulation of ErbB2/neu initiates molecular changes that predispose cells to the malignant phenotype, however additional factors are required to promote the loss of epithelial integrity characteristic of the malignant phenotype. HGF has been identified as the synergistic factor for ErbB2/neu induced EMT (Chapter IV, Fig.1-3). HGF promotes epithelial remodeling in MDCK cells, however in the context of a deregulated ErbB2/neu, HGF disrupts MDCK tubular epithelial structures and promotes cell invasion in collagen and on Matrigel. This is accompanied by reduced E-cadherin protein levels and increased Erk activation (Chapter IV, Fig.5 and data not shown). This correlates with an enhanced epithelial-mesenchymal transition and provides a model for the progression of aggressive carcinomas (Figure 1).

The abovementioned observations demonstrate that the different responses to HGF depend on the cell context. Consistently, Birchmeier et al. (1997) have shown that MDA435 breast cancer cells transfected with HGF form tumors with metastasis to the lung in the absence of E-cadherin, whereas when E-cadherin is reintroduced to the cells HGF promotes three- dimensional epithelial structures (Birchmeier et al., 1997).

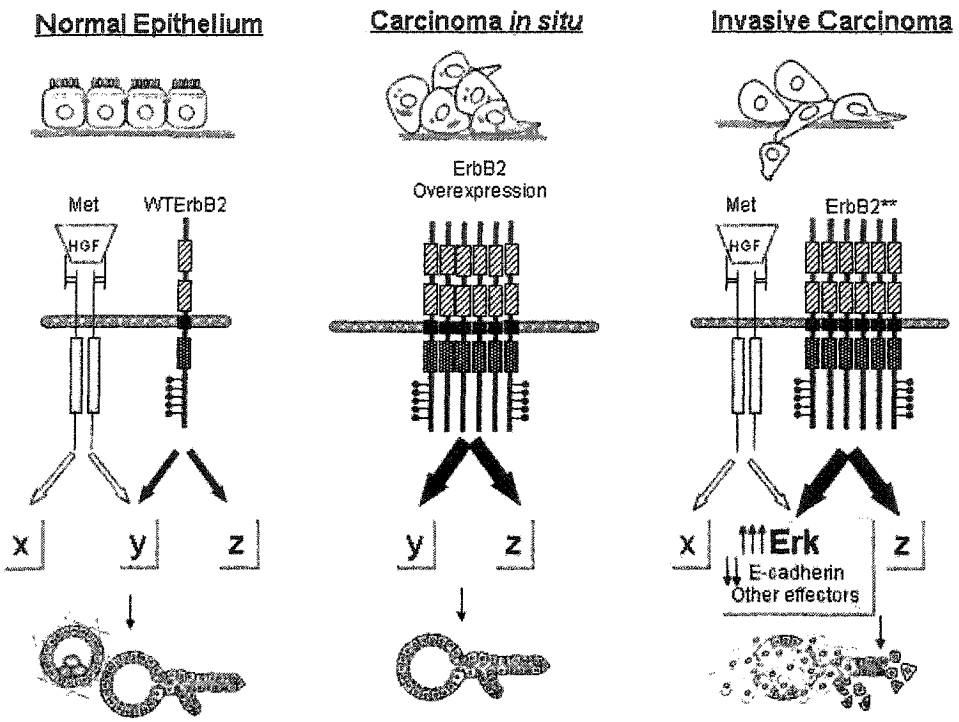


Figure 1: HGF synergizes with oncogenic ErbB2/Neu to promote loss of differentiation and invasion. According to this model, ErbB2 overexpression and activation initiates cellular changes that predispose cells to the malignant phenotype. In this context, HGF promotes the disruption of epithelial organization and enhances cell invasion.

The synergy described in this model is specific to HGF and ErbB2. EGF or heregulin fail to alter epithelial organization of deregulated ErbB2/neu-expressing cells (data not shown). This observation is particularly significant for breast cancer where HER2/ErbB2 overexpression is associated with poor prognosis. Met and HGF have also been identified as prognostic markers (Jin et al., 1997; Nagy et al., 1996; Tuck et al., 1996; Yamashita et al., 1994). Tissue microarray of breast tumors show a tight correlation between HGF and Met expression, with elevated levels of Met associated with poor prognosis (Kang et al., 2003). Correlation between HGF and ErbB2 overexpression has not been shown yet, and Met expression in breast tumors has been associated with poor outcome independent from HER2/ErbB2 (Tolgay Ocal et al., 2003).

However, this does not exclude the possible implication of Met and HGF in the progression of HER2/ErbB2-related malignant disease.

4.2 Cooperation between oncogenes

Synergistic induction of epithelial-mesenchymal transition has been reported in cultured cell models and transgenic mice. A classical model is TGFβ, which triggers different cellular outcomes depending on the cell context. For example, TGFβ promotes growth arrest in hepatocytes and Ha-Ras transformation does not alter epithelial polarity. However TGFβ induces EMT in these transformed cells, with induction of MMP9, CD44 and Snail, the E-cadherin repressor, in addition to E-cadherin downmodulation (Gotzmann et al., 2002). Other examples of TGFβ cooperation with Ras, Ha-Ras and EGF have been described in primary cultured cells (Grande et al., 2002; Janda et al., 2002a; Janda et al., 2002b; Oft et al., 1996). In addition, cooperative induction of mammary tumors between ErbB2/neu and mutant p53 or TGFα has been reported in transgenic mice, resulting in shortened tumor latency (Li et al., 1997; Muller et al., 1996).

Cross talk between EGFR and Met in transformed cells has also been reported, involving TGFα and EGF-induced Met phosphorylation and association with EGFR (Jo et al., 2000). In our model, deregulated ErbB2/neu does not induce Met phosphorylation or upregulation of HGF (Khoury et al., 2001; unpublished data). ErbB2/neu is known to potentiate signaling downstream from EGFR family members by altering receptor recycling and degradation (Gulliford et al., 1997; Huang et al., 1999; Wang et al., 1999; Waterman et al., 1998; Worthylake et al., 1999). Whether deregulated ErbB2 retards Met

internalization in MDCK cells, contributing to signal deregulation and subsequent induction of EMT, is not known.

4.3 Reduced E-cadherin protein levels

Loss of differentiation during cancer progression is accompanied by the breakdown of cellular junctions, a prerequisite for cell dissociation. E-cadherin, a major component of adherens junctions is downregulated in many human cancers and in established cancer cell lines (Ji et al., 1997). Therefore, a role for E-cadherin as a tumor suppressor gene has been proposed. E-cadherin gene expression is repressed by the transcription factor Snail which is upregulated in tumors and carcinoma cell lines (Blanco et al., 2002; Cano et al., 2000). Other repressors include Slug (Hajra et al., 2002), Snail-induced δEF1/ZEB1 (Sekido et al., 1994) and SIP1/ZEB2 (Comijn et al., 2001). In addition, post-translational downmodulation occurs involving protein internalization and degradation, and proteolytic cleavage.

Synergistic induction of EMT by HGF and deregulated ErbB2/neu is accompanied by reduced levels of E-cadherin protein (Chapter IV, Fig.5). Real-time PCR analysis suggests post-transcriptional downmodulation (data not shown). Proteolytic cleavage of E-cadherin by matrilysin and stromelysin-1 induces cellular changes characteristic of epithelial-mesenchymal transition (Lochter et al., 1997; Noel et al., 2000). Deregulated ErbB2 and HGF induce sustained Erk activation (Chapter IV, data not shown) which may upregulate metalloprotease expression and hence promote cleavage of E-cadherin (McCawley et al., 1998). Alternatively, phosphotyrosine-dependent internalization and degradation of E-cadherin could occur following HGF

treatment of MDCK cells expressing deregulated ErbB2/neu. E-cadherin endocytosis is mediated by Hakai, an E3 ubiquitin-ligase (Fujita et al., 2002) that interacts with E-cadherin following Src-induced tyrosine phosphorylation. Src is activated downstream of deregulated ErbB2/neu.

5. ZEB1 and deregulated ErbB2/neu

Deregulated ErbB2/neu promotes a partial EMT in MDCK epithelial cells, accompanied by the redistribution of E-cadherin into the cytoplasm, without altering the steady-state levels of E-cadherin protein. Nevertheless, direct immunofluorescence reveals the presence of ZEB1, a transcriptional repressor of E-cadherin in MDCK cells. Interestingly, ZEB1 is present in all MDCK cells irrespective of their morphology. However, the expression pattern is different in the presence of deregulated ErbB2/neu. ErbB2/neu-expressing MDCK and breast carcinoma cell lines show a unique subnuclear punctuate pattern, suggesting that ErbB2/neu might be involved in the translocation of ZEB1 into subnuclear compartments (Fig.6, Chapter IV). A model is proposed (Figure 2) where ZEB1 translocation might be required for the assembly of a repressor complex including CtBP, another modulator of EMT (Grooteclaes et al., 2003; Grooteclaes and Frisch, 2000; van Grunsven et al., 2003). HGF might modulate the repressor complex assembly and activity, resulting in the modulation of gene expression toward an enhanced epithelial-mesenchymal transition. It was previously shown that ZEB1 is required for differentiation of the central nervous system and the muscle. ZEB1 is also involved in TGFβ-induced growth arrest and transcription (Postigo, 2003).

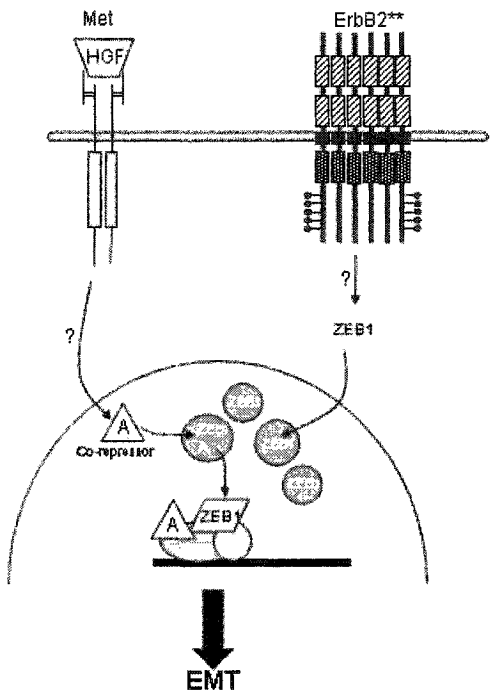


Figure 2: Hypothetical model for the regulation of ZEB1 transcriptional activity downstream from a deregulated activated ErbB2/Neu. ErbB2/Neu might induce ZEB1 translocation to a subnuclear compartment (red circles) where ZEB1 participates in the assembly of a transcription complex. HGF might modulate this process by promoting the recruitment of a corepressor/coactivator, resulting in the modulation of gene expression toward a potent EMT.

In the mammary gland, HGF is released by the stroma surrounding the breast epithelial cells where Met and ErbB2 are expressed. Both Met and ErbB-2 are involved in normal tissue differentiation, and the deregulation of either receptor is implicated in the development of neoplastic disease. Hence cross-talk between ErbB2/Neu and HGF-activated Met might be an important factor for the progression of malignant disease.

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